

# RNA and sex determination in *Caenorhabditis elegans*

## Post-transcriptional regulation of the sex-determining *tra-2* and *fem-3* mRNAs in the *Caenorhabditis elegans* hermaphrodite

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The *Caenorhabditis elegans* hermaphrodite sequentially produces sperm and oocytes from a single pool of precursors. Therefore, the hermaphrodite's germ line is the site of two major cell fate decisions: a germ cell precursor first undergoes a mitosis/meiosis decision and then a sperm/oocyte decision. While the mitosis/meiosis decision is governed by Notch/GLP-1 signalling, the sperm/oocyte decision relies on post-transcriptional regulation of two key mRNAs, *tra-2* and *fem-3*. This review focuses on factors that are required for the silencing of these mRNAs, which results in the sequential production of sperm and oocytes. Most factors that regulate the expression of *tra-2* and *fem-3* are homologous to proteins involved in RNA regulation in yeast, mammals or *Drosophila*, suggesting that at least some of the molecular mechanisms regulating the two worm mRNAs have been conserved throughout evolution.

### Introduction

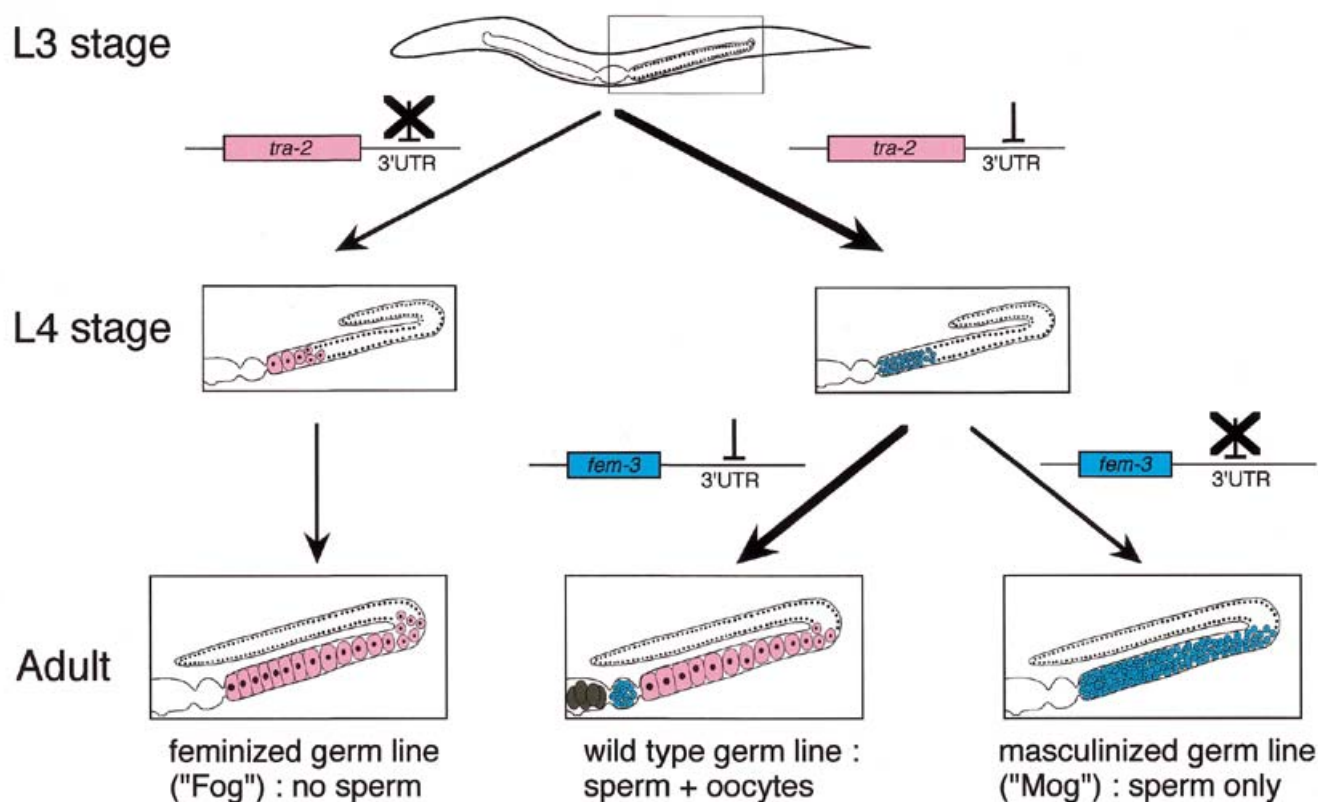
During the last two decades, numerous examples have shown that post-transcriptional regulation of gene expression is important in many organisms, particularly during embryogenesis and larval development (Wickens *et al.*, 2000). Post-transcriptional regulation can occur at any step between transcription and translation. Thus it can affect RNA processing, RNA transport, stability or translation initiation. Post-transcriptional control not only allows a quicker response to the environment compared with *de novo* transcription, but also permits regulated gene expression in the absence of transcription (Mathews *et al.*, 2000). This may be particularly important during early embryogenesis, when transcription is often repressed. Furthermore, regulation at the RNA level allows precise control of protein

