Role of the *C. elegans* U2 snRNP protein MOG-2 in sex determination, meiosis, and splice site selection

Simone Zanetti, Marco Meola, Arlette Bochud, Alessandro Puoti *

*Department of Biology, University of Fribourg, Switzerland*

In *Caenorhabditis elegans*, germ cells develop as spermatids in the larva and as oocytes in the adult. Such fundamentally different gametes are produced through a fine-tuned balance between feminizing and masculinizing genes. For example, the switch to oogenesis requires repression of the *fem-3* mRNA through the *mog* genes. Here we report on the cloning and characterization of the sex determination gene *mog-2*. MOG-2 is the worm homolog of spliceosomal protein U2A. We found that MOG-2 is expressed in most nuclei of somatic and germ cells. In addition to its role in sex determination, *mog-2* is required for meiosis. Moreover, MOG-2 binds to U2B′RNP-3 in the absence of RNA. We also show that MOG-2 associates with the U2 snRNA in the absence of RNP-3. Therefore, we propose that MOG-2 is a bona fide component of the U2 snRNP. Albeit not being required for general pre-mRNA splicing, MOG-2 increases the splicing efficiency to a cryptic splice site that is located at the 5′ end of the exon.

**Introduction**

How does a germ cell decide between mitotic proliferation and entry into meiosis? What are the factors that are necessary to differentiate a germ cell as a sperm or an oocyte? Such fundamental questions are addressed in the protandric nematode *Caenorhabditis elegans*. Genes involved in germline sex determination have been identified through genetic screens for animals that were defective in gamete production. For example, *fem-3 loss-of-function (lf) alleles lead to feminized hermaphrodites that omit spermatogenesis (Hodgkin, 1976).* Genes involved in germline sex determination have been identified through genetic screens for animals that were defective in gamete production. For example, *fem-3 loss-of-function (lf) alleles lead to feminized hermaphrodites that omit spermatogenesis (Hodgkin, 1986).* In contrast, *fem-3 gain-of-function (gf) mutant hermaphrodites continue spermatogenesis throughout their entire lives (Barton et al., 1987).* In the latter, post-transcriptional repression of the *fem-3* mRNA is abolished through mutations in the 3′ untranslated region (3′ UTR; Ahrlinger and Kimble, 1991). The *fem-3* mRNA is repressed through a number of regulatory proteins, among which the FBF and the MOG proteins are the most prominent examples (Graham and Kimble, 1993; Zhang et al., 1997). The Puf proteins FBF-1 and FBF-2 bind directly to a regulatory region of the *fem-3* 3′ UTR (Zhang et al., 1997). Although all *mog* genes cloned to date code for proteins that are homologous to splicing factors, splicing defects have not been found in the respective mutants (Belfiore et al., 2004; Kasturi et al., 2010; Puoti and Kimble, 1999, 2000).

RNA splicing is a key event in the processing of the primary transcript to mRNA. Splicing takes place in the spliceosome, a large dynamic nuclear complex consisting of five snRNAs, snRNP proteins, and non-snRNP proteins (Moore et al., 1993). U1, U2, U4/U6 and U5 snRNAs play crucial roles in RNA–RNA interactions with the pre-mRNA (Konarska and Sharp, 1987). While U1 snRNP binds to the 5′ splice site of the intron, the U2 snRNP associates with the branchpoint (Black et al., 1985). In humans, U1 snRNA binds to the snRNP protein U1A, while U2 snRNA interacts with the snRNP protein U2B′ (Scherly et al., 1990b). In vertebrates, specific binding of U2B′ to U2 snRNA requires yet another RNA-binding protein, U2A′ (Mattaj et al., 1986; Scherly et al., 1990b). Splicing is essentially identical in *C. elegans* and in vertebrates, but several differences need to be mentioned. First, as in other free-living nematodes, *C. elegans* introns are much shorter than in vertebrates, with a median length between 48 and 52 nucleotides (Blumenthal and Steward, 1997). Second, although *C. elegans* introns almost exclusively start with a GU and end with an AG dinucleotide, no branchpoint consensus and no obvious polypyrimidine tract have been identified (Blumenthal and Steward, 1997). Finally, in contrast to human U2B′, the worm homolog RNP-3 binds specifically to U2 snRNA without requiring the *C. elegans* ortholog of U2A′, also known as SAP-1 (spliceosomal-AI-ide Protein 1; Saldi et al., 2007).

In this study, we report the cloning and the initial characterization of the *mog-2* gene. *mog-2* encodes SAP-1, the worm homolog of U2A′ (Caspary and Seraphin, 1998; MacMorris et al., 2003; Polycarpou-Schwarz et al., 1996). We show that MOG-2/SAP-1 functions in splicing and that it is synthetically required for meiosis. Furthermore, we show that MOG-2...
binds to RNP-3 and to U2 snRNA in vivo. Finally, we identify a motif in MOG-2 that is crucial for RNP-3 binding and possibly for U2 snRNA binding and sex determination.

