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PRP-17 and the pre-mRNA splicing pathway are preferentially required for the proliferation versus meiotic development decision and germline sex determination in *Caenorhabditis elegans*

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Abstract

In *C. elegans*, the decision between germline stem cell proliferation and entry into meiosis is controlled by GLP-1 Notch signaling, which promotes proliferation through repression of the redundant GLD-1 and GLD-2 pathways that direct meiotic entry. We identify *prp-17* as another gene functioning downstream of GLP-1 signaling that promotes meiotic entry, largely by acting on the GLD-1 pathway, and which also functions in female germline sex determination. PRP-17 is orthologous to the yeast and human pre-mRNA splicing factor PRP17/CDC40 and can rescue the temperature-sensitive lethality of yeast *PRP17*. This link to splicing led to an RNAi screen of predicted *C. elegans* splicing factors in sensitized genetic backgrounds. We found that many genes throughout the splicing cascade function in the proliferation/meiotic entry decision and germline sex determination indicating that splicing *per se*, rather than a novel function of a subset of splicing factors, is necessary for these processes.

Keywords

germline development; proliferation; meiotic entry; germline sex determination; pre-mRNA splicing; PRP-17; GLP-1 Notch signaling

INTRODUCTION

Stem cells are unique, as they are relatively unspecialized with the potential to generate multiple daughter cell types, including those that can enter a differentiation pathway and those that can proliferate providing a population of self-renewing cells. A balance between stem cell self-renewal and differentiation is crucial for animal tissue development and maintenance. If too many cells differentiate, the self-renewing stem cell population is depleted, and in total fewer differentiated cells are made. Conversely, if too many cells continue to proliferate at the expense of differentiation, then tissue formation and maintenance halts and a tumor may result.

The *C. elegans* germline is an excellent model for studying regulation of cell fate decisions, including the switch from stem cell proliferation to meiotic development (Crittenden *et al*. 1994; Hansen *et al*. 2004b; Crittenden *et al*. 2006). The germline of the late larval/ adult hermaphrodite is spatially organized with a population of proliferating stem cells at the distal end followed more proximally by the transition zone where cells have switched to the

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earliest stages of meiotic prophase and will develop along the male pathway forming sperm, in late larvae, or the female pathway forming oocytes, in the adult (Figure 1A). The conserved GLP-1 Notch signaling pathway regulates the balance between proliferation and entry into meiosis (Seydoux and Schedl 2001; Kimble and Crittenden 2005; Hansen and Schedl 2006). Membrane-bound LAG-2 ligand (Henderson *et al.* 1994; Tax *et al.* 1994), expressed in the somatic distal tip cell (DTC) (Fitzgerald and Greenwald 1995), binds to the Notch receptor GLP-1 (Austin and Kimble 1987, Austin and Kimble 1989; Yochem and Greenwald 1989), expressed in the plasma membrane of distal germ cells (Crittenden *et al*. 1994). Ligand binding likely causes cleavage of the intracellular portion of GLP-1 and its subsequent translocation to germ cell nuclei, where it forms a ternary complex with the transcription factor LAG-1 (Christensen *et al*. 1996; Mumm and Kopan 2000) and the transcriptional coactivator LAG-3/SEL-8 (Doyle *et al*. 2000; Petcherski and Kimble 2000), resulting in the transcription of genes that promote germ cell proliferation and/or inhibit entry into meiosis. As germ cells transit proximally, away from the DTC, the GLP-1 Notch signal decreases and they switch to meiotic development. If the GLP-1 signaling pathway is inactivated, all germ cells prematurely enter into meiosis and differentiate, depleting the stem cell population (Glp phenotype) (Austin and Kimble 1987; Lambie and Kimble 1991). Conversely, constitutive activation of GLP-1, as in the strong gain-of-function (gf) allele, *glp-1(oz112gf)*, results in the failure of germ cells to differentiate (enter meiosis), and instead cells proliferate throughout the gonad, forming a tumor (Tum phenotype)(Berry *et al.* 1997). Weak *glp-1(gf)* mutations, such as *glp-1(ar202gf)*, display two types of ectopic proliferation. One type, called a “late-onset tumor”, is characterized by a proliferation zone that increases in size over time, extending past the usual ~20 cell diameter proliferative zone observed in wild type (Berry *et al*. 1997; Pepper *et al*. 2003). The second type is known as proximal proliferation (Pro phenotype), where the most proximal germ cells fail to enter meiosis and continue proliferating, while more distal germ cells enter meiosis similar to wild-type (Pepper *et al.* 2003).

Genetic evidence indicates that the GLD-1 and GLD-2 pathways, named after their founding members, function redundantly downstream of GLP-1 Notch to promote entry of germ cells into meiosis and/or inhibit proliferation (Kadyk and Kimble 1998; Hansen and Schedl 2006). GLD-1 is a KH domain-containing RNA binding protein that acts as a translational repressor (Jones and Schedl 1995; Jan *et al*. 1999; Lee and Schedl 2001). GLD-2 is the catalytic portion of a poly(A) polymerase that acts as a translational activator (Wang *et al*. 2002). In single *gld-1* and *gld-2* null mutants, germ cells enter meiosis normally, indicating that either gene is sufficient for meiotic entry. However, animals that lack both *gld-1* and *gld-2* gene activities exhibit germline tumors, which are epistatic to a *glp-1* null allele (Kadyk and Kimble 1998). This type of tumor is known as a “synthetic tumor.” In addition to *gld-1* and *gld-2*, the genes *nos-3* and *gld-3* have been shown to function in these pathways. NOS-3, one homolog of Drosophila Nanos (Kraemer *et al*. 1999; Subramaniam and Seydoux 1999), functions in the GLD-1 pathway (Hansen *et al*. 2004a). Like *gld-1(null)*, a null allele of *nos-3* forms a synthetic tumor in the absence of *gld-2* or *gld-3* activity. GLD-3, a BicC related RNA binding protein, functions in the GLD-2 pathway (Eckmann *et al*. 2004) and appears to physically interact with GLD-2 (Eckmann *et al* 2002). Additionally, mutations in the *mog* splicing factor genes (see below) display a synthetic tumorous phenotype with *gld-3* null, suggesting that they are also involved in the regulation of the proliferation/meiotic entry decision in *C. elegans* (Belfiore *et al*. 2004); however, the relationship of the *mog* genes to the GLP-1 signaling pathway is unknown.

The generation of male or female gametes from stem cell daughters depends on the germline sex determination pathway. In the hermaphrodite, germline sexual development consists of two phases: a transient period of spermatogenesis followed by oogenesis. Whether spermatogenesis or oogenesis occurs depends, in part, upon the relative activities of genes

that promote the male fate (e.g. *fem-3*, *fog-1*, and *fog-3*) and genes that promote the female fate (e.g. *tra-2*, *tra-3* and the *mog* genes) (reviewed in Ellis and Schedl 2006). *fem-3* and the germline sex determination terminal regulators *fog-1* and *fog-3* are active during the L3/L4 stage to promote spermatogenesis (Hodgkin 1986; Barton and Kimble 1990; Ellis and Kimble 1995), due in part to transient translational repression of *tra-2* (Goodwin *et al*. 1993; Jan *et al*. 1999). The switch from spermatogenesis to oogenesis depends, in part, upon the translational repression of *fem-3* (Ahringer and Kimble 1991; Ahringer *et al*. 1992). Loss-of-function mutations in the *mog* genes cause a "Mog" (*m*asculinization *o*f the *g*ermline) phenotype in hermaphrodites, producing excess sperm at the expense of oocyte production (Graham and Kimble 1993; Graham *et al*. 1993), indicating that the *mog* genes function to promote the female/oocyte fate in hermaphrodites. Genetic epistasis analysis placed the *mog* genes upstream of *fem-3*, *fog-1*, and *fog-3* (Graham and Kimble 1993; Graham *et al*. 1993; Barton and Kimble 1990; Ellis and Kimble 1995), suggesting the possibility that the *mog* genes may negatively regulate these female-promoting genes. Reporter assays have shown that *mog* gene activity, in addition to being present in the germline, is also detected in the soma, where it acts through the *fem-3* 3′ UTR to repress reporter constructs (Gallegos *et al*. 1998). These findings suggested that the MOGs, directly or indirectly, promote the female fate by translational repression of *fem-3*.

One process required for eukaryotic gene expression is pre-mRNA splicing, which removes the intervening sequences of nuclear pre-mRNAs. The splicing reaction takes place in a large dynamic protein-RNA complex, the spliceosome, which assembles onto a nascent pre-mRNA in a step-wise fashion to align splice sites and catalyze the two-step splicing reaction (reviewed in Krämer, 1996; Moore and Proudfoot, 2009; Wahl *et al*. 2009) (also see Figure 5). Most *C. elegans* genes contain introns (*C. elegans* Sequencing Consortium *et al*. 1998), indicating a general requirement for splicing in gene expression. The splicing mechanism in *C. elegans* occurs similarly to that in other higher eukaryotes and many factors involved in the splicing process are highly conserved among these species (Blumenthal and Steward 1997; Thomas *et al*. 1990; Blumenthal, 2005; Zahler, 2005). Interestingly, MOG-1, MOG-4, and MOG-5 are orthologous to the yeast and human pre-mRNA splicing factors PRP16, PRP2, and PRP22, respectively (Puoti and Kimble 1999; Puoti and Kimble 2000), which suggests that they could function in splicing in *C. elegans* to regulate the proliferation/meiotic entry and sex determination decisions. Additionally, *mog-6*/*cyn-4*, which encodes a U box-containing peptidyl-prolyl cis-trans isomerase, shares many genetic and molecular properties with the *mog* genes (Belfiore *et al.* 2004); while the human ortholog Cyp-60 has not been shown to be a stable component of the splicing machinery, a number of peptidyl-prolyl cis-trans isomerases function during splicing (Wahl *et al.* 2009). To date, no steady-state defect in splicing of a set of tested genes has been detected in *mog-1* or *mog-6* mutants (Puoti and Kimble 1999; Belfiore et al 2004), although this is technically challenging given rapid nuclear degradation of incorrectly/unspliced mRNAs.

Here we describe a new regulator of *C. elegans* germline development, *prp-17*, that shares genetic similarities with the *mog* genes. We find that *prp-17* functions downstream of GLP-1 Notch signaling to promote entry into meiosis and/or inhibit germ cell proliferation and demonstrate that it also functions to promote the oocyte fate decision in *C. elegans* sex determination. Like MOG-1, −4 and −5, which are orthologous to yeast and human pre-mRNA splicing factors, reciprocal best BLAST analysis reveals that *C. elegans* PRP-17 is orthologous to the yeast and human pre-mRNA splicing factor PRP17/CDC40. Furthermore, we show that *C. elegans prp-17* can rescue the temperature-sensitive lethality of a null allele of yeast *PRP17*, suggesting that the *C. elegans* protein functions in splicing. Finally, we find that many *C. elegans* splicing factor orthologs function in the meiotic entry and sex determination decisions, implicating a role for pre-mRNA splicing *per se* in the regulation of these aspects of *C. elegans* germline development.