dosage, as is the case for mammalian tumour necrosis factor- $\alpha$  and *Caenorhabditis elegans xol-1*, which are regulated both transcriptionally and post-transcriptionally (Han *et al.*, 1991; Nicoll *et al.*, 1997).

Post-transcriptional regulation has been studied in many organisms, including *C. elegans*, in which several genes that are post-transcriptionally regulated have been identified. These genes code for the heterochronic RNAs *lin-14*, *lin-28* and *lin-41* (Lee *et al.*, 1993; Moss *et al.*, 1997; Slack *et al.*, 2000), the *xol-1* RNA that is responsible for primary sex determination (Nicoll *et al.*, 1997; Skipper *et al.*, 1999), the maternal RNAs *glp-1* and *apx-1* that are required for embryonic polarity (Evans *et al.*, 1994; Mickey *et al.*, 1996) and the germline sex determination *fem-3* and *tra-2* mRNAs (feminization; sexual transformer; Ahringer and Kimble, 1991; Goodwin *et al.*, 1993). This review focuses on insights recently gained from studies on germline cell fate decisions mediated by the regulation of the *tra-2* and *fem-3* RNAs whose products play distinct roles in specifying gametes as sperm or oocytes. In both cases, regulation is achieved via elements that are present in the 3' untranslated regions (UTRs) of the RNAs.

*Caenorhabditis elegans* exists in two sexual forms, males and hermaphrodites. Hermaphrodites develop through four larval stages before reaching adulthood. They produce their first germ cells during the fourth larval stage (L4), and these differentiate into sperm. Approximately 160 sperm are made in each of the two gonadal arms at this time. After the L4-to-adult molt, spermatogenesis is turned off and all of the gametes that form subsequently develop into oocytes (for a review see Schedl, 1997). The sequential production of two fundamentally different germ cells requires post-transcriptional repression of two

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**Fig. 1.** Development of the germ line in wild-type and mutant *C. elegans* hermaphrodites. The hermaphroditic gonad consists of two arms that extend during development and are connected through the vulva (top). The L3 worm is outlined with its anterior to the left. The right gonadal arm is boxed and shown to scale in L4 larvae and adults. Undifferentiated germ cell nuclei are shown as black dots, while mature sperm and oocytes are shown in blue and pink, respectively. Development of a wild-type hermaphrodite follows the thick arrows. Repression is shown by an inverted T. Suppression of repression is shown by a crossed inverted T. During the L3 stage, no mature gametes are present and the gonad arms start to reflex (top). At L4, wild-type hermaphrodites produce sperm (right) while *tra-2* (*gf*) mutants make oocytes (left). The onset of spermatogenesis is dependent on repression of *tra-2* through its 3'UTR. Wild-type adults contain both sperm and oocytes because spermatogenesis switches to oogenesis after the L4-to-adult molt (bottom, centre). This switch requires repression of the *fem-3* mRNA through its 3'UTR. While *tra-2* (*gf*) mutants continue oogenesis, leading to a feminized germ line (Fog, bottom, left), *fem-3* (*gf*) mutants do not switch to oogenesis and accumulate excess sperm (Mog, bottom, right).

mRNAs, *tra-2* and *fem-3*; the former must be repressed for spermatogenesis, and the latter for oogenesis (Figure 1). While TRA-2 is a large transmembrane protein that has some similarity to the Patched receptor (Kuwabara and Kimble, 1995), no functional motifs have been found in the FEM-3 protein. One model proposes that FEM-3 is a cytoplasmic protein that binds to the intracellular portion of membrane-bound TRA-2 (Mehra *et al.*, 1999) and that the release of FEM-3 from TRA-2 in response to the extracellular ligand HER-1 results in male development (Kuwabara and Kimble, 1992). In this model, the sperm/oocyte switch depends on the physical interactions between the TRA-2 and FEM-3 proteins and thus requires a finely tuned balance between TRA-2 and FEM-3. Regulation at the RNA level might be critical to achieving the proper stoichiometry.