Materials and Methods

Strains

All strains were maintained by standard procedures at 20 °C unless specified. Deletion alleles *sac*-1(ok1221)/mln1(dpy-10(e128)mln1) and *rnp-3*(ok1424) are from the CGC. *gld-2*(q497); *mog-2*(ok1221) double mutants are from strain *gld-2*(q497);*ht2[qls48](I);*mog-2*(ok1221) II and *mog-2*(q75) *gld-3*(q730) double mutant came from *lin-31*(n301) *mog-2*(q75) *gld-3*(q730);*mln1(dp-10(e128)mln1). Pre-

mRNA processing and decay were analyzed in *smg-1*(r861) II, *mog-2*(q75) II, *mog-2*(ok1221) II, *smg-1*(r861) II, *mog-2*(q75) II;*rnp-3*(ok1424)*/nt1q[glq51](IV);* animals. *mln1, h72* and *nt1q* are semi-dominant fluorescent markers. Other genotypes used in 3' splice site recognition: *dpy-10(e128) II, smg-1*(r861) II, *dpy-10(e128) II, *mog-2*(q75) *gld-3*(q730) II, *unc-4*(e120)*dpy-10(e128) II, *smg-1*(r861) II; *mog-2*(q75) *gld-3*(q730) II, *dpy-10(e128) II; *em-3*(q95) III; *mog-6*(q465) *dpy-10(e128) II was obtained from *mog-6*(q465) *dpy-10(e128) II; *mln1* and *mog-5*(q449)*dpy-10(e128) II* is from *mog-5*(q449) *dpy-10(e128) II; *unc-85* (e1414) II.

Cloning of *mog-2* and accession number

*mog-2* has been mapped on chromosome II, between *lin-31* and *unc-85* (Graham et al., 1993). We further mapped *mog-2* by analyzing recombinants between *mog-2* and two single nucleotide polymorphisms in cosmids R03H10 (position 9489) and ZK430 (position 13198). By sequencing candidate genes, we found that ORF H20J04.8 has been assigned to *mog-2* cDNA sequence data.

Phenotype analyses

Fertile hermaphrodites were allowed to lay eggs at the appropriate temperature for 4 h. Developmental stages were determined by the size of the germ line. Plates were scored for dead eggs 30 h after being laid and sterile adults were observed 24 h after the vulva had formed. For sperm counting, worms were grown at 15 °C until L4 and then switched to 25 °C. To avoid larval arrest at 25 °C, we used homozygous siblings of *mog-2*/*mln1* heterozygotes. Sperms were identified by their nuclear morphology upon staining with DAPI (4,6-diamino-2-phenylindole).

RNA interference

Sense and antisense RNA corresponding to the entire coding region of *mog-2* were generated using T3 and T7 RNA polymerases (Stratagene). RNA interference was performed by injecting dsRNA (0.5 μg/μl) into either young N2 or *gld-3*(q730)/*mln1* adults. Worms were shifted from 20 °C to 25 °C 12 h post injection. *mog-2*(q75) and *mog-2*(q75);*rnp-3*(ok1424) hermaphrodites were grown at 20 °C on HT115 bacteria producing gld-1, nos-3 or *rnp-2* double-stranded RNA. L3-L4 larvae were then switched to 25 °C. Adults were scored for germline defects by DAPI staining.

MOG-2 antibodies and immunostaining

Polyclonal antibodies were produced against Glutathione-S-Transf erase (GST)-tagged MOG-2. For Western blotting, approxi mately 100 μg of protein extracted from adult worms were loaded. Equal loading was verified by staining with Ponceau S or with α-Tubulin antibodies (1:2000, Sigma). Blots were incubated in Blotto/ Tween with anti-MOG-2 antibodies (1:800). Secondary HRP IgG conjugates (Sigma) were used at a dilution of 1/25,000.

For immunostaining, anti-MOG-2 (1:100), anti-GLD-1 (1:100), anti-UAF-1 (1:75) and anti-GLP-1(1:8) antibodies were diluted in PBT, 5% BSA. Worms were fixed with 1.5% formaldehyde (Sigma) in 100 mM phosphate buffer at pH 7.5 followed by cold methanol for 5 °C. FITC or Cy3-conjugated secondary antibodies were used at a dilution of 1:1000 (Jackson ImmunoResearch). Immunostaining with anti-PGL-1 (1:500) or anti-MOG-2 (1:100) was performed on entire larvae as described (Rettinger et al., 1996). All incubations were performed overnight at 4 °C, followed by several washes. Stained worms were mounted with Vectashield containing 2 μg/ml DAPI and observed under fluorescence.