RESULTS

prp-17(oz273) enhances the *glp-1(oz264)* phenotype at 20°

To isolate negative regulators of GLP-1 Notch signaling or positive regulators of entry into meiosis, we conducted an F2 screen for recessive, loss-of-function enhancers of a temperature sensitive, weak *glp-1* gain-of-function allele, *oz264. glp-1(oz264)* homozygous animals display wild-type phenotypes at 15° and 20° (Figure 1B), but at 25°, hermaphrodite germlines exhibit late-onset tumors and proximal proliferation (Kerins 2006). In a pilot screen of about 500 haploid genomes for enhancement of this phenotype at 15° we isolated the *oz273* mutation. Based on results described below, *oz273* is an allele of the gene *prp-17. prp-17(oz273); glp-1(oz264)* adult hermaphrodites display a late-onset tumorous phenotype, characterized by proliferating germ cells that extend to the loop region, and proximal proliferation. Both groups of cells are positive for a marker of proliferating cells, showing nucleoplasmic REC-8 staining (Figure 1D) (Pasierbek et al. 2001; Hansen *et al*. 2004b). This differs from a wild-type adult hermaphrodite, where only the most distal germ cells, extending ~20 cell diameters from the distal tip, proliferate (Crittenden *et al*. 1994; Hansen *et al*. 2004b) and are positive for REC-8. Although most cells appear to be proliferating, some cells are positive for the HIM-3 meiotic marker, showing chromosome axis staining (MacQueen and Villeneuve 2001; Zetka *et al.* 1999), and the DAPI image shows a few sperm, indicating that some level of differentiation occurs in these mutants. This phenotype is highly penetrant and significantly enhances *glp-1(oz264)*, as 96.9% of gonad arms from *prp-17(oz273); glp-1(oz264)* hermaphrodites are tumorous, whereas *glp-1(oz264)* hermaphrodites do not exhibit overproliferation at 20° (Figure 1B, Table 1). Based on these phenotypic data, we conclude that *prp-17* has a function in the proliferation/meiotic development decision.

prp-17(oz273) promotes both the female fate in germline sex determination and oocyte development

In a *glp-1(+)* genetic background at 20°, 98.0% of *oz273* hermaphrodite germlines show a Mog phenotype of excess spermatogenesis at the expense of oogenesis, similar to the previously characterized *mog* genes (Graham and Kimble 1993)(Figure 1C, Table 1). This same phenotype can be seen at 15° (data not shown). At 25°, *oz273* homozygotes show severe developmental defects, including larval arrest, slow growth, underdeveloped germlines, and 7.4% larval lethality (n = 54 animals, data not shown), indicating that the *oz273* mutant displays temperature-sensitivity. The masculinized phenotype suggests that *prp-17* plays a role in the switch from spermatogenesis to oogenesis in the developing hermaphrodite. To begin to understand where *prp-17* functions in the sex determination pathway, we analyzed *oz273* mutants in *fog-1, fog-3,* and *fem-3* null genetic backgrounds. *fog-1*, *fog-3* and *fem-3* are terminal regulators of germline sex determination and promote the male fate; loss of *fog-1*, *fog-3* or *fem-3* gene activity results in animals that produce only oocytes (Fog) (Barton and Kimble 1990; Ellis and Kimble 1995; Hodgkin 1986). *fog-1(q241) prp-17(oz273), fog-3(q443) prp-17(oz273)* and *prp-17(oz273); fem-3(e1996)* animals display a Fog phenotype with no evidence of spermatogenesis (Table 1). Given that the *oz273* mutation is loss-of-function/probable null (see below), these observations indicate that *prp-17*, like the *mog* genes (see DISCUSSION), functions upstream of *fog-1*, *fog-3* and *fem-3* to promote the oocyte fate. Additionally, oocytes in these animals exhibit abnormal differentiation (variable size, irregular membrane organization), although they proceed to diakinesis (Figure 2A). *prp-17* is thus necessary for oocyte development, as well as general viability and development at 25°.

C. elegans prp-17 functions to promote initiation of meiotic development

C. elegans prp-17(oz273) was originally isolated as an enhancer of the weak *glp-1* gain-of-function overproliferation phenotype, suggesting the wild-type gene functions either upstream of or in parallel to GLP-1 signaling as a negative regulator, or downstream of or in parallel to GLP-1 signaling to promote entry of germ cells into meiosis. To help distinguish between these possibilities, we took advantage of the GLD-1 and GLD-2 pathways, which function redundantly downstream of GLP-1 to promote entry into meiosis (Kadyk and Kimble 1998; Hansen *et al* 2004b). In both *gld-1(null)* and *gld-2(null)* single mutants, germ cells enter meiosis normally; however, the *gld-1(null) gld-2(null)* double mutant shows overproliferation due to a meiotic entry defect (Kadyk and Kimble 1998; Hansen *et al*. 2004b). *gld-3* and *nos-3* function in the GLD-2 and GLD-1 pathways, respectively, and form synthetic tumors with null mutants in the parallel pathway (Eckmann *et al* 2004; Hansen *et al*. 2004a). Like a *gld-1(null) gld-2(null)* double mutant, a *prp-17(oz273)* double mutant with *gld-2(q497)* null also shows meiotic entry defects/overproliferation (Table 1). This same phenotype can be seen in *prp-17(oz273); gld-3(q730)* mutants (Figure 2B), which display robust Pro and late-onset tumorous phenotypes. To determine if the *prp-17(oz273)* overproliferation phenotype with *gld-2(null)* and *gld-3(null)* was dependent on *glp-1* function, we examined each double mutant in the absence of *glp-1* activity. Similar to the tumorous *gld-1 gld-2; glp-1* triple null mutant, *prp-17(oz273); gld-3(q730); glp-1(q175)* animals are tumorous (Figure 2C). Thus, *prp-17* cannot simply function as a negative regulator of GLP-1, but instead must function downstream of or in parallel to GLP-1 to promote entry into meiosis and/or inhibit proliferation. In this case, the synthetic tumorous phenotype of *prp-17(oz273)* with *gld-2(null)* and *gld-3(null)* argues that PRP-17 functions, at least in part, out side the GLD-2 pathway, possibly to promote the GLD-1 pathway. Surprisingly, proliferation in *gld-2(q497) prp-17(oz273); glp-1(q175)* requires GLP-1 activity (Table 2); the reason for this difference in behavior for *gld-2* and *gld-3* is not known. We also examined the interaction of *prp-17* with null mutants in the GLD-1 pathway, *gld-1* and *nos-3*. Because *gld-1(q485)* single mutant hermaphrodites show an overproliferation phenotype due to a defect in meiotic progression during oogenesis (Francis *et al*. 1995), we examined *gld-1(q485)* males, which are phenotypically wild-type. *gld-1(q485) prp-17(oz273)* males display weak overproliferation phenotypes (Table 2), while *prp-17(oz273); nos-3(oz231)* hermaphrodites display normal meiotic entry. The synthetic tumorous phenotype with *gld-1* indicates that PRP17 also functions outside the GLD-1 pathway, although the weakness of the phenotype, and the absence of an interaction with *nos-3*, suggests that this function is less important. Together, the genetic data indicates that *prp-17* functions downstream or in parallel to GLP-1 signaling, likely largely to promote the GLD-1 pathway, with a minor role outside of the GLD-1 pathway, possibly in the GLD-2 pathway or in a minor third pathway that has been proposed (Hansen et al. 2004b).

The *oz273* mutation is a deletion in the gene F49D11.1, whose protein is orthologous to the yeast and human splicing factor PRP17/CDC40

Meiotic mapping with morphological and SNP markers narrowed the region of the *oz273* mutation to an ~20 kb interval suitable for candidate gene testing. We then used feeding RNAi to screen candidate genes for their ability to enhance the overproliferation phenotype of *rrf-1(pk1417); glp-1(oz264)* and cause a Mog phenotype in *rrf-1(pk1417)* animals at 20°. *rrf-1* is an RNA-directed RNA polymerase that functions in RNAi in the soma, but not the germline. Therefore, the *rrf-1(pk1417)* null mutant is largely resistant to RNAi in the soma, but sensitive to germline RNAi (Sijen *et al*. 2001), which greatly reduces pleiotropic somatic phenotypes and allows germline-specific RNAi phenotypes to be obtained. RNAi of the three genes in the ~20 kb region revealed one gene that phenocopied the *oz273* mutation. RNAi of F49D11.1 formed tumors in *rrf-1(pk1417); glp-1(oz264)* animals, and also caused

a Mog phenotype in *rrf-1(pk1417)* animals at 20° (data not shown). PCR amplification and sequencing of genomic F49D11.1 DNA from *oz273* mutants revealed an 873 bp deletion that includes the first 173 amino acids encoded by exon 2, according to the predicted amino acid sequence (Figures 3A, 3B). When we performed RT-PCR on feminized *oz273* animals, we were surprised to detect message in mutants; however, the size did reflect a deletion when compared to wild-type cDNA (data not shown). Sequencing of the mutant RT-PCR product revealed a cryptic splice site that occurs 10 bp downstream of the deletion, resulting in a predicted frameshift and premature stop codon. The combination of the 173 amino acid deletion, frameshift and premature stop suggests that the *oz273* allele is likely null (also see below).

The reciprocal best BLAST method aligned the F49D11.1 predicted amino acid sequence with both yeast and human CDC40/PRP17, proteins involved in the second catalytic step of pre-mRNA splicing (Vijayraghavan *et al.* 1989; Zhou and Reed 1998). The most conserved region of the protein is the C-terminus, which also includes six WD-40 repeats, the only functional protein motifs of PRP17 identified by Pfam (Finn *et al*. 2006) (Figure 3C). The *oz273* deletion eliminates all of the WD-40 repeats and also a four-residue motif shown to be critical for splicing in yeast (Lindsey-Boltz et al, 2000) (Figure 3B), suggesting the predicted mutant *C. elegans* protein is non-functional.

C. elegans prp-17 can functionally rescue the yeast *prp17* null mutant

Since *C. elegans prp-17* showed high conservation with yeast *prp17*, we asked if it could rescue the temperature sensitive lethal phenotype of the yeast *prp17* null mutant strain. In yeast, *prp17* null mutants are viable at 23°, but cannot survive at 30° and 37°, due to a splicing defect in the second catalytic step of pre-mRNA splicing (Vijayraghavan *et al.* 1989). Rescue of this temperature sensitive lethality in yeast by *C. elegans prp-17* would be consistent with PRP-17 functioning in splicing in *C. elegans*. A yeast strain where the *PRP17* gene has been deleted, prp17Δ BJ2168 (Lindsey and Garcia-Blanco 1998), was transformed with the plasmid pG1-HACeprp-17 and plated on selective medium at 25°, 30°, and 37° and scored after 3–5 days for growth. The vector pG1 alone was used as a negative control, and both pG1-HAyprp17 and pG1-HAhprp17 (kindly provided by Usha Vijayraghavan) were transformed as positive controls. At 25°, all transformed strains grow well (Figure 4). *C. elegans prp-17* successfully rescues the yeast mutant strain at 30°, but not at 37°; similarly, human *PRP17* rescues the yeast mutant strain at 30°, but not at 37° (Lindsey *et al.* 1998; also this paper, Figure 4). The comparable pattern of rescue for *C. elegans prp-17* and human *PRP17* indicates that, like the human *PRP17* gene (Lindsey *et al*. 1998), *C. elegans prp-17* is a functional ortholog of yeast *PRP17*.