### Repression of *tra-2* for the onset of spermatogenesis

Genetic studies have shown that *tra-2* promotes female development in that *tra-2* loss-of-function (*lf*) mutations transform XX

animals into non-mating pseudomales that do not produce oocytes (Hodgkin and Brenner, 1977). Insight into *tra-2* regulation came from analysis of *tra-2* gain-of-function (*gf*) mutants that do not produce sperm during L4, but instead produce only oocytes (Doniach, 1986; Figure 1). *tra-2* expression is regulated by two repeated elements in the 3'UTR that were identified through dominant *tra-2* (*gf*) mutations. These elements, the TGEs (for *tra-2* and *G*li *E*lements, formerly named DREs), consist of two repeats of 28 nucleotides that are separated by a four-nucleotide spacer and that are disrupted by small deletions in *tra-2* (*gf*) alleles (Goodwin *et al.*, 1993). Single copies of TGEs are also found in the 3'UTR of other mRNAs such as the *tra-2* mRNA from *Caenorhabditis briggsae* and the human oncogene *GLI*, suggesting that regulation through the TGE is conserved in other species (Jan *et al.*, 1997).

Factors that bind directly to the TGEs or that negatively regulate *tra-2* have been sought and have provided insights into the mechanisms that control *tra-2* expression. RNA gel shift experiments performed with crude worm extracts revealed a binding activity—the direct repeat factor (DRF)—which specifically

recognizes one TGE in the *tra-2* 3'UTR (Goodwin *et al.*, 1993). DRF is a good candidate for a post-transcriptional repressor of *tra-2*, and its components are being characterized. These potentially include the GLD-1 (germ line development) protein, which was identified in a yeast three-hybrid screen as a specific binding factor for the TGEs (Jan *et al.*, 1999). In addition, the F-box-containing protein FOG-2 (feminization of the germ line) was found to interact directly with GLD-1 and has been proposed to act as a bridge to assemble a *tra-2* mRNA–multi-protein complex, which could repress *tra-2* translation (Clifford *et al.*, 2000). GLD-1, a cytoplasmic protein that belongs to the STAR (signal transduction and activation of RNA) family has a single KH RNA-binding motif that is required for inhibition of *tra-2* translation through the TGEs (Figure 2A; Jan *et al.*, 1999). Consistent with their role in regulating *tra-2* activity, GLD-1 and FOG-2 are both required for spermatogenesis and are expressed in the hermaphrodite germ line (Jones and Schedl, 1995; Clifford *et al.*, 2000).

In another approach, a genetic screen for loss-of-function alleles that mimic a *Tra-2* (*gf*) phenotype identified the *laf-1* gene (lethal and fog). Like *gld-1*, *laf-1* is required for *tra-2* repression and works through the *tra-2* 3'UTR. *laf-1* is needed for spermatogenesis and for the repression, *in vivo* and *in vitro*, of transgenes bearing TGEs in their 3'UTRs (Goodwin *et al.*, 1997). In addition, double mutant analyses indicate that *laf-1* acts upstream of *tra-2*, as would be predicted for a regulator of *tra-2*. The molecular identity of *laf-1* is not known yet, and hence direct binding of LAF-1 to the *tra-2* 3'UTR has not been tested. Future experiments will determine whether or not LAF-1 is another component of DRF.