MOG-2 interaction assays and screen

*mog-2*, *rnp-3* and *rnp-2* full-length cDNAs were either introduced into pBTM116 (Bartel and Fields, 1995) to create LexA protein fusions, or into pACTII to make GAL4 activation domain fusion proteins. The plasmid form of a random-primed cDNA library (AACT-RE2) was screened for MOG-2 binding proteins using a MOG-2::LexA fusion construct on minimal medium lacking leucine, tryptophan, and histidine and supplemented with 2.5 mM of 3-aminotriazole. Two-hybrid assays were performed in strain L40. All constructs were sequenced and tested for the presence of the fusion protein by Western blotting using either anti-LexA or anti-GAL4 activation domain antibodies (Upstate Biotechnology).

For in-vitro protein–protein binding assays, full-length *rnp-2* cDNA was cloned into pCITE (Novagen), *'S*Met-labeled RNP-3 protein was produced using TNT-coupled reticulocyte lysate (Promega), *mog-2 (+)* and *mog-2*(q75) cDNAs were introduced into pGEX. Binding assays were performed as described (Belfiore et al., 2004).

For immunoprecipitations, mixed-stage worms were lysed in a mortar in liquid nitrogen, collected with homogenization buffer (10 mM KCl, 1.5 mM MgCl2, 1 mM DTT, 10 mM Tris–HCl pH 8.0, 50 mM sucrose, 0.05% Nonidet P-40) and treated with RNasin (0.025 U/μl). Protein G Dynabeads (Invitrogen) were coupled with anti-MOG-2 antibodies and incubated 1 h at 4 °C with worm extract in IP Buffer (100 mM KCl, 0.1 mM DTT, 20 mM Tris–HCl pH 8.0, 0.1% Nonidet P-40, 0.2 mM EDTA). Beads were washed with 1× PBS and eluted as indicated in the manufacturer’s instructions with 1× NuPAGE LDS sample buffer. Mock and IP were treated with RNase-free DNase (0.05 U/μl), with Proteinase K, and finally incubated 5° at 95 °C. Phenol/chloroform precipitates were analyzed by Northern blotting with a U2 snRNA full-length probe.

Northern analysis and RT-PCR

Poly(A)+ RNA and total RNA were extracted and analyzed as described (Puoti and Kimber, 1999). RT-PCR was performed on total RNA from adult worms (Puoti and Kimber, 1999). For RT-PCR, 1 μg of DNase-treated total RNA and 100 ng of random primers were processed as described (Belfiore et al., 2004). As a positive control we used genomic DNA and random-primed single-stranded cDNAs from wild type animals. Sequences of primers are available on request. After 21 cycles of PCR, *dpy-10(e128)* cDNAs were separated on a 2% agarose gel and probed with a template that equally recognizes both spliced and unspliced variants. Quantification was performed with an Amersham Biosciences Phosphoimager.

Results and discussion

The MOG-2 protein and the Mog-2 mutant phenotype

The *mog-2*(q75) allele was isolated in a screen for recessive sterile mutants (Graham et al., 1993). *mog-2* has been mapped on chromosome
between lin-31 and unc-85 (Graham et al., 1993). We have found that mog-2 corresponds to ORF H20J04.8, which codes for Spliceosomal-Associated Protein SAP-1 (Fig. 1A; MacMorris et al., 2007; Saldi et al., 2007). MOG-2 contains 3 Leucine-rich repeats (LRR;Bairoch, 1991), while its yeast homolog harbors six LRRs (Caspar and Seraphin, 1998). LRRs are implicated in protein–protein interactions (Kobe and Deisenhofer, 1993). The mog-2(q75) allele has a 5-nucleotide deletion at the end of the first exon of ORF H20J04.8. The use of a cryptic splice acceptor site generates a 24-nucleotide in-frame deletion. The predicted mutant MOG-2(q75) protein therefore lacks 8 amino acids (Fig. 1B). Interestingly, the deleted amino acids belong to a most conserved motif (Fig. 1C). We found that the mutant MOG-2(q75) protein is produced at low levels (Fig. 3B,C) and therefore asked whether mog-2(q75) behaved like a genetic null allele. We thus compared the phenotypes of mog-2(q75) with a deletion allele that removes the entire first exon. This allele, sap-1(ok1221), is likely null and named mog-2(ok1221) for simplicity in the rest of this study (Fig. 1A). We found that the phenotypes caused by mog-2(q75) or mog-2(ok1221) are very similar, indicating that both alleles are null. Furthermore, mog-2(q75)/mog-2(ok1221) trans-heterozygotes are indistinguishable from the respective homozygotes (data not shown). While mostly fertile at 15 °C, more than 20% of mog-2(q75) hermaphrodites developed as somatic females that made excess of sperm and no oocytes at 20 °C (the Mog phenotype, masculinization of the germ line; Fig. 1E,F). Similarly, RNA interference against mog-2 led to Mog animals (51%, n=149; Fig. 1D). At 25 °C, most germ lines are fully masculinized with only 1% of fertile progeny producing sperm and oocytes (Fig. 1E,F,G). In masculinized mog-2(q75) animals we counted 180 ± 88 (n=20) sperm per gonadal arm, while mog-2(ok1221) had 227 ± 111 (n=20) sperm. Regarding the extent of masculinization, both mog-2 alleles are therefore comparable. In addition, we observed that the number of germ cells depends on the developmental stage when animals are shifted to restrictive temperature. If shifted from 15 °C to 25 °C, late-L4 larvae developed as Mog adults containing 300 or more sperm (q75: 276 ± 90, n=20 and ok1221: 333 ± 92, n=20; Graham et al., 1993). In contrast, when mid-L3 to mid-L4 larvae were shifted to 25 °C, they developed into adults with smaller germ lines containing 137 ± 46 (n=14) and 155 ± 42 (n=14) sperm for mog-2(q75) and mog-2(ok1221), respectively (Fig. 1F). In addition to germline defects, both mog-2 alleles cause slow growth and maternal effect lethality (Fig. 1H and Graham et al., 1993). At 25 °C, 100% of the progeny of homozygous mog-2 mutants died as embryos, without showing signs of morphogenesis (Fig. 1H). When larvae were shifted at 25 °C before they had reached mid-L3, they typically arrested somatic and germline development (data not shown). The only difference we observed between the two mog-2 alleles was their growth kinetics. Both mog-2 alleles grew significantly slower than wild type siblings at 15 °C and 20 °C. Furthermore, mog-2(q75) larvae required more time to develop into adults than mog-2(ok1221) suggesting that the former produce a defective protein that interferes with growth (Fig. 1F). Finally, we show that mog-2 is required maternally for early embryonic development, and zygotically for germline and somatic development (Fig. 1H).
*mog-2* is required for the switch from mitosis to meiosis