Proteins that function throughout the splicing cascade are also involved in the *C. elegans* proliferation/meiotic entry and sex determination decisions

Since *C. elegans prp-17* behaves as a functional ortholog of yeast *PRP17*, the *prp-17* mutant germline phenotypes may be caused by a pre-mRNA splicing defect. Other *C. elegans* genes that encode splicing factor orthologs, *mog-1*, *mog-4*, *mog-5*, as well as *mog-6*, share genetic characteristics with *prp-17*. Like *prp-17*, the *mog* genes have a Mog mutant phenotype, function to promote the oocyte fate in sex determination, acting upstream of *fem-3*, *fog-1* and *fog-3* (Graham and Kimble 1993; Graham *et al.* 1993; Belfiore *et al.* 2004). Mog gene mutants/ RNAi also form synthetic tumors with *gld-3* null (Belfiore *et al.* 2004); however, this result does not distinguish whether overproliferation is due to a defect in meiotic entry or meiotic prophase progression (Hansen et al. 2004b). We find that mutation or RNAi of *mog-1*, *mog-4*, *mog-5* or *mog-6* enhanced the weak *glp-1* gain-of-function overproliferation phenotype (Supplemental Figure 1 and data not shown) indicating that overproliferation is due to a defect in meiotic entry rather than meiotic progression (Hansen *et al.* 2004b).

Furthermore, we find that overproliferation in *gld-3(q730); mog-1(q223)* and *gld-3(q730) mog-6(q465)* is independent of *glp-1* activity (triple mutant has a tumorous phenotype, Supplemental Figure 2 and data not shown). Thus, like *prp-17*, *mog-1* and *mog-6* functions downstream, or in parallel, to GLP-1 signaling, likely at least in part in the GLD-1 pathway to promote meiotic entry.

The yeast and human orthologs of PRP-17, MOG-1, MOG-4 and MOG-5 all function in later steps of the splicing cascade (King and Beggs 1990; Kim and Lin 1993; Schwer and Guthrie 1992; Company *et al*. 1991; Schwer and Gross 1998; McPheeters *et al.* 2000) (Figure 5, Table 2). Previous studies suggested that MOG-1, −4, −5 and −6 might not function in splicing in *C. elegans* or might have splicing independent functions related to sex determination (Puoti and Kimble 1999; 2000, Belfiore *et al.* 2004). Thus, similarly, one possibility is that PRP-17 and the MOG proteins may have a non-splicing function that promotes meiotic development. This led to the question: how broad is the requirement for splicing factor function in the proliferation/ meiotic entry decision and germline sex determination? We reasoned that if loss of function of general factors throughout the splicing process phenocopies the *prp-17* and *mog* mutants, then it suggests that splicing activity may indeed be disrupted in these mutants, implicating a role for pre-mRNA splicing in the *C. elegans* meiotic entry and sex determination decisions. Alternatively, if the meiotic entry and sex determination phenotypes result from depletion of only a distinct subset of splicing factors (*i.e*., factors that function in only one step of splicing), it would be consistent with the subset of factors functioning together in a very specific aspect of splicing or together having some non-splicing activity that is necessary for these processes.

To address these issues, we conducted a feeding RNAi screen of 114 *C. elegans* genes that encode orthologs of a set of yeast and human proteins implicated in pre-mRNA splicing (Jurica and Moore 2003). We screened for the same phenotypes that characterize the *prp-17(oz273)* mutation: enhancement of *rrf-1(pk1417); glp-1(oz264)*, synthetic tumor formation with *rrf-1(pk1417); gld-3(q730)*, and a Mog phenotype in *rrf-1(pk1417)* animals at 20°. GFP RNAi was used as a negative control, and *prp-17*, *mog-1*, *mog-4*, and *mog-5* RNAi were used as positive controls. In an initial screen of the 114 annotated splicing factors tested (Supplemental Table 1), 51 displayed at least one of the phenotypes scored and were then analyzed in more depth. Of these 51 genes, 47 exhibited a statistically significant difference in penetrance of phenotypes compared to the corresponding GFP RNAi negative control (a small percentage of *rrf-1(pk1417); glp-1(oz264); GFP(RNAi)* animals display overproliferation phenotypes at 20° - see MATERIALS AND METHODS) (Table 2). 31 factors enhance *rrf-1(pk1417); glp-1(oz264)* significantly more than GFP control RNAi and 38 show overproliferation with *rrf-1(pk1417); gld-3(q730)*. 11 show a Mog phenotype in an *rrf-1(pk1417)* background.

The fact that the yeast and human orthologs of *C. elegans* PRP-17, MOG-1, MOG-4, and MOG-5 function in late splicing steps led us to wonder if overproliferation and the Mog phenotypes were specific to the disruption of these later splicing steps. However, our RNAi data indicates that disruption in any splicing step can cause these phenotypes (Table 2, Figure 5). For example, RNAi of the *C. elegans* ortholog of PRP40/CA150, which has been shown to function in the formation of Complex E (an early step), enhances *glp-1(oz264)*. The *C. elegans* gene Y65B4A.1 encodes an ortholog of a component of the U2 snRNP, HTATSF1, which functions in the formation of Complex A (Figure 5)(Krämer 1996; Jurica and Moore 2003; Wahl et al. 2009); RNAi of this gene displays a Mog phenotype in the *rrf-1* background, enhances *rrf-1;glp- 1(oz264)*, and forms synthetic tumors in the *rrf-1;gld-3(null)* background (Figures 2D, 2E; Table 2). Yeast and human PRP31 functions later, in the formation of the U4/U5-U6 complex. RNAi of this *C. elegans* ortholog both enhances *rrf-1;glp-1(oz264)* and causes a Mog phenotype in a *glp-1(+)* background. *C.*

elegans DDX-23, which is ortholgous to yeast Prp28 and functions to initiate the catalytic activation of the spliceosome, was shown by Konishi *et al.* (2008) to function in germline sex determination, giving a Mog mutant phenotype. RNAi of F01F1.7 *ddx-23* enhances *rrf-1;glp-1(oz264),* and forms synthetic tumors in the *rrf-1; gld-3(null)* background (Table 2). Disruption of late splicing steps can also cause meiotic entry and sex determination phenotypes, as observed with the *C. elegans* ortholog of yeast and human PRP43, which is involved in the release of the lariat-intron from the spliceosome, forming the post-splicing complex. These results indicate that there is no correlation between where each factor functions in the splicing pathway and the RNAi phenotype, suggesting that general disruption of splicing can cause overproliferation and/or Mog phenotypes.

The number of splicing factor genes displaying one or more of the RNAi phenotypes is likely to be a significant underestimate given the high false negative rate in our experiments: *prp-17, mog-1, −5* and *−6* null mutants or RNAi are known to enhance weak *glp-1(gf)* alleles and cause a synthetic tumor with *gld-3(null)* at high penetrance (Table 1; Belfiore *et al.* 2004; Supplemental Figure 1 and data not shown), yet RNAi of these genes in the corresponding genetic backgrounds showed significant experiment-to-experiment variation in penetrance. Additionally, strong splicing factor knockdown phenotypes (lethality, small germlines) may have masked the overproliferation phenotype. We also observed that RNAi of certain genes displayed only one of the phenotypes scored (Table 2). While these single defects might suggest that a gene is showing a specific genetic interaction, because of the high false negative rate we think it is more likely that these genes are functioning similarly to *prp-17, mog-1, −5,* and *−6* to promote both meiotic entry and the female germ cell fate.

To determine if the proliferation/meiotic entry decision and sex determination phenotypes are specifically due to a disruption of splicing function, or if any decrease in general levels of gene expression can cause the observed RNAi phenotypes, we RNAi-depleted a small sample of both core RNA polymerase II and core ribosomal protein genes in *rrf-1(pk1417); glp-1(oz264), rrf-1(pk1417),* and *rrf-1(pk1417); gld-3(q730)* animals. We reasoned that if we observed overproliferation and germline masculinization after RNAi of one or both of these gene groups, then the phenotypes may not be due to a defect in splicing *per se,* but rather due to defects in general levels of gene expression. RNAi of RNA polymerase II and ribosomal protein genes, while showing other highly penetrant defects, did not phenocopy RNAi of splicing factors (Supplemental Table 2), consistent with a splicing defect as the causative agent in producing the mutant phenotypes.

DISCUSSION

The role of *prp-17* and splicing factors in the GLP-1 Notch signaling pathway

The GLP-1 Notch pathway functions in *C. elegans* to promote germ cell proliferation by inhibiting the redundant GLD-1 and GLD-2 pathways that promote meiotic development (Kadyk and Kimble 1998; Hansen *et al* 2004b). We have provided evidence that *prp-17* functions downstream, or in parallel, of GLP-1 in the germ cell proliferation versus meiotic entry decision. *prp-17(oz273);gld-3(*null*)* animals have an overproliferation phenotype that is independent of *glp-1* activity. *prp-17(oz273)* forms strong synthetic tumors with *gld-2* and *gld-3* null indicating that PRP-17 acts in large part outside of the GLD-2 pathway, likely in the GLD-1 pathway, to promote meiotic entry. However, *prp-17(oz273)* also forms weak synthetic tumors with *gld-1* null, indicating that PRP-17 also functions outside of the GLD-1 pathway, possibly in the GLD-2 pathway.

We and Mantina *et al.* (2009) identified K02F2.3 *teg-4,* the *C. elegans* ortholog of splicing factor SAP130 (human, also known as SF3B3) and Rse1 (yeast), which is a component of splicing Complex A, as functioning in the proliferation/ meiotic entry decision. Extensive

genetic analysis to place *teg-4* in this decision by Mantina et al. (2009) yielded essentially identical results to those reported here for *prp-17*; TEG-4 functions downstream of GLP-1 signaling, largely to promote the GLD-1 pathway. Thus for the four splicing factors that have been examined, PRP-17, TEG-4 SAP130, MOG-1 PRP16 and MOG-6 CYP-60, appear to function together in the proliferation/ meiosis decision.

prp-17 and the *mog* genes in *C. elegans* germline sex determination

C. elegans germline sex determination depends upon the balance between the levels of *tra-2* and *fem-3* activity. In L3/L4 stage hermaphrodites, *tra-2* is translationally repressed, and *fem-3* expression ultimately activates the terminal regulators *fog-1* and *fog-3*, allowing spermatogenesis to occur (reviewed in Goodwin and Ellis 2002; Ellis and Schedl 2006). Oogenesis begins in the adult in response to the translation of *tra-2* mRNA and translational repression of *fem-3*. The *mog-1*, *mog-4*, and *mog-5* genes were isolated in genetic screens for recessive mutations that cause failure of the sperm-oocyte switch (Graham and Kimble 1993; Graham *et al.* 1993). Double null mutants with the *mog* genes and *fem-3*, *fog-1*, and *fog-3* produce only oocytes (Graham and Kimble 1993; Graham *et al.* 1993; Ellis and Kimble 1995), suggesting the *mogs* function upstream of these genes to effect the sperm-oocyte switch, possibly by negatively regulating one or more of these genes. A reporter transgene bearing the *fem-3* 3′ UTR was used to examine the possible role of the *mogs* in *fem-3* translational repression (Gallegos *et al*. 1998). In wild-type animals, the reporter was poorly expressed; however, derepression was observed in *mog* mutant backgrounds. Thus, the *mog* genes appear to be required, directly or indirectly, for *fem-3* 3′ UTR repression, at least in the soma (Gallegos *et al*. 1998). This conclusion has been applied to *mog* function in the germline, although the mechanism by which MOG proteins mediate *fem-3* mRNA translational repression (in both the soma and germline) is unclear.