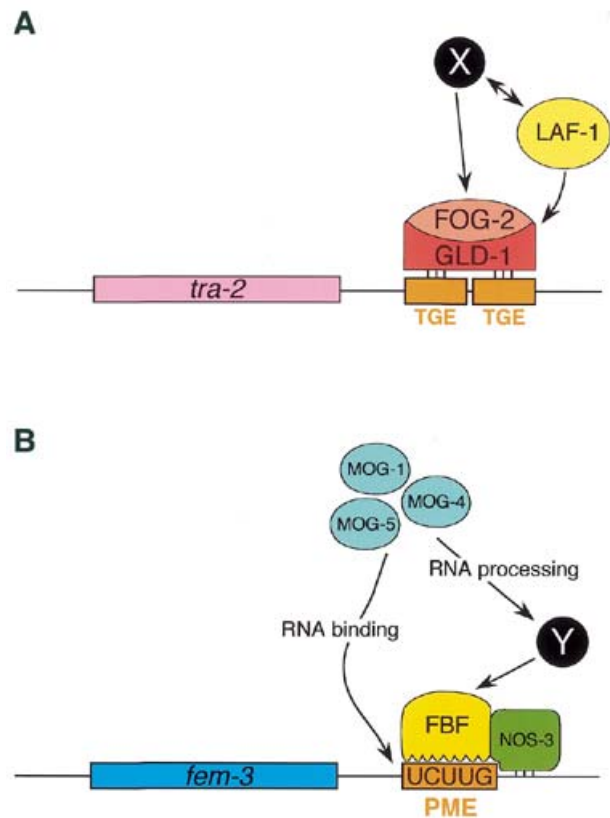
Although polyribosome analysis has shown that *tra-2* is regulated at the level of translation, and that repression via its 3'UTR acts through the TGE by shortening of the *tra-2* mRNA poly(A) tail (Thompson *et al.*, 2000), the precise action of DRF on *tra-2* remains to be elucidated.

In addition to regulation at the RNA level, the activity of TRA-2 might also be controlled post-translationally. In fact, *tra-3* has been shown to encode a calpain-like protease that cleaves TRA-2 and generates a peptide with a feminizing activity that could bind and inactivate FEM-3 (Barnes and Hodgkin, 1996; Goodwin *et al.*, 1997; Sokol and Kuwabara, 2000).

## Repression of *fem-3* for the sperm/oocyte switch

*fem-3* is necessary for spermatogenesis in both the male and the hermaphrodite; *fem-3(lf)* mutants never produce sperm (Kimble *et al.*, 1984). Conversely, *fem-3(gf)* mutants do not switch to oogenesis and thus accumulate excess sperm (Barton *et al.*, 1987). These findings indicate that *fem-3* inhibition is required for the sperm/oocyte switch.

The first evidence that *fem-3* might be regulated at the RNA level came from *fem-3(gf)* mutants that were obtained as extragenic suppressors of *fem-1(lf)* and *fem-2(lf)* (Barton *et al.*, 1987). The molecular lesions in all 19 *fem-3(gf)* alleles are point mutations or small deletions in the 3'UTR, and disrupt a five-nucleotide element named the PME (point mutation element; Ahringer and Kimble, 1991). Thus, the PME may act as a *cis*-acting repressor of *fem-3*. In addition to its localization in the 3'UTR, three additional lines of evidence argue that the PME is respon-



**Fig. 2.** Trans-acting regulators of *tra-2* and *fem-3*. (A) *tra-2* regulation. The GLD-1 protein binds specifically to the TGEs in the 3'UTR of the *tra-2* mRNA. FOG-2 interacts with RNA bound to GLD-1. Although a single GLD-1 protein is shown here, the TGEs could be bound by several molecules of GLD-1. LAF-1 is an additional factor that regulates *tra-2* either by binding directly to *tra-2* or by acting on GLD-1 or another putative factor (X). Some or all of the trans-acting factors that regulate *tra-2* might form a protein complex named DRF. (B) *fem-3* regulation. The model shows FBF and its interacting partner NOS-3 forming a cytoplasmic complex on the *fem-3* mRNA. FBF specifically recognizes the PME through its eight Puf repeats, while NOS-3 binds RNAs non-specifically. Although the function of NOS-3 is redundant with those of NOS-1 and NOS-2, the latter proteins have not been included in this complex since they do not bind to FBF. On the other hand, at least three nuclear MOG proteins might act on *fem-3* either directly or indirectly, via the FBF/NOS-3 complex and/or additional factors (Y). This action may involve RNA processing and RNA binding.

sible for the post-transcriptional rather than transcriptional regulation of *fem-3*. First, the steady-state levels of *fem-3* mRNA are similar in wild-type animals and *fem-3(gf)* mutants. Secondly, a *fem-3* RNA-binding activity can be titrated by expressing a heterologous RNA that contains the wild-type *fem-3* 3'UTR, resulting in masculinized germ lines in hermaphrodites. Such masculinization does not occur if a gain-of-function mutated *fem-3* 3'UTR is used, indicating that the wild-type *fem-3* 3'UTR is specifically bound by one or several factors that are required for the sperm/oocyte switch (Ahringer and Kimble, 1991). Thirdly, the somatic expression of a reporter construct bearing a heat shock promoter, the *lacZ* gene and the 3'UTR of *fem-3* is normally repressed in wild-type worms, but it is de-repressed if a mutation disrupts the PME in the transgene (Gallegos *et al.*,