In hermaphrodites, the switch from mitotic proliferation to meiosis is promoted through two redundant branches that function downstream of GLP-1/Notch. These two branches include GLD-1 and GLD-2. While GLP-1 is necessary for germline proliferation, GLD-1 and GLD-2 function redundantly in meiosis (Austin and Kimble, 1987; Kadyk and Kimble, 1998). Remarkably, most genes that are necessary for meiosis also function in germline sex determination (Belfiore et al., 2004; Eckmann et al., 2004; Hansen et al., 2004a; Kasturi et al., 2010; Kerins et al., 2010). The adult *C. elegans* germline germline features different regions with distinct stages of germ cell development. In the distal part, nuclei are mitotic and proliferate. In the transition zone, nuclei start entering into meiosis, stay in pachytene and finally differentiate as spermatids or oocytes (for review see Schedl, 1997). In previous studies, we have found that *mog-1*, *mog-3*, *mog-4*, *mog-5*, and *mog-6* are synthetically required with *gld-2* or *gld-3* for the switch from mitosis to meiosis (Belfiore et al., 2004; Kasturi et al., 2010). *mog::gld* double mutant germ lines contain mitotic nuclei in both their distal and proximal ends, while germ nuclei in the central region are mitotic with signs of gametogenesis (Pepper et al., 2003). To test if *mog-2* functions in parallel with *gld-2*, we constructed a *gld-2(mg-2) double mutant*. We found that its germ line contains proliferating nuclei in both distal and proximal parts (Fig. 2A,B). Proximal proliferation was confirmed by staining for the mitotic marker phosphohistone H3 (Fig. 2B,C). *gld-3(mg-2) double mutants* arrested as L1 larvae and could not be used to analyze germline phenotypes. The arrested L1 larvae expressed PGL-1 only in the germline primordium (Fig. 2D,E), indicating that, in contrast to *mep-1* mutants, arrested *gld-3(mg-2) larvae* make no ectopic germ cells (Uhavaithaya et al., 2002). To confirm mitotic and meiotic states, we treated larval stages, when spermatogenesis switches to oogenesis (Fig. 3A). While the *mog-2* transcript is absent in *mog-2(ok1221)* animals, it is present at low levels in *mog-2(q75)* (Fig. 3B). The presence of *mog-2(q75)* was confirmed by RT-PCR and sequencing of the cDNA. Furthermore, *mog-2(q75)* mutants produced low levels of the mutated MOG-2 protein that lacks 8 amino acids, as predicted by the cDNA sequence (Fig. 3C). No MOG-2 protein was found in *mog-2(ok1221)* mutants. Although the MOG-2(q75) protein is produced, the