As in the proliferation/meiotic entry decision, *C. elegans prp-17* behaves genetically similarly to the *mog* genes in germline sex determination. The Mog phenotype of the *prp-17(oz273)* mutant indicates *prp-17* is required for the sperm-oocyte switch. Like the *mog* genes (Graham and Kimble 1993; Graham *et al.* 1993; Ellis and Kimble 1995), *fog-1*, *fog-3* and *fem-3* null mutants are epistatic to *prp-17(oz273)*, placing *prp-17* upstream of these genes.

Although *fog-1 prp-17(oz273)* or *fog-3 prp-17(oz273)* double mutants produce only oocytes, the oocytes are abnormal and underdeveloped. Defective oocytes are also observed in *fog-1;mog(null)* double mutants (Graham *et al*. 1993). These findings suggest that like the *mog* genes, not only is *prp-17* required for the sperm/oocyte switch, but it is also required for oocyte development. *prp-17* likely functions together with the *mog* genes in these processes.

C. elegans prp-17 as a pre-mRNA splicing factor

Sequence analysis suggests that PRP-17 is an ortholog of the yeast and human splicing factor, PRP17/CDC40. Similarly, MOG-1, MOG-4, and MOG-5 also are orthologous to the yeast and human splicing factors PRP16, PRP2, and PRP22, respectively. These factors have been extensively studied in yeast, due to the organism's relatively low abundance of annotated intron-containing genes (Spingola *et al*. 1999; David *et al* 2000), making it a simpler system to conduct splicing assays. In yeast, all four genes were isolated as temperature-sensitive mutants that blocked pre-mRNA splicing at various stages (Vijayraghavan *et al.* 1989). These and other studies have more precisely identified the role of each gene in the splicing cascade (Figure 5). Both Prp16 and Prp17 are required for the second catalytic step in pre-mRNA splicing (Vijayraghavan *et al.* 1989; Zhou and Reed 1998; Schwer and Guthrie 1992). Prp2 functions just prior to the first catalytic step,

restructuring the spliceosome to an active conformation (King and Beggs 1990; Kim and Lin 1996). Prp22 both functions in the second catalytic reaction and is required for spliceosome disassembly (Schwer and Gross 1998; McPheeters *et al*. 2000). As mentioned before, Prp17 contains six WD-40 repeats, however, Prp16, Prp2, and Prp22 belong to a different family of proteins, the DExH-box family of RNA helicases (Burgess *et al*. 1990; Chen and Lin 1990; Company *et al.* 1991). Although the yeast and human homologs have demonstrated roles in pre-mRNA splicing, the biochemical functions of these proteins in *C. elegans* has only been inferred based on orthology, and splicing targets of these factors have yet to be discovered. However, in the case of *prp-17,* the ability of the *C. elegans* gene to rescue the temperature sensitive lethal splicing defect of yeast *prp17(null)* (Vijayraghavan *et al*. 1989) strongly suggests functional orthology as a splicing factor.

Pre-mRNA splicing and *C. elegans* germline development

The shared genetic characteristics among *prp-17* and the *mog* genes, plus the orthology of their gene products to annotated yeast and human pre-mRNA splicing factors led us to ask if their mutant germline meiotic entry and sex determination phenotypes are a reflection of a general defect in pre-mRNA splicing in *C. elegans*. We found that RNAi of 47/114 splicing factor orthologs confer overproliferation and/or Mog sex determination phenotypes (Table 2) and these gene products are distributed throughout the splicing process (Figure 5). These results are supported by genetic analysis of individual splicing factor orthologs TEG-4 and DDX-23 (Konishi *et al.* 2008; Mantina *et al.* 2009). The number of splicing genes we identified is likely an underestimate given the high frequency of RNAi false negatives. Together, these results strongly suggest that a general disruption in splicing, not specific steps or a specific complex, causes overproliferation and/or Mog sex determination phenotypes, arguing against the possibility that a subset of splicing factors has an independent non-splicing related function in these aspects of *C. elegans* germline development.

Given the essential nature of mRNA splicing in gene expression, why are we observing specific effects on the meiotic entry and sex determination processes in the *mog* mutants and the RNAi screen? First, we propose that splicing is reduced but not eliminated in the *prp-17* and *mog* mutants and following RNAi of the splicing factors. Each step of pre-mRNA splicing is accomplished by large protein-RNA complexes (Krämer 1996; Wahl *et al.* 2009), rather than individual factors, creating redundancy (Frank *et al*. 1992; Fu *et al*. 1992; Mayeda *et al*. 1992; Zahler *et al*. 1992; Ben-Yehuda *et al*. 2000). Loss of certain factors, while decreasing the overall efficiency of splicing can be compensated by other factors in the splicing complex; examples of such factors may be genes such as *prp-17* and *mog-1* identified in genetic screens where strong loss-of-function/ possibly null mutants display meiotic entry and sex determination phenotypes in adults. Other factors may have a more essential function in splicing, such genes are less likely to be identified in mutant screens because of strong developmental/arrest defects, but a function in the meiotic entry and sex determination processes can be uncovered by RNAi partial knockdown in the *rrf-1* background that reduces somatic effects. Second, we argue that meiotic entry and germline sex determination are processes that are preferentially sensitive to reduction in splicing pathway function. The overproliferation and the Mog phenotypes are highly specific and require, respectively, the production of cell cycle progression and spermatogenesis gene products whose corresponding pre-mRNAs must be spliced during their synthesis. Preferential sensitivity is thus suggested by reduction in splicing causing cell fate changes, yet not noticeably disrupting cell cycle progression and spermatogenesis. Sensitized backgrounds were employed to show that reduction in splicing function causes overproliferation; however, this is required in part because redundant pathways promote meiotic entry and elimination of a single pathway does not result in a meiotic entry defect

(reviewed in Kimble and Crittenden 2005; Hansen and Schedl 2006). Additionally, RNAi of both RNA Pol II and ribosomal protein genes, while showing penetrant phenotypes, does not cause overproliferation and/or Mog sex determination phenotypes; although this is a negative result, it suggests that these phenotypes are not generated by a general disruption of gene expression.

We propose three hypotheses by which disruption throughout the splicing cascade can preferentially affect genes in the proliferation/meiotic development decision and the sex determination pathway. Here we assume that one or more genes in the proliferation/meiotic entry decision and in the sex determination pathway are preferentially affected by a disruption in splicing. The first hypothesis proposes that meiotic entry and sex determination genes undergo canonical pre-mRNA splicing and that although *C. elegans* splicing factors are required for the efficient splicing of all mRNAs, the pre-mRNA of one or more genes that promote meiotic entry and sex determination are inherently spliced inefficiently. For example, a pre-mRNA that has multiple inefficiently spliced introns may be significantly more affected by a decrease in splicing factor activity (through loss-of-function mutations or RNAi) than other mRNAs. Therefore, in wild-type animals, splicing of this group of pre-mRNAs is sufficiently efficient to produce enough gene product for normal development; however, when there is a decrease in splicing factor activity in mutants or following RNAi, splicing efficiency is reduced to a level too low for adequate production of gene product, leading to meiotic entry and sex determination phenotypes.

The second hypothesis proposes that one or more gene products involved in meiotic entry and the sperm-oocyte switch arise from alternative splicing of their respective pre-mRNAs, and that these alternative splice events are inherently inefficient. Depletion of splicing factors reduces the efficiency of the alternative splice events, therefore disrupting the processes of meiotic entry and the sperm-oocyte switch. An estimated 13% of annotated *C. elegans* genes are alternatively spliced (Zahler 2005), suggesting that alternative splicing plays an important role in *C. elegans* gene expression. Thus it is possible that alternative splicing plays a role in the proliferation/meiotic entry decision and sex determination pathway. Evidence for alternative splicing in *C. elegans* somatic sex determination already exists: *xol-1*, the major regulator of male fate, is alternatively spliced to produce three transcripts, one of which is necessary and sufficient for wild-type function (Rhind *et al*. 1995). Drosophila sex determination is also regulated by alternative splicing. For example, one way the gene *Sxl (sex-lethal)* controls sex determination is by continuously directing female-specific splicing of its own pre-mRNA: removal of a translation-terminating exon occurs in females, but not in males (Bell *et al*. 1988).

The third hypothesis proposes that pre-mRNA splicing in *C. elegans* may be necessary for the efficient assembly of ribonucleoprotein (RNP) complexes that subsequently regulate mRNA translation in the cytoplasm. For example, various studies in metazoans have shown that some splicing factors also function as part of a complex of conserved proteins known as the exon-junction complex, or EJC (Blencowe *et al*. 1998; Mayeda *et al*. 1999; Kataoka *et al*. 2000; Le Hir *et al*. 2000; McGarvey *et al*. 2000; Zhou *et al*. 2000; Luo *et al*. 2001), which is recruited to positions near the exon junctions of mRNAs as a consequence of splicing. The EJC has multiple roles in mRNA metabolism, such as mRNA export and nonsense-mediated decay, as well as cytoplasmic localization of mRNAs (Hachet and Ephrussi 2004). It is possible that the EJC (or another unknown protein complex) also functions in preparing messages for translational control. In this model, disruption throughout the splicing pathway would adversely affect assembly of this complex, preventing efficient translational regulation of target mRNAs. Translational regulation is important for both the proliferation/meiotic entry decision and for germline sex determination in *C. elegans* (Kimble and Crittenden 2005; Hansen and Schedl 2006; Ellis

and Schedl 2006). Interestingly, RNAi of the EJC protein, RNPS1 (Mayeda *et al*. 1999), enhances the tumorous phenotype of *glp-1(oz264)* (Table 2).

If the first or second hypothesis holds true, it appears that *C. elegans* germline meiotic entry and sex determination is mediated by pre-mRNA splicing. Evidence for cellular and developmental processes being regulated by pre-mRNA splicing exists in other systems. In birds and mammals, expression of two different isoforms of the cardiac troponin T (cTNT) gene is developmentally regulated by alternative splicing during striated muscle development (Cooper and Ordahl 1985; Jin and Lin 1989; Greig *et al*. 1994; Ryan and Cooper 1996). In yeast, splicing is implicated in the control of cell cycle progression. *PRP17/CDC40* was first isolated as a cell division cycle mutant, (Kassir and Simchen 1978), and it is thought to regulate cell cycle progression through the splicing of *ANC1* (Dahan *et al.* 2004). Mutants of other splicing factor genes, such as *PRP22, PRP16, PRP8, SYF1,* and *SYF3*, have also been shown to cause cell cycle progression defects (Hwang and Murray 1997; Biggins *et al.* 2001; Shea *et al.* 1994; Russell *et al.* 2000).