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1998). In contrast to the distribution of *tra-2*, that of *fem-3* mRNA in polyribosomes has not been studied so far, and as a consequence, it remains to be shown whether *fem-3* is actually regulated at the level of RNA processing, nuclear export, stabilization or translation. Furthermore, good antibodies against TRA-2 and FEM-3 have not been obtained so far. Thus, it has not been shown that the protein levels of TRA-2 and FEM-3 actually change during gametogenesis.

Factors that act *in trans* to *fem-3* via its 3'UTR and that are required for the regulation of the sperm/oocyte switch have been sought via both genetic and biochemical means. The rationale behind the genetic screens that were undertaken was that, if such *trans*-acting factors are required for *fem-3* repression, a mutation leading to the loss of the repressor should generate a phenotype that is similar to *Fem-3(gf)*. Genetic screens identified six *mog* genes (masculinization of the germ line) that act upstream of *fem-3* and are required for the sperm/oocyte switch in hermaphrodites (Graham and Kimble, 1993; Graham *et al.*, 1993). Additional evidence for *mog* function in *fem-3* regulation came from the observation that protein expression from the *lacZ::fem-3 3'UTR* transgene was higher in each of the six *mog* mutants than in wild-type worms, indicating that the *mog* genes are required for *fem-3* repression through its 3'UTR *in vivo* (Gallegos *et al.*, 1998). Of the six *mog* genes, *mog-1*, *mog-4* and *mog-5* were cloned and found to encode nuclear proteins of a particular DEAH-box RNA helicase subfamily. These proteins are the *C. elegans* homologues of yeast splicing factors Prp16p, Prp2p and Prp22p, respectively, suggesting that *fem-3* regulation could involve pre-mRNA splicing as well (Puoti and Kimble, 1999, 2000).

In addition to the genetic approach, yeast two- and three-hybrid screens were used to identify *trans*-acting factors that either bind to known *fem-3* regulators via protein–protein interactions or that bind directly to the *fem-3* 3'UTR, respectively. To identify factors that bind specifically to the *fem-3* 3'UTR, Zhang *et al.* (1997) used a yeast three-hybrid screen for RNA-binding proteins. Specifically, two 37-nucleotide tandem repeats containing the PME were used as RNA bait. This screen yielded two distinct proteins, FBF-1 and FBF-2 (*fem-3* binding factor), which share 91% amino acid identity and that are probably redundant. RNA interference directed against both *fbf-1* and *fbf-2* led to masculinized hermaphrodites that accumulate excess sperm and do not switch to oogenesis, suggesting that *fbf* is required for the switch from spermatogenesis to oogenesis (Zhang *et al.*, 1997).

In another approach, protein–protein interaction screens using the yeast two-hybrid system identified additional *trans*-acting factors. The *fem-3*-binding protein FBF-1 was used as a bait for interacting proteins (Kraemer *et al.*, 1999). The *C. elegans* protein NOS-3 (Nanos) was identified as a binding partner for both FBF-1 and FBF-2 in the yeast two-hybrid system and *in vitro*. NOS-3 binds directly to FBF proteins but has an additional general RNA-binding activity that is not required for its binding to FBF. NOS-3 contains two unusual zinc fingers in its C-terminus and has at least two orthologues in *C. elegans*, NOS-1 and NOS-2, which are required for germ-line viability (Subramaniam and Seydoux, 1999). Similarly to FBF, the three *nos* genes are likely to have redundant functions since sterility or occasional excess sperm was only observed in *nos* double or triple mutants (Kraemer *et al.*, 1999; Subramaniam and Seydoux, 1999).