Fig. 2. *mog-2* functions in meiosis. (A,B) Proximal proliferation in *gld-2(mg-2)* germ lines. Mitotic nuclei are present in both distal and proximal parts. Anti-phospho-histone H3 (PH3) staining of mitotic nuclei (arrows). (C) Wild type adult germ lines are mitotic only at the distal end (arrows). (D,E) *mog-2(mg-2)* double mutants stained with DAPI and anti-PGL-1. The germline primordium is indicated (arrow). (F,G) In wild type germ lines, mitosis marker GLP-1 is restricted to the distal part (between the two arrows). (H) A *gld-2(mg-2)* germ lines express GLP-1 well beyond the distal part. (I) Meiosis marker GLD-1 is expressed from the transition zone to the end of the pachytene region in both wild type and *mog-2* mutants (between the two arrows). In the mutant, nuclei exit meiosis then re-enter mitosis at the proximal end. M, mitotic region; TZ, transition zone. Asterisks indicate the distal end of the germ line. Bar, 10 μm.
corresponding mutant behaves as a genetic null allele (Fig. 1H). MOG-2 is expressed in many somatic and germline nuclei. Here we focus on its distribution in the germ line. MOG-2 was detected in all germline nuclei, including those in mitosis (M), in meiosis (TZ, pachytene) and in oocytes (Fig. 3D). However, MOG-2 was not found in spermatids. In L4 larvae, which transiently produce sperm, we found MOG-2 in mitotic and meiotic nuclei, except in secondary spermatocytes (Fig. 3E). Similarly, male germ lines also expressed MOG-2 in all nuclei except in secondary spermatocytes and mature sperm (Fig. 3F). No signal was detected in mutant mog-2(ok1221) germ lines (Fig. 3G). mog-2(q75) did not stain for MOG-2, possibly because the protein levels are too low to be detected (Fig. 3C, not...
shown). Taken together, our results show that MOG-2 is a nuclear protein that is present in most embryonic and somatic cells. In the germ line, MOG-2 was found in all nuclei, except in secondary spermatocytes and spermatids. Previous reports show that fem-3 is post-transcriptionally repressed, and that the mog genes are necessary for this repression (Ahringer and Kimble, 1991; Gallegos et al., 1998). The distribution of MOG-2 in the hermaphroditic germ line suggests that MOG-2 is present in the whole oogenic lineage, but also to some extent in nuclei that are committed to spermatogenesis. Our results suggest that MOG-2 is present, possibly to repress fem-3, until germ cells have completed their second meiotic division.

MOG-2 is a component of U2 snRNP

U2A, the vertebrate homolog of MOG-2, is a component of the U2 snRNP (Mattaj et al., 1986; Scherly et al., 1990a; Sillekens et al., 1989). The U2 snRNP functions in spliceosome assembly by interacting with the branchpoint located near the 3′ splice site (Moore et al., 1993; Padgett et al., 1984). In addition to U2A, the vertebrate U2 snRNP includes the U2 snRNA, the RRM protein U2B′, and other proteins (Jurica and Moore, 2005). In mammals and trypanosomones, U2A′ binds to U2B′ in the absence of the U2 snRNA. Nevertheless, U2A′ is required for efficient binding of U2B′ to the U2 snRNA (Boelen et al., 1991; Caspary and Seraphin, 1998; Polycarpou-Schwarz et al., 1996; Preusser et al., 2009; Scherly et al., 1990b; Simpson et al., 1991). The C. elegans genome encodes two homologous proteins, RNP-2 and RNP-3, which correspond to U1A and U2B′, respectively. While RNP-2/U1A binds to the U1 snRNA, RNP-3/U2B′ interacts with U2. Nevertheless, in the absence of RNP-3, RNP-3 can bind to U2, indicating that both proteins play redundant roles in U2 snRNA binding (Saldi et al., 2007).

In Drosophila, U1A and U2B′ are represented by one single protein, SNF which binds to both U1 and U2 snRNA (Albrecht and Salz, 1993; Polycarpou-Schwarz et al., 1996). However, dU2A′, the Drosophila homolog of U2A′, significantly enhances the binding specificity of SNF to the U2 snRNA (Nagengast and Salz, 2001). Furthermore, Drosophila U2A′ interacts with SNF, associates with the U2 snRNPs, and is essential for viability (Nagengast and Salz, 2001). Similarly, yeast Lea1p/U2A′ and Yib9p/U2B′ are essential for growth and viability at 37°C, but have no independent function (Caspary and Seraphin, 1998). The situation is different in C. elegans, where U2A′ is neither required for U2B′ binding to the U2 snRNA, nor for viability (Saldi et al., 2007).