We propose a simple genetic model, with respect to the first hypothesis, to explain how pre-mRNA splicing may regulate *C. elegans* meiotic entry and germline sex determination (Figure 6). Meiotic entry pathways, such as the GLD-1 pathway, require efficient splicing of its associated genes to function properly. We propose that splicing factors positively regulate genes in the GLD-1 pathway to promote initiation of meiotic development (Figure 6A). *prp-17* and *mog* mutants, as well as RNAi of splicing factor genes, reduces the activity of the GLD-1 pathway. However, because of redundancy with the GLD-2 pathway an overproliferation phenotype is not observed unless a sensitized background is employed. Splicing *per se* also functions in the sperm-oocyte switch in *C. elegans* sex determination. Work from Gallegos *et al* (1998) suggests that the *mog* genes are required for translational repression of the *fem-3* 3′ UTR, however the mechanism of this repression is unclear. As of yet, there is no evidence for a direct interaction between the general splicing MOG proteins and the mature *fem-3* mRNA. Therefore, it is possible that PRP-17, the MOGs, and other splicing factors indirectly regulate *fem-3* mRNA translation through splicing of direct translational repressors of *fem-3* (Figure 6B). Depletion of these splicing factors therefore leads to inefficient splicing of these repressors, which ultimately leads to inappropriate translation of *fem-3* mRNA. These models shed new light on the control of *C. elegans* germline development and an important next step is to identify the gene products that are preferentially affected by disruption of the splicing pathway.

EXPERIMENTAL PROCEDURES

General Methods and Strains

Standard methods were used to maintain and manipulate *Caenorhabditis elegans* (Brenner 1974). All strains were derived from the wild-type *C. elegans var. Bristol* strain N2 (Brenner 1974) with the exception of the natural isolate CB4856 (Hodgkin and Doniach, 1997). Experiments were conducted at 20° unless otherwise noted. Mutant combinations were constructed using standard methods and germline phenotypes analyzed by Nomarski microcopy, staining of dissected gonads (Jones et al. 1996) with DAPI and identification of proliferative germ cells by nucleoplasmic staining with the anti-REC-8 antibody (kindly provided by Joseph Loidl; Paierbek *et al.* 2001) and meiotic prophase germ cells by chromosome axis staining with the anti-HIM-3 antibody (kindly provided by Monique Zetka; Zetka *et al.* 1999).

Enhancer screen

oz273 was isolated in a screen for tumorous enhancers of a weak *glp- 1* gain-of-function allele, *oz264. glp-1(oz264)* was originally isolated as a tumorous enhancer of *teg-1(oz230)* at 20° (Kerins 2006). In a *teg-1(+)* genetic background, *glp-1(oz264)* homozygous hermaphrodites display late-onset tumorous and proximal proliferation phenotypes at 25°, but appear wild-type at both 20° and 15° (Kerins 2006), similar to previously characterized weak *glp-1(gf)* alleles (Pepper *et al*. 2003). The *oz264* molecular lesion is a G to A nucleotide change, which in turn causes a single amino acid alteration (G to E) at position 528 in GLP-1 (WP CE00237, WormBase.org). This amino acid is the first residue in the second LNG repeat of the extracellular portion of the GLP-1 protein, adjacent to another weak *glp-1(gf)* mutation, *ar202* (G529E) (Pepper *et al*. 2003). We used the *glp-1(oz264)* strain at 20° to identify recessive tumorous enhancers. Fourth larval stage (L4) *glp-1(oz264)* hermaphrodites were mutagenized with a final concentration of 62.5mM EMS for 4 hours at 20°, placed on NGM plates and allowed to self at 15°. Approximately one week later, L4 hermaphrodites were cloned to NGM plates at 15°. 6–10 days later, F2 progeny were screened for tumorous phenotypes. About 500 haploid genomes were screened.

Mapping

Three-factor mapping—Standard mapping techniques were used to delineate the genetic location of *oz273* relative to *lin-11(n566) unc-75(e950)* and *unc-29(e193) dpy-24(s71)*. The Mog phenotype was genetically mapped between *dpy-24* (4.73 cM) and *unc-75* (9.30 cM) on LG I.

SNP mapping—We further localized the position of the *oz273* mutation relative to SNPs. Most SNP mapping was performed with SNPs listed in the Washington University Genome Sequencing Center *C. elegans* SNP Database (Wicks *et al*. 2001). Balanced heterozygotes with the *oz273* mutation marked in cis with either *dpy-24(s71)* (genotype: *dpy-24(s71) oz273/hT2::GFP[qIs48]*) or *unc-75(e950)* (genotype: *oz273 unc-75(e950)/ hT2::GFP[qIs48]*) were mated to males from the polymorphic CB4856 (Hawaiian) strain. Non-GFP hermaphrodites were selected to individual plates and allowed to self-fertilize. Among the F2 progeny, Dpy non-Mog or Unc non-Mog recombinant animals were recovered, allowed to self-fertilize, and then individual Dpy non-Mog or Unc non-Mog fertile animals were selected until a homozygous line was obtained. Genomic DNA was prepared for each recombinant, as well as for both the N2 and Hawaiian strains, and PCR amplified. 1 kb regions of sequence surrounding each SNP used were amplified with primers from the database, or primers designed independently. The resultant PCR products were tested for the presence of N2 or Hawaiian DNA at each SNP by either sequence analysis or restriction digests. Sequencing intergenic regions between candidate genes identified one additional SNP, *ozP1*, which is a G to T base substitution in CB4856 at position 10952238 (or 2297 within cosmid Y95D11A) on LG I. The *oz273* mutation lies in a 19.9 kb region to the right of SNP F49D11(18106) and to the left of *ozP1*.

RNAi and sequencing

Gene identification—We used WormBase to search for candidate genes within the ~20 kb region on LG I determined by SNP mapping. Each candidate was tested with feeding RNAi (Timmons and Fire 1998) using the Ahringer RNAi bacteria library (Fraser *et al*. 2000; Kamath and Ahringer 2003). Each RNAi colony was grown overnight at 30° in 2 mL LB with 100μg/mL of ampicillin and 10μg/mL of tetracycline. Cultures were diluted 1:50 in the same medium, and grown 6 hours at 37°, or overnight at 30°, and then seeded onto agar plates containing NGM, 50μg/mL of ampicilin, and 1 mg/mL of β-lactose. The RNAi bacteria were induced at room temperature for 3 days for dsRNA expression. 3–5

synchronized L4 hermaphrodites of the genotypes *rrf-1(pk1417)*, *rrf-1(pk1417);glp-1(oz264)*, and N2 were placed on individual plates and allowed to lay eggs at 20°. Parental animals were removed the next day, and the F_1 generation was scored for a Mog phenotype in *rrf-1(pk1417)* and N2 animals, and a tumorous phenotype in *rrf-1(pk1417);glp-1(oz264)* animals. Animals of the same genotypes feeding on GFP RNAi were used as a negative control. *ozIs2[gld-1::gfp::FLAG(+)];gld-1(q485)* (Schumacher *et al.* 2005) animals feeding on GFP RNAi were used as a positive control for dsRNA induction. The positive candidate, F49D11.1, was sequenced in *oz273* mutant animals to determine the molecular lesion.

RNA interference screen of *C. elegans* splicing factors—A list of *C. elegans* splicing factor orthologs was compiled using the Proteome Bioknowledge® Library (Costanzo *et al*. 2001) based on a list of known yeast and human splicing factors (Jurica and Moore 2003). Using the RNAi feeding bacteria libraries from both Marc Vidal and Julie Ahringer (Rual *et al* 2004; Fraser *et al*. 2000; Kamath and Ahringer 2003), bacteria was grown and dsRNA expression was induced as described above. Three gravid adults of the genotypes *rrf-1(pk1417)*, *rrf-1(pk1417); glp-1(oz264)*, and *rrf-1(pk1417); gld-3(q730)/ mIn1[dpy-10(e128)mIs14]* were placed on individual plates and allowed to lay eggs at 20°. Parental animals were removed the next day, and the F_1 (note: this is essentially a P_0 feeding experiment, since maternal gene activity is not being RNAi depleted) generation was scored two days past L4 stage for a Mog phenotype in *rrf-1(pk1417)* animals, and a tumorous phenotype in *rrf-1(pk1417); glp-1(oz264)* and *rrf-1(pk1417); gld-3(q730)* animals. The same three genotypes were also fed GFP RNAi bacteria as a negative control. The same positive control for dsRNA induction described above was used. Statistical analysis (z-score test) was used to determine which splicing factors showed greater enhancement of *rrf-1(pk1417); glp-1(oz264)* than the GFP control.

Note: From 12 independent RNAi experiments, *rrf-1(pk1417); glp-1(oz264)* hermaphrodites exhibited, on average, 15.8% overproliferation in the presence of GFP RNAi (See Table 2 legend). However, *rrf-1(+); glp-1(oz264)* hermaphrodites feeding on OP50 bacteria display 0% overproliferation (n = 104 arms, scored 2 days past L4). Therefore it appears that *rrf-1* may affect the sensitized *glp-1(oz264)* genetic background. *rrf-1* does not affect the *gld-3(q730); GFP(RNAi)* phenotype. This apparent interaction between *rrf-1* and *glp-1(oz264)* has yet to be further investigated; however, the effect of *rrf-1(pk1417)* on our RNAi screen most likely contributes to an underestimate of the number of splicing factors that enhance *glp-1(oz264)*.

Yeast complementation assay—To make pG1-HACeprp-17, full length *prp-17* cDNA was amplified from N2 cDNA using primers designed to create a BamHI restriction site, start codon, an HA epitope tag 5′ to the coding sequence, and a SalI restriction site 3′ to the coding sequence. The PCR product was then cloned into the yeast expression vector, pG1, which contains the yeast glyceraldehyde-3-phosphate dehydrogenase promoter and the yeast *TRP1* gene (Schena *et al.* 1991). The subsequent ligations were transformed into One ShotR TOP10 (Invitrogen) competent cells. The presence of the *prp-17* insert in individual transformants were verified by restriction digest and sequencing of the *prp-17* coding region.

Control, pG1, and Prp17 expressing plasmids, pG1-HACeprp-17 (worm), pG1-HAyprp17 (yeast), and pG1-HAhprp17 (human) (Lindsey and Garcia-Blanco 1998) were individually transformed into the yeast strain *prp17*ΔBJ2168 (Lindsey and Garcia-Blanco 1998) and plated onto medium lacking tryptophan at 25° for 3–4 days. Individual colonies were picked and streaked onto new Trp^- plates and incubated at 25° for 4 days. Yeast from these plates were grown in 2 mL of Trp^- liquid medium for 4 days, their OD_{600} was normalized to 1.0,

and 3μL of 10-fold serial dilutions were plated onto Trp^- plates at 25°, 30°, and 37°. Pictures were taken 3–6 days later at each temperature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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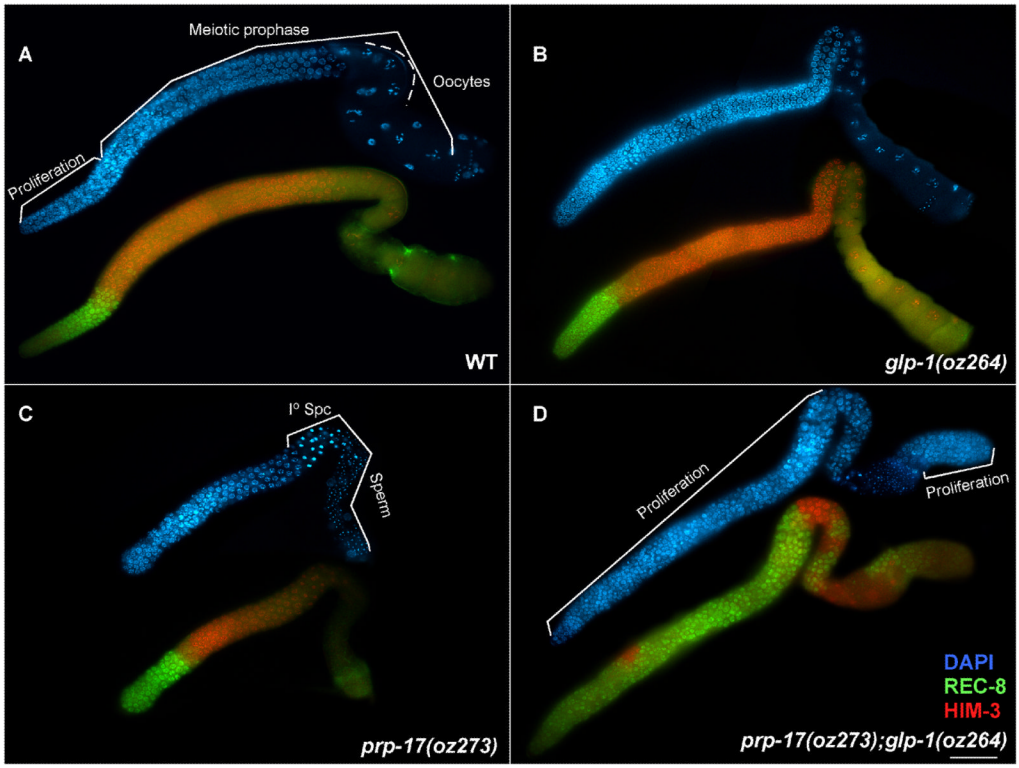