## FBF-like and NOS-like repressors

FBF is a member of a large protein family that is characterized by the presence of eight Puf (Pumilio and EBF) repeats of ~37 amino acids and its *Drosophila* counterpart is the Pumilio (Pum) protein (Zhang *et al.*, 1997). While FBF represses *fem-3* expression by acting on the *fem-3* 3'UTR, Pum is necessary to repress the translation of the *hunchback* (*hb*) mRNA through binding to the NREs (Nanos response elements), which are located in the *hb* 3'UTR (Wharton and Struhl, 1991). Repression of *hb* involves deadenylation of the mRNA and requires the formation of a protein complex that includes at least Pum, Nanos (Nos) and the NREs (Wharton and Struhl, 1991; Wreden *et al.*, 1997). In *Drosophila*, the interaction between Nos and Pum is dependent on the presence of the *hb* mRNA (Sonoda and Wharton, 1999). Additional components such as the *Drosophila* 'Brain Tumor' (Brat) protein, which forms a quaternary complex by associating with *hb* RNA-bound Pum and Nos (Sonoda and Wharton, 2001), might be required for the repression of *hb* mRNA. Intriguingly, one Brat homologue in *C. elegans* is LIN-41, which is required for the switch from L4 to adult and which post-transcriptionally regulates the heterochronic mRNA *lin-29* (Slack *et al.*, 2000). *Drosophila nos* encodes a general RNA-binding protein with two unusual CCHC zinc fingers that are conserved in the *C. elegans* NOS-1, NOS-2 and NOS-3 proteins. Unlike *Drosophila* Nos and Pum, *C. elegans* FBF and NOS-3 associate even in the absence of an RNA molecule. Moreover, no interaction has so far been reported between LIN-41 and either FBF or NOS-3. However, the sequence similarities between NOS-3 and *Drosophila* Nos and their cooperation with FBF and Pum, respectively, suggest that similar molecular mechanisms govern *fem-3* and *hb* repression.

*Drosophila* Nos was first discovered to be essential, together with Pum, for posterior patterning of the *Drosophila* embryo (Lehmann and Nusslein-Volhard, 1991), while FBF and NOS-3 were identified as regulators of *fem-3* and hence implicated in the sperm/oocyte switch in *C. elegans* hermaphrodites. However, recent studies have shown that Pum is also required for germ cell development and migration in the embryo and for maintenance of the germline stem cells in the adult ovary (Forbes and Lehmann, 1998; Asaoka-Taguchi *et al.*, 1999). Similarly, the *C. elegans nos* and *fbf* genes might also have functions beyond those in the sperm/oocyte switch since *nos-1* to *-3*, and some *C. elegans pum* homologues including *fbf*, appear to be required for the proliferation and survival of germ cells (Kraemer *et al.*, 1999; Subramaniam and Seydoux, 1999).

Concerning *cis*-acting elements, Tadauchi *et al.* (2001) have shown that Mpt5p, a Puf family member in *Saccharomyces cerevisiae* also regulates mRNA through 3'UTR elements, in this case, that of the *HO* mating type switch gene. Like Pum, yeast Mpt5p negatively regulates *HO* by destabilizing the mRNA, perhaps by promoting its deadenylation (Tadauchi *et al.*, 2001). Interestingly, sequence conservation is also found in the 3'UTRs of *fem-3*, *hb* and *HO* mRNAs. In fact a four-nucleotide sequence (UUGU) is found twice in each NRE, once in the *HO* 3'UTR and once in the *fem-3* 3'UTR, where it almost overlaps with the PME (ucUUGU; Zamore *et al.*, 1997; Tadauchi *et al.*, 2001). Together with the similarities found between Puf family members and their interaction with Nos-like proteins, the identification of a partially conserved *cis*-acting element

suggests that Puf/Nos-dependent RNA regulation may represent an ancient mechanism for post-transcriptional regulation of gene expression.

### Future perspectives

The *cis*-acting regulatory elements in *fem-3* and *tra-2* were discovered 10 years ago. At present, several factors that are required for the regulation of *tra-2* and *fem-3* are known, and their initial characterization indicates that the regulation of the two mRNAs in *C. elegans* involves *trans*-acting factors and *cis*-acting elements that may have similar or related functions in other species. Although a molecular function has been proposed for most of the *trans*-acting regulators that have been identified, the molecular mechanisms through which they regulate *tra-2* or *fem-3* are not well understood. Searches for additional components, the establishment of *in vitro* systems, and comparisons with other model systems such as *Drosophila* and yeast are likely to bring the answers.

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*Front from left: Paolo Pugnale, Marco Belfiore, Zariffa Saudan & Anne-Catherine Schläppi. Back in the centre: Alessandro Puoti*

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