We searched for binding partners of MOG-2 by yeast two-hybrid screening. We found that MOG-2 strongly interacts with RNP-3/U2B′ but not with RNP-2/U1A (Fig. 4A). Interestingly, the MOG-2(q75) deletion abrogates binding to RNP-3 (Fig. 4A). In vitro, a MOG-2::GST fusion protein was found to interact with RNP-3 independently of RNA. Again, this interaction requires the eight amino acids that are deleted in MOG-2(q75) (Fig. 4B). No RNP-3 was retained on beads loaded with plain GST. Our binding data indicate that MOG-2 belongs to the U2 snRNP and confirm that RNP-2 and RNP-3 are the C. elegans homologs of U1A and U2B′, respectively. Furthermore, we provide evidence that the interaction between RNP-3/U2B′ and MOG-2/U2A′ is conserved in C. elegans and that although RNP-2 can substitute RNP-3 in U2 RNA binding, only RNP-3 is able to interact with MOG-2. We therefore tested interactions with other proteins that function in C. elegans sex determination but found that MOG-2 binds neither to FBF-1 nor to MOG-3 (Fig. 4A). Remarkably, MOG-2 does not associate with MEP-1, which otherwise interacts with all other MOG proteins cloned to date (Fig. 4A; Belfiore et al., 2002, 2004; Kasturi et al., 2010). MOG-2 mutants are characterized by slow growth, embryonic lethality and sterility (Graham et al., 1993; Saldi et al., 2007). Removal of rpm-3 resulted in slow growth and reduced viability, while mog-2(ok1221);rpm-3(Ok1424) double mutants are more severely affected (Saldi et al., 2007). Similarly, we found that at permissive temperature, mog-2(q75);rpm-3(Ok1424) double mutants arrested larval development at L2, without showing signs of oogenesis or spermatogenesis. In arrested larvae, germine and somatic nuclei were enlarged, indicating cell degeneration as a possible cause of arrested development (Fig. 4C). Furthermore, in such double mutants, the severity of the maternal-lethal phenotype was increased at 20°C, with 31% (n = 322) of mog-2(q75);rpm-3(ok1424) animals dying as embryos, compared to 16% for mog-2(q75) single mutants. The phenotype was even stronger in mog-2(q75);rpm-3(ok1424);rpm-2(RNAi) animals in which 45% (n = 170) of the progeny arrested as embryos at 20°C. Therefore, the functions of mog-2 and rpm-3 overlap only partially.

In vitro binding studies and crystal structure analysis suggest that human U2A′ binds to the U2 snRNA (Boelen et al., 1991; Price et al., 1998). To test the role of MOG-2 in the spliceosome, we analyzed its ability to associate with the U2 snRNPs. Native protein extracts were prepared from either wild type or mutant rpm-3(ok1424 null), mog-2(q75), and mog-2(ok1221 null) nematodes. The extracts were incubated with beads coupled to anti-MOG-2 antibodies and the bound RNAs were analyzed by Northern blotting. U2 snRNA was detected in the total lysate of all extracts, and immunoprecipitated with anti-MOG-2 antibodies thereby indicating that it binds to MOG-2 (Fig. 4D). In the absence of RNP-3, MOG-2 still bound to the U2, but to a lesser extent (Fig. 4D). Reduced U2 snRNA binding by MOG-2(q75) can be explained as follows: on one hand the mutant MOG-2(q75) protein is produced in smaller amounts than wild type MOG-2 (Fig. 3C). In addition, U2 snRNA is present at reduced levels in a U2B′/RNP-3 mutant in yeast (Caspary and Seraphin, 1998) and in C. elegans (data not shown). No U2 snRNA was detected in mog-2(q75) mutant extracts, indicating that the deleted motif in MOG-2 is necessary for U2 snRNA binding (Fig. 4D). However, alternative possibilities need to be considered: first, we found that MOG-2(q75) does not interact with RNP-3 in the yeast two-hybrid system, indicating that both proteins could be necessary for robust U2 snRNA binding. Second, MOG-2 levels are significantly lower in the mog-2(q75) mutants as compared to wild type (Fig. 3C). As expected, U2 snRNA was not co-immunoprecipitated in the absence of MOG-2 in a mog-2(ok1221) null mutant (Fig. 4D). We propose that in the absence of RNP-3, MOG-2 can still associate with the U2 snRNA. Similar results have been obtained with Drosophila U2A′, which interacts with U2 snRNPs in the absence of SNF (Nagengast and Salz, 2001). Similarly, the trypanosome U2A′ homolog U2-40 K has been shown to be a component of the U2 snRNP, but the requirement for U2B′ has not been tested (Nagengast and Salz, 2001; Preusser et al., 2009). Our data show that in C. elegans, MOG-2/U2A′ is able to bind to the U2 snRNA in vivo and that this interaction does not require RNP-3/U2B′. MOG-2 might therefore interact with the U2 snRNA either directly, or through other proteins such as RNP-2/U1A, which functions redundantly with U2B′ in C. elegans (Saldi et al., 2007). However, our data from the yeast two-hybrid system tend to exclude RNP-2 in this interaction. The fact that the C. elegans MOG-2 is able to associate with the U2 snRNPs in the absence of RNP-3 supports an earlier finding that Drosophila SNF is not essential for U2A′ binding to U2 snRNPs. Therefore, U2B′-independent association of U2A′ to the U2 snRNPs is conserved at least between C. elegans and Drosophila (Nagengast and Salz, 2001).