Figure 1. The *prp-17(oz273)* phenotype

Fluorescent images of single adult hermaphrodite gonad arms that have been dissected away from the body. The top image in each panel depicts DAPI staining (blue) to visualize DNA morphology. The bottom image shows the proliferative cell nuclei (green) detected with antibodies specific to REC-8, while meiotic prophase nuclei (red) are detected with HIM-3 (see Hansen *et al*. 2004b). Animals were grown continuously at 20°C. Distal end is to the left. Scale bar = 20 μm. (A) Wild-type hermaphrodite. The ~20 cell diameter proliferative zone consists of REC-8-positive, HIM-3-negative germ cells, whereas cells that have entered meiotic prophase are HIM-3-positive, REC-8-negative. Proliferation, meiotic prophase, the loop region (where gametogenesis begins, dotted line), and oocytes are labeled. (B) The *glp-1(oz264)* hermaphrodite at 20° appears wild-type. (C) *prp-17(oz273)* mutants are Mog, characterized by an absence of oocytes and a vast excess sperm in the proximal gonad and 1° spermatocytes (Spc) in the loop region and extending into the distal gonad. (D) *prp-17(oz273);glp-1(oz264)* hermaphrodite has a tumorous germline, with REC-8-positive cells throughout most of the gonad.

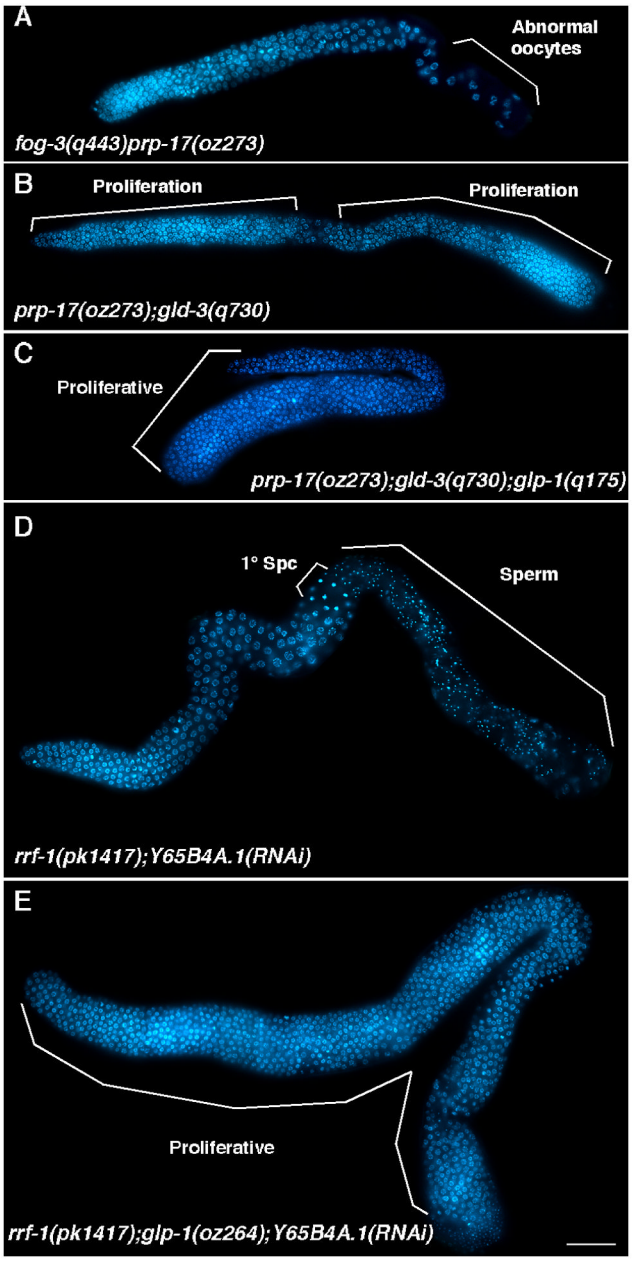

Figure 2. *prp-17* and HTATSF1 function in meiotic entry and sex determination

Fluorescent images of single adult hermaphrodite gonad arms that have been dissected away from the body and stained with DAPI to visualize DNA morphology. Distal end is to the left. Scale bar = 20 μm. (A) The *fog-3(q443) prp-17(oz273)* hermaphrodite does not make sperm, but does make oocytes (shown in proximal region), although abnormal. (B) *prp-17(oz273); gld-3(q730)* hermaphrodites show a late-onset tumor and proximal proliferation, indicated by DAPI staining (regions of proliferation are noted, based on REC-8/HIM-3 staining, which is not shown). (Actual genotype: *prp-17(oz273); gld-3(q730); unc-32(e189)*) (C) *prp-17(oz273); gld-3(q730); glp-1(q175)* hermaphrodites are tumorous, showing proliferating cells throughout the germline. (Actual genotype: *prp-17(oz273); gld-3(q730); unc-32(e189) glp-1(q175)*) (D) and (E) Y65B4A.1*(RNAi)* (human ortholog: HTATSF1) phenocopies *prp-17(oz273)* in both *glp-1(+)* and *glp-1(oz264)*

genetic backgrounds. (D) *rrf-1(pk1417);* Y65B4A.1*(RNAi)* hermaphrodites are Mog, exhibiting excess sperm, 1° spermatocytes (Spc) and no oocytes. (E) *rrf-1(pk1417); glp-1(oz264);* Y65B4A.1*(RNAi)* animals show overproliferation throughout the germline.

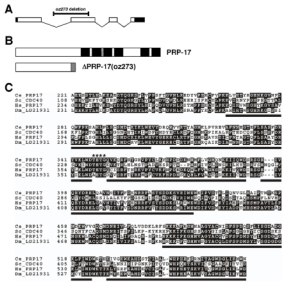

Figure 3. The *prp-17* gene and gene product

(A) The *prp-17* message contains 4 exons (boxes). 5′ and 3′ UTRs are shown in black. The *oz273* mutation deletes the first 521 base pairs of exon 2. (B) Top: the predicted PRP-17 protein contains 567 amino acids. The main functional motifs, six WD-40 repeats, are shown in black. The hatched box indicates a 4-amino acid motif shown to be essential for splicing in yeast (Lindsey-Boltz et al, 2000) (see C). Bottom: the predicted truncated protein produced in *prp-17(oz273)* mutants. The *oz273* deletion mutation creates a premature STOP codon upstream of all functional motifs. The shaded box indicates aberrant amino acids (and premature STOP codon) created by the predicted cryptic splice site of the mutant. (C) Alignment of the conserved C-terminal 221–567 amino acids of the *C. elegans* PRP-17 protein (accession no. NP_492851.1) with yeast (*S. cerevisiae*, accession no. NP_010652.1), human (accession no. NP_056975.1), and fly (*D. melanogaster*, accession no. NP_651005.1) orthologs. Residues in black are identical in two or more orthologs. WD-40 repeats are underlined. Asterisks denote the 4-amino acid motif described in (B).

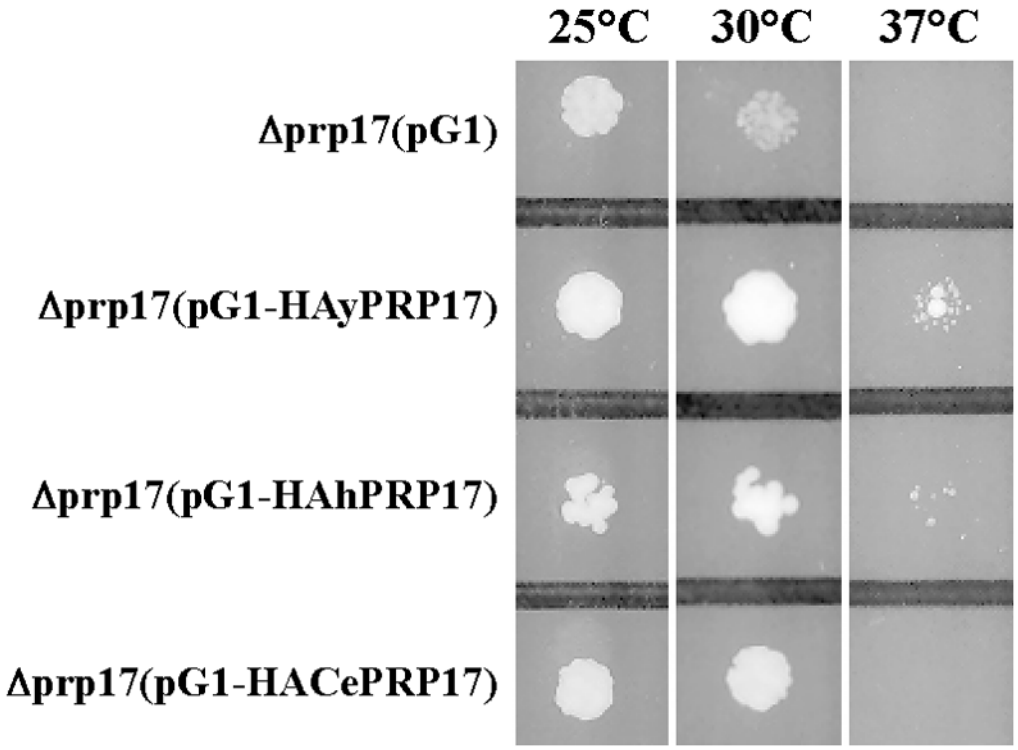

Figure 4. Complementation of a yeast *PRP17* knockout strain

The yeast strain prp17Δ BJ2168 was transformed with the high copy plasmid pG1 alone or with pG1 containing full-length yPRP17, hPRP17, or CePRP17. 10-fold serial dilutions were made of each transformation, which were then grown on medium lacking tryptophan for 3–6 days at 25, 30, and 37°C. The figure shows 1:100 dilutions of each transformation grown for 4 days at each temperature.

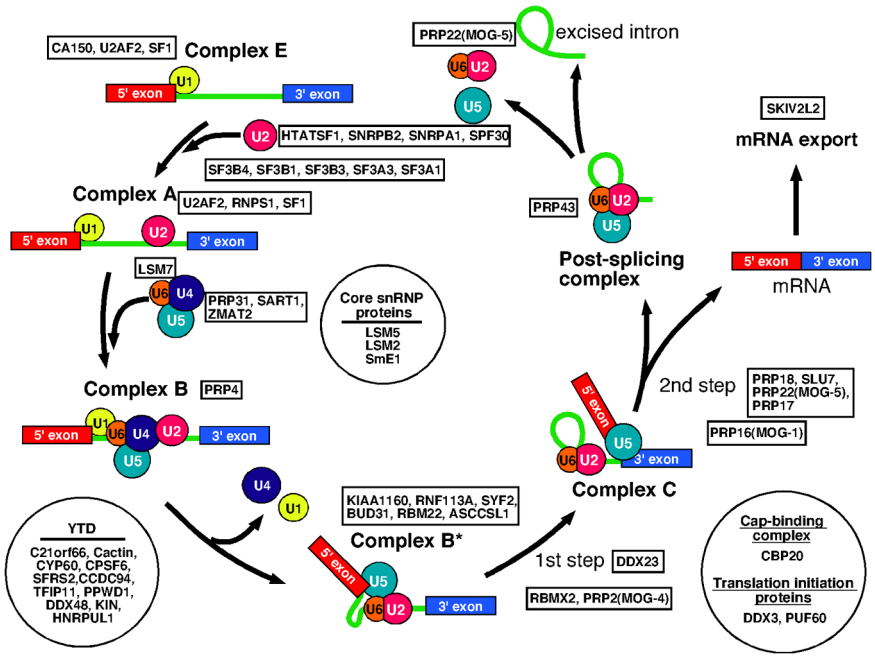

Figure 5. The pre-mRNA splicing pathway and *C. elegans* genes that function in the proliferation/meiotic development decision and germline sex determination

Adapted from Ohi *et al.* 2005. Schematic drawing of the pre-mRNA splicing pathway. The assembly of an activated spliceosome onto a nascent transcript is necessary for pre-mRNA splicing to occur. Assembly begins when U1 and U2 bind to the 5′ and 3′ splice sites, respectively (Complexes E and A). The U5/U4/U6 tri-snRNP then binds (Complex B), followed by rearrangement of the spliceosomal complex which releases U1 and U4 (Complex B*). The first catalytic step of splicing occurs next, resulting in the release of the intron at the 5′ splice site, and subsequent formation of the lariat intermediate (Complex C). The second catalytic step then occurs, completely excising the intron and associated splicing complexes (post-splicing complex), resulting in fully spliced exons, which then undergo further processing and export from the nucleus. The post-splicing complex undergoes disassembly, and the cycle can begin again. The *C. elegans* splicing factor orthologs that display overproliferation and/or Mog phenotypes (Table 2) are listed near the step(s) where the yeast/human proteins have been shown to act (boxes), or listed in circles (to indicate those that function in processes not shown). YTD = the splicing step(s) in which these genes function is yet to be determined.

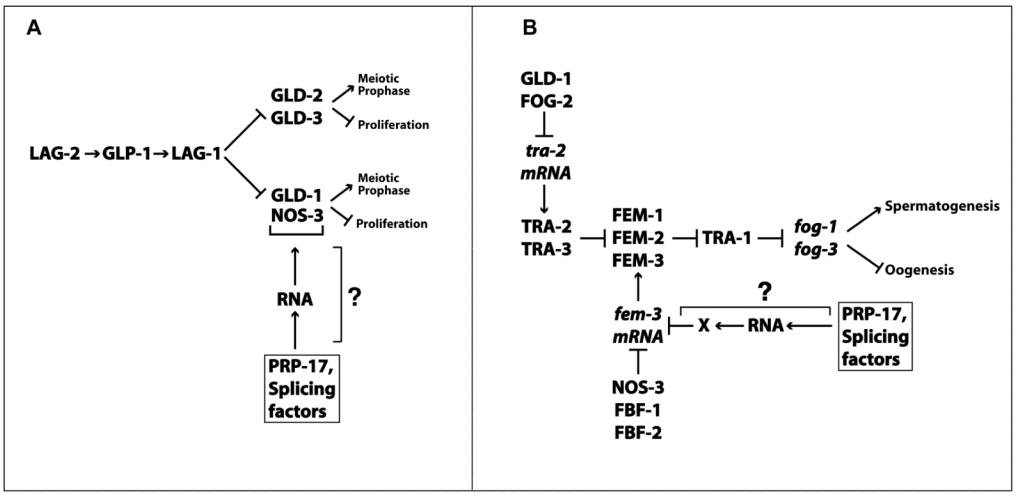

Figure 6. Genetic models for PRP-17 and splicing factors in the *C. elegans* proliferation/meiotic development decision and germline sex determination

Positive interactions are depicted with arrows, while negative interactions are depicted with T-bars. Proteins are shown in capital letters; gene names are shown in lower case italics. (A) The core GLP-1 Notch signaling pathway shown with the downstream GLD-1 and GLD-2 pathways that promote entry into meiosis (Kimble and Crittenden 2005; Hansen and Schedl 2006). PRP-17 and other *C. elegans* splicing factor orthologs promote meiotic development by positively regulating the GLD-1 pathway through the splicing of mRNAs of genes that function in this pathway. Because the relevant direct splicing targets of PRP-17/spliceosome have not yet been identified, we show general positive regulation on the GLD-1 pathway as a whole. (B) The core germline sex determination pathway in *C. elegans* (Ellis and Schedl 2006). In hermaphrodites GLD-1 and FOG-2 act to promote spermatogenesis by repression of *tra-2* mRNA in early larvae; the switch to oogenesis occurs upon repression of *fem-3* mRNA and derepression of *tra-2* mRNA in late larvae. PRP-17 and other splicing factor orthologs may promote the oocyte fate in *C. elegans* germline sex determination via splicing of the mRNA of an unknown gene (or genes) X, which functions as a translational repressor of *fem-3* mRNA during hermaphrodite oogenesis. In (A), PRP-17 and the splicing factors have been placed downstream of GLP-1 signaling as triple mutants of *glp-1* null, *gld-3* null with *prp-17*, *mog-1* or *mog-6* mutations (described here) and *teg-4* (Mantina *et al.* 2009) are tumorous, demonstrating that *glp-1* activity is not required for the overproliferation phenotype. Previous work indicated that the GLD-2 polyA polymerase and the GLD-3 Bicaudal-C related RNA binding protein function together to promote entry into meiosis (Kadyk and Kimble,1998; Wang et al. 2002; Eckmann et al. 2004; Hansen et al. 2004a and b). Therefore, we were surprised to find that the *gld-2(q497) prp-17(oz273); glp-1(q175)* triple mutant is not tumorous, but rather has the *glp-1* premature meiotic entry phenotype, with similar results in triple mutants with *mog-1* and *mog-6* (data not shown) as well as for *teg-4* (Mantina *et al.* 2009). There are other situations where *gld-2* and *gld-3* do not behave identically in the proliferation versus meiosis decision. *gld-3(q730)* has a stronger meiotic entry defect in combination with *gld-1* or *nos-3* null alleles than does *gld-2(q497)* (Eckmann *et al.* 2004; Hansen *et al.* 2004a and b). The genetic behavior of the *pas-5* proteasome subunit shows a similar set of interactions with *gld-2*, *gld-3* and *glp-1* as the splicing factor mutants: the *pas-5; gld-3; glp-1* mutant is tumorous while *gld-2 pas-5; glp-1* mutant shows the *glp-1* premature meiotic entry phenotype (MacDonald *et al.* 2008). How might GLD-2 and GLD-3 function together yet display some different genetic behaviors? One possibility is that GLD-3 may have a separate function, outside of its role with GLD-2, in promoting entry into meiosis, possibly in the proposed third pathway that

acts in parallel to the GLD-1 and GLD-2 pathways (Hansen *et al.* 2004b). Alternatively, although the *gld-2(q497)* allele used these experiments is a stop mutation (Wang *et al.* 2002), it may not fully eliminate *gld-2* activity. In contrast, the *gld-3(q730)* allele is a deletion that likely eliminates all *gld-3* activity. While the reasons for differences in behavior of *gld-2* and *gld-3* remain to be resolved, the finding that the tumorous phenotype of the *gld-3* null with splicing factor mutants is independent of *glp-1* activity strongly supports the proposal that the splicing factors function, at least in part, downstream of GLP-1 signaling.

Table 1

Genetic interactions between *prp-17* and genes that function in the proliferation/meiotic entry decision and germline sex determination[a]

	% gonad arms with indicated phenotype						
Genotype (20°C)	**Wild type (sperm + oocytes)**	**Mog**	**Tum[b]**	**Pro**	**Fog**	**Glp**	**n= [c]**
WT [d]	100	0	0	0	0	0	many
glp-1(oz264)	100	0	0	0	0	0	106
prp-17(oz273) [e]	0	98	0	0	0	0	99
prp-17(oz273); glp-1(oz264) [f]	0	2	97	0	0	0	97
fog-1(q241) prp-17(oz273)	0	0	0	0	99[k]	0	61
fog-3(q443) prp-17(oz273)	0	0	0	0	100	0	67
prp-17(oz273); fem-3(e1996)	0	0	0	0	98[k]	0	53
prp-17(oz273); nos-3(oz231)	0	100	0	0	0	0	69
gld-1(q485) prp-17(oz273) [g] (males)	79	0	6	15	0	0	52
gld-2(q497) prp-17(oz273)	0	0	100	0	0	0	103
prp-17(oz273); gld-3(q730)	0	0	71	29	0	0	51
gld-1(q485) prp-17(oz273); glp-1(q175) [h]	0	0	0	0	0	100	60
gld-2(q497) prp-17(oz273); glp-1(q175) [i]	0	0	0	0	0	100	53
prp-17(oz273); gld-3(q730); glp-1(q175) [j]	0	0	100	0	0	0	21

[a] Animals were grown at 20° and scored by Nomarski (except gld-3-containing strains which were scored by DAPI staining under fluorescent microscopy) one day past L4 stage. Overproliferation phenotypes were confirmed, but not quantitated, by REC-8 and HIM-3 staining.

[b] Tumorous = both late-onset tumorous and fully tumorous

[c] n = number of gonad arms

[d] WT is both N2 and *unc-32(e189)*.

[e] 2% of animals show a small, sickly germline phenotype.

[f] 1% of animals show an abnormal gonad development phenotype

[g] Males were examined; WT phenotype indicates normal male germline development

[h] Actual genotype = *gld-1(q485) prp-17(oz273); unc-32(e189) glp-1(q175null)*

[i] Actual genotype = *gld-2(q497) prp-17(oz273); unc-32(e189) glp-1(q175null)*

[j] Actual genotype = *prp-17(oz273); gld-3(q730); unc-32(e189) glp-1(q175null)*

[k] Remaining 1 or 2% of gonads have small germlines with little differentiation.