We found that MOG-2 and UAF-1 co-localize in the nuclei of germ cell precursors, oocytes, but not in sperm. Surprisingly, both proteins do not localize to nuclear speckles, as expected for splicing factors, but are evenly distributed throughout the nucleoplasm (Fig. 4E; Zamore and Green, 1991). Neither MOG-2 nor UAF-1 are found in the nucleolus. Almost not a proof, the co-localization of MOG-2 with a well-characterized splicing factor such as UAF-1 indicates that the former could function in pre-mRNA splicing.

MOG-2 is involved in pre-mRNA splicing

MOG-1, MOG-4, and MOG-5 are the worm homologs of well-characterized yeast splicing factors (Puoti and Kimble, 1999, 2000).
However, no defects in general splicing were found in the corresponding *C. elegans* mutants (Belfiore et al., 2004; Kasturi et al., 2010; Puoti and Kimble, 1999). The identity of MOG-2 clearly indicates a role in RNA splicing. We therefore analyzed by Northern blotting several candidates involved in sex determination, including *flb*, *nos-3* and the three *fem* genes, but they all appeared to be correctly spliced (data not shown). We therefore focused on RNAs that are alternatively spliced. As a first attempt, we tested the splicing of *unc-52*, which is alternatively processed in mutants of the RRM domain protein MEC-8 (Lundquist et al., 1996). We detected alternatively spliced products in which either exons 15 or 16 were joined to exon 19. However, we did not observe differences between wild type and *mog-2* mutants, indicating that MOG-2 does not alter *unc-52* alternative splicing (data not shown).

Most introns in *C. elegans* are as small as 50 nucleotides and could remain unnoticed in larger transcripts (Blumenthal and Thomas, 1988), therefore we amplified fragments of selected cDNAs using primers designed to span small introns. In *C. elegans*, the 5’ donor splice site consensus is AG/GURARU and the 3’ splice acceptor site is UUXCAG/R (intronic nucleotides are in italics, the cleavage site is represented by the slash, R stands for purine; Blumenthal and Steward, 1997). The nucleotides shown in bold are conserved in *C. elegans* and other species. Using specific oligonucleotides that span 122 introns in 32 different genes, we analyzed discrete regions encompassing small introns that would have escaped observation upon Northern blot analysis. Most introns analyzed were correctly spliced in both *mog-2* mutant allels, as well as in *mog-2; rnp-3* double mutants. These include the *mrg-1* and *drslh-1* RNAs, which are predicted to produce alternatively spliced variants (Stein et al., 2001). To ensure that incompletely-spliced RNAs are not degraded by nonsense-mediated decay, we included *smg-1* mutants in our analysis. However, the processing of such introns was similar in wild type, *smg-1*, and *smg-1; mog-2* mutants (data not shown). Similarly, the first intron of the *fem-3* transcript was correctly processed in all mutant backgrounds analyzed, indicating that absence of *mog-2* does not generate a gain-of-function isoform of *FEM-3* (Fig. 5A, top row). Therefore, if *mog-2* was involved in splicing, it could control the splicing of another mRNA, which regulates *fem-3*. *nos-3* is implicated in the sperm-oocyte switch and could therefore represent a target of *mog-2* (Wang et al., 2002). We analyzed its third intron and found that *mog-2* does not affect the processing of this particular intron (Fig. 5A).

A similar observation was made for the first intron of the *daz-1* RNA, which is also required for germline sex determination (Karashima et al., 2000). We found, however, that a significant proportion of a 50-nucleotide intron of *ama-1* was not excised in *smg-1; mog-2* and *mog-2; rnp-3* double mutants (Bird and Riddle, 1988). Such spliced variant was found neither in *smg-1*, nor in wild type animals (Fig. 5A 3rd row). Failure in removing this intron of 43 nucleotides resulted in a truncated MOG-1 protein that lacks most of its sequence, including conserved motifs that are essential for MOG-1 function. It should be noted that normally processed transcripts were...
found in all cases, in addition to the spliced variant. Therefore, proteins other than mog-2 and RNP-3 must function in the recognition of such introns. Our data show that mog-1 splicing is slightly reduced in the absence of mog-2. Therefore, it is conceivable that the phenotype observed in mog-2 mutants is caused, at least in part, by the reduction of mog-1 function. What makes the two introns of ama-1 and mog-1 different from the other 120 introns that have been analyzed in our survey? Both the 5′ intron splice donor and the 3′ intron splice acceptor signals are not significantly divergent from those found in most C. elegans introns. Also the sizes of both introns are within those commonly found in C. elegans (Blumenthal and Steward, 1997). Therefore, mog-2 must play a role in recognizing other signals within the intron or exon.