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Table 2

C. elegans splicing factor orthologs that show meiotic entry and sex determination phenotypes by RNAi

Observed RNAi phenotype(s)	Predicted gene[a]	*C. elegans* locus	Yeast[b]	Human[c]	Splicing step/ general process[d]	% Overproliferation w/ *rrf-1;glp-1(oz264)* (n) *e*	% Overproliferation with *rrf-1;gld-3* (n)	% Mog with *rrf-1* (n) *f*	Experiment # *g*
Enhances *glp- 1(oz264)*, Synthetic tumor with *rrf-1;gld- 3(0)*, Mog with *rrf- 1*	F26A3.2		Cbc2	CBP20	Cap-binding complex	41.5 (94)	25.9 (27)	1.1 (95)	3
	Y65B4A.1		Cus2	HTATSF1	U2	67.3 (98)	54.1 (74)	1.2 (85)	2
	C08B11.5	*sap-49*	Hsh49	SF3B4	U2, Complex Δ	46.7 (90)	77.3 (44)	5.3 (94)	2
	W08D2.7		Mtr4	SKIV2L2	mRNA export	31.6 (95)	17.2 (87)	2.1 (95)	1
	F43G9.12			C21orf66	YTD	33.3 (93)	63.8 (58)	1.0 (97)	1
	W03H9.4			Cactin	YTD	25 (100)	75.0 (40)	31.9 (72)	3
Enhances *glp- 1(oz264)*, Synthetic tumor with *rrf-1;gld- 3(0)*	F28F8.3	*lsm-5*	Lsm5	LSM5	Core snRNP protein	49.5 (99)	17.5 (40)	0.0 (108)	4
	T10G3.6	*gut-2*	Lsm2	LSM2	Core snRNP protein	46.8 (94)	48.1 (54)	0.0 (104)	8
	K08D10.4	*rnp-2*	Msl1	SNRPB2	U2	25.5 (94)	46.4 (56)	0.0 (98)	5
	T08A11.2		Hsh155	SF3B1	U2, Complex A	26.5 (98)	57.4 (54)	0.0 (105)	2
	K02F2.3	*teg-4*	Rse1	SAP130	U2, Complex A	28.3 (99)	1.5 (68)	0.0 (86)	3
	ZK593.7	*lsm-7*	Lsm7	LSM7	U6	80 (100)	81.6 (76)	0.0 (100)	4
	Y110A7A.8		Prp31	PRP31	U4/U6-U5	48.9 (90)	42.1 (38)	0.0 (92)	12
	F19F10.9		Snu66	SART1	U4/U6-U5	31.3 (96)	16.0 (50)	0.0 (96)	1
	C36B1.5		Prp4	PRP4	Complex B	44 (91)	53.1 (64)	0.0 (98)	12
	F53B7.3		Isy1	KIAA1160	Complex B*	39 (100)	82.6 (92)	0.0 (106)	12
	K01G5.1		Cwc24	RNF113A	Complex B*	42.7 (96)	14.1 (85)	0.0 (102)	7
	K04G7.11		Syf2	SYF2	Complex B*	34.3 (102)	27.1 (96)	0.0 (101)	8
	F01F1.7	*ddx-23*	Prp28	DDX23	1st step	56.6 (99)	28.8 (59)	0.0 (100)	4
	F32B6.3		Prp18	PRP18	2nd step	38.9 (95)	6.3 (64)	0.0 (95)	1
	C07E3.1		Spp382	TFIP11	YTD	19.6 (97)	1.1 (94)	0.0 (102)	7
	Y87G2A.6	*cyp-15*	Cpr3	PPWD1	YTD	20.8 (96)	1.3 (75)	0.0 (98)	7
	Y65B4A.6		Fal1	DDX48	YTD	34.5 (110)	34.1 (41)	0.0 (103)	7
	Y52B11A.9		Rts2	KIN	YTD	42.9 (105)	8.6 (58)	0.0 (100)	6
Enhances *glp-1(oz264)*	ZK1127.9		Prp40	CA150	Complex E	87.5 (96)	0.0 (46)	0.0 (104)	4

Observed RNAi phenotype(s)	Predicted gene[a]	*C. elegans* locus	Yeast[b]	Human[c]	Splicing step/ general process[d]	% Overproliferation w/ *rrf-1;glp-1(oz264)* (n) *e*	% Overproliferation with *rrf-1;gld-3* (n)	% Mog with *rrf-1* (n) *f*	Experiment # *g*
	K02F3.11	*rnp-5*		RNPS1	Complex A	49.0 (98)	0.0 (73)	0.0 (100)	11
	Y54E10A.9	*vbh-1*	Ded1	DDX3	Translation initiation	40.0 (100)	0.0 (101)	0.0 (88)	10
	D1046.1			CPSF6	YTD	27.6 (87)	0.0 (96)	0.0 (99)	6
	EEED8.7	*rsp-4*	Pub1	SFRS2	YTD	26.3 (99)	0.0 (n/a)	0.0 (n/a)	5
Mog with *rrf-1*, Synthetic tumor with *rrf-1;gld- 3(0)*	F56D2.6		Prp43	PRP43	Post splicing complex	ns (98)	78.7 (47)	12.2 (90)	5
	Y47G6A.20	*rnp-6*	Pab1	PUF60	Translation initiation	ns (83)	54.4 (57)	1.1 (93)	8
	F37C12.1		Yju2	CCDC94	YTD	ns (104)	5.8 (69)	1.0 (102)	10
Mog with *rrf-1*	Y92C3B.2	*uaf-1*		U2AF2	Complex E, Complex A	ns (91)	0 (50)	1.6 (64)	2
	C07A9.2		Cwc14	BUD31	Complex B*	ns (102)	0.0 (54)	1.2 (84)	10
Synthetic tumor with *rrf-1;gld- 3(0)*	Y49E10.15	*snr-6*	Sme1	SmE1	Core snRNP protein	ns (99)	16.7 (48)	0.0 (100)	1
	Y116A8C.32		Msl5	SF1	Complex E, Complex A	ns (106)	14.4 (83)	0.0 (97)	8
	Y71D11A.2	*smr-1*		SPF30	U2	ns (100)	12.2 (82)	0.0 (90)	12
	T13H5.4		Prp9	SF3A3	U2, Complex A	ns (106)	55.2 (67)	0.0 (97)	11
	W07E6.4	*prp-21*	Prp21	SF3A1	U2, Complex A	ns (93)	45.2 (62)	0.0 (100)	11
	ZK686.4		Snu23	ZMAT2	U4/U6-U5	ns (106)	17.1 (47)	0.0 (103)	2
	T11G6.8		Ecm2	RBM22	Complex B*	ns (93)	37.5 (48)	0.0 (93)	1
	Y46G5A.4	*phi-10*	Prp44	ASCCSL1	Complex B*	ns (102)	2.4 (42)	0.0 (91)	3
	C30B5.4		Ist3	RBMX2	Before 1st step	ns (96)	5.3 (76)	0.0 (102)	11
	K07C5.6		Slu7	SLU7	2nd step	ns (100)	16.0 (81)	0.0 (95)	10
	Y41E3.11		Sec31	HNRPUL1	YTD	ns (100)	9.7 (72)	0.0 (102)	9
Controls [h]	C04H5.6	*mog-4*	Prp2	PRP2	Before 1st step	48 (102)	50 (34)	12.9 (101)	5
	K03H1.2	*mog-1*	Prp16	PRP16	2nd step	33.7 (95)	9.6 (94)	0.0 (101)	1
	EEED8.5	*mog-5*	Prp22	PRP22	2nd step, s.d. [i]	31.9 (94)	0.0 (34)	0.0 (86)	1
	F59E10.2	*mog-6*		CYP60	YTD	39.6 (96)	0.0 (100)	0.0 (108)	9

*a, b, c*The predicted C. elegans orthologs were found using the Proteome Bioknowledge Library (Costanzo et al. 2001; Hodges et al. 2002), based on a list of known yeast and human splicing factors from Jurica and Moore 2003.

[d] The splicing step, complex, or general process in which each predicted gene is known to be involved is listed (see Krämer 1996; Jurica and Moore 2003; Wahl et al. 2009 for reviews). YTD: role in splicing is yet to be determined.

[e] A z-score test was used to determine if enhancement of rrf-1; glp-1(oz264) was significantly different than that of the GFP control. P-values less than 0.05 at a 95% confidence interval were considered significant. RNAi of genes that did not significantly enhance rrf-1; glp-1(oz264) are designated with "ns"- not significant.

[f] The Mog phenotype, a vast excess of sperm in the proximal region and 1° spermatocytes in the distal region, is never observed in wild-type or control RNAi hermaphrodites. Thus, though the Mog phenotype was observed at a low frequency, we believe that it is a significant result.

[g] Genes that were tested in the same experiment are designated with an experiment number. The values for the prp-17 and GFP controls and number of animals scored () for each experiment are as follows:

Experiment 1: rrf-1; glp-1(oz264); GFP(RNAi) showed 16.5% (91) overproliferation; rrf-1;glp-1(oz264); prp-17(RNAi) showed 23.1% (91) - not significant

Experiment 2: rrf-1; glp-1(oz264); GFP(RNAi) showed 12.5% (72) overproliferation; rrf-1; glp-1(oz264); prp-17(RNAi) showed 17.6% (91) - not significant

Experiment 3: rrf-1; glp-1(oz264); GFP(RNAi) showed 11.5% (104) overproliferation; rrf-1; glp-1(oz264); prp-17(RNAi) showed 12.0% (100) - not significant

Experiment 4: rrf-1; glp-1(oz264); GFP(RNAi) showed 22.7% (97) overproliferation; rrf-1;glp-1(oz264); prp-17(RNAi) showed 48.3% (89) - significant

Experiment 5: rrf-1; glp-1(oz264); GFP(RNAi) showed 14.9% (101) overproliferation; rrf-1; glp-1(oz264); prp-17(RNAi) showed 24.3% (111) - significant

Experiment 6: rrf-1; glp-1(oz264); GFP(RNAi) showed 16.5% (97) overproliferation; rrf-1; glp-1(oz264); prp-17(RNAi) showed 22.1% (95) - not significant

Experiment 7: rrf-1; glp-1(oz264); GFP(RNAi) showed 10.0% (100) overproliferation; rrf-1; glp-1(oz264); prp-17(RNAi) showed 22.9% (96) - significant

Experiment 8: rrf-1; glp-1(oz264); GFP(RNAi) showed 23.0% (100) overproliferation; rrf-1;glp-1(oz264); prp-17(RNAi) showed 26.0% (100) - not significant

Experiment 9: rrf-1; glp-1(oz264); GFP(RNAi) showed 21.9% (96) overproliferation; rrf-1; glp-1(oz264); prp-17(RNAi) showed 25.2% (95) - not significant

Experiment 10: rrf-1; glp-1(oz264); GFP(RNAi) showed 7.0% (100) overproliferation; rrf-1; glp-1(oz264); prp-17(RNAi) showed 11.8% (102) - not significant

Experiment 11: rrf-1; glp-1(oz264); GFP(RNAi) showed 26.2% (103)overproliferation; rrf-1; glp-1(oz264); prp-17(RNAi) showed 28.9% (104) - not significant

Experiment 12: rrf-1; glp-1(oz264); GFP(RNAi) showed 13.1% (99) overproliferation; rrf-1;glp-1(oz264); prp-17(RNAi) showed 23.3% (90) - significant

[h] Controls should enhance rrf-1 ;glp-1(oz264), form synthetic tumors with rrf-1;gld-3(0), and cause a Mog phenotype with rrf-1.

[i] s.d. = spliceosome disassembly