mog-2 is required for the recognition of a weak splice site

The U2 snRNP recognizes the branchpoint and possibly interacts with the 3′ splice site (Moore et al., 1993). However, among the transcripts analyzed, we did not find divergences among 3′ splice acceptor sites. We therefore tested the dpy-10(e128) allele, which modifies the invariant AG of the 3′ splice site in the second intron (AG to AA; Aroian et al., 1993). In dpy-10(e128) mutants, three products were found: the unspliced variant, low amounts of dpy-10 mRNA that has been spliced to the mutated AA site and dpy-10 transcripts that have been spliced to a cryptic AG site that is located two nucleotides downstream of the mutated AA. The presence of a fraction of spliced dpy-10(e128) mRNA indicates that the AG dinucleotide at the end of intron 2 is not essential for splicing, because a small fraction of dpy-10(e128) mRNA was spliced to the mutated AA site (Aroian et al., 1993). We asked if the selection of the cryptic splice site was dependent on mog activity. To do so, we compared RT-PCR products between several mutants, and calculated the ratio between the unspliced and spliced variants. In three independent experiments, we found that in the absence of mog-2, the unspliced variant was two times more abundant, compared to smg-1, dpy-10 (Fig. 5B, compare lanes 1–4). An even more pronounced effect was observed in mog-6 dpy-10 animals. However, the ratio between the unspliced and spliced cDNAs was close to that found in single dpy-10(e128) mutants when tested with either mog-5, fem-3 or unc-4 (Fig. 5B, compare lane 1 with lanes 5–8). When dpy-10(e128) was spliced, the splicing occurred in most cases at a cryptic slice site [AG(+)2] located 2 nucleotides downstream of the regular splice site instead of the mutated [AA(0)] position (Aroian et al., 1993; Fig. 5C). Therefore, MOG-2 and MOG-6 lead to the activation of a nearby cryptic splicing acceptor. Since normal splicing still occurs in a fraction of the dpy-10(e128) mRNA, it is likely that other proteins or splice signals are required. These remain to be uncovered.

Since alternatively spliced daz-1 and nos-3 mRNAs were found in both smg-1 and mog-2 animals (Fig. 5A; not shown), we tested mog-2 for a role in nonsense-mediated decay. The spliced variant of rpl-7A is a hallmark for defective nonsense-mediated decay and is therefore detected in smg mutants (Mitrovich and Anderson, 2000). While an alternatively spliced variant of the rpl-7A was detected in smg-1 and smg-2, it was observed neither in wild type, nor in mog-2 animals (Fig. 5D). Therefore, mog-2 does not function in RNA surveillance.

Taken together, our data suggest that mog-2 and mog-6 activate a cryptic splice site in the mutant dpy-10(e128) RNA. However, both genes are not sufficient, because a small fraction of the transcript is spliced even in their absence. Then, how do defective mog genes lead to a mutant phenotype? The simplest explanation is that in the absence of mog, the dose of correctly spliced mRNAs decreases, thereby shifting the balance in germline sex determination towards the male fate.
Conclusion
Post-transcriptional regulation of the fem-3 RNA requires the action of the mog genes (Gallegos et al., 1998; Puoti et al., 2001). The mog genes code for proteins that have homologs that are either directly or indirectly involved in pre-mRNA splicing (Belfiore et al., 2004; Kasturi et al., 2010; Kerins et al., 2010; Konishi et al., 2008; Mantina et al., 2009; Puoti and Kimble, 1999). In addition to regulating sex determination, such genes could function in the decision between mitosis and entry into meiosis. In Drosophila, the U1A/RNP-2 and U2B′/RNP-3 homolog SNF functions in sex determination by establishing the autoregulatory feedback loop to maintain female-specific splicing of Sxl (Oliver et al., 1988; Steinnemann-Zwickly, 1988). SNF is required for both germline and somatic sex determination, but its function in the soma is visible only when levels of Sxl are low. In the germ line, SNF is necessary for germ cell differentiation (Albrecht and Salz, 1993; Oliver et al., 1988). Similarly to SNF, MOG-2 could function in pre-mRNA splicing, perhaps by promoting the processing of particular transcripts. We found a few abnormally spliced mRNAs, among which mog-1 is an interesting candidate. mog-1 codes for the worm homolog of Prp16 (Puoti and Kimble, 1995), which not only acts as a general splicing factor, but is also required for the proofreading of spliced products in Saccharomyces cerevisiae (Schwer and Gross, 1998; Schwer and Guthrie, 1991). An important next step is to find additional RNA targets that lead to a Mog phenotype if alternatively spliced. High throughput sequencing techniques are likely to uncover such targets.

Acknowledgments
We thank Judith Kimble and the National Bio Resource Project for providing mog-2 mutant strains. Some nematode strains used in this work were supplied by the CGC, which is funded by the NIH. Antibodies are from the Kimble, Schedl and Nonet laboratories. We thank the University of Fribourg for the financial support.

References