Department of Molecular Biology,
Rowan University-SOM,
Stratford, NJ, 08084

C. briggsae TRA-2 interacts with TRA-1 to prevent spermatogenesis

Yongquan Shen and Ronald E. Ellis

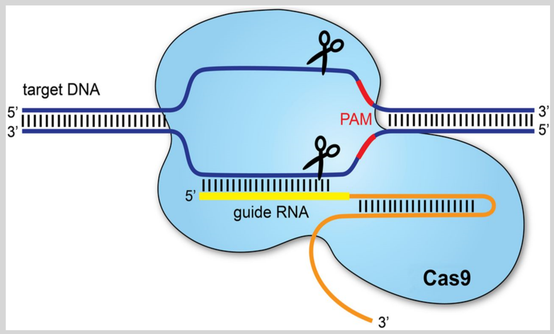
Rowan University

Abstract

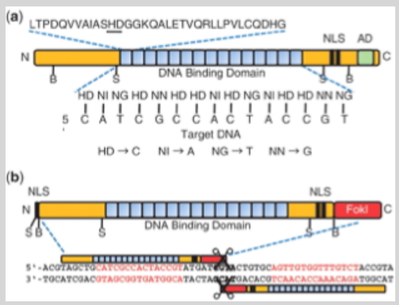
We are studying *C. briggsae* TRA-1, to learn how this Gli transcription factor regulates sexual identity in worms. To begin, we generated *cbr-tra-1(v455),* which has an OLLAS tag inserted near the N-terminus, and *cbr-tra-1(v424),* which has one near the C-terminus. Both alleles develop normally, so neither tag affects function. On western blots we see that *Cbr*-TRA-1 is cleaved to form a product that is slightly larger than its *C. elegans* counterpart. This product is predicted to be a repressor that blocks the transcription of male genes. Finally, sequence alignments reveal a small conserved domain near the putative cleavage site. To analyze TRA-1 function, we used gene editing to make the following mutations: (1) *cbr-tra-1(v197)* is a frameshift *upstream* of the cleavage site. When its mRNA is stabilized, the truncated product makes a functional TRA-1 repressor, since *XX* animals develop normal hermaphrodite bodies. However, they make *only* oocytes and no sperm. (2) *cbr-tra-1(v405)* and *cbr-tra-1(v406)* are frameshifts located *downstream* of the cleavage site. they develop normal hermaphrodite bodies, so TRA-1 repressor is functioning. However, these mutations also cause animals to make oocytes instead of sperm, which implies that full-length TRA-1 normally promotes spermatogenesis. (3) *cbr-tra-1(v197v383)* alters 30 residues in the domain that binds TRA-2, and disrupts interactions between *C. briggsae* TRA-2 and TRA-1 in yeast two-hybrid assays. These *XX* mutants make significantly more sperm than the wild type. Furthermore, this mutation restores spermatogenesis to many *she-1(v35) XX* animals. We conclude that TRA-2 normally binds TRA-1 to block spermatogenesis. Analyses of *tra-2(null); fem-3(null); she-1(null)* animals support this model. This result is surprising, since *C. elegans tra-2* mutations that block the interaction, cause oogenesis. (4) We are now making mutants which alter the putative cleavage domain without affecting upstream or downstream regions to see if full-length *Cbr*-TRA-1 activates male genes like Gli/Ci in humans and flies. (5) we and others in our lab found that mutations in several chromatin regulators alter the sperm/oocyte decision. Taken all the results together, we propose that full-length TRA-1 promotes spermatogenesis by working with chromatin regulatory factors, whereas the cleaved form of TRA-1 represses spermatogenesis. In *C. briggsae,* TRA-2 blocks the function of full-length TRA-1.

Using gene editing to study *Cbr*-TRA-1 function in nematodes

Genome editing with CRISPR/Cas9 or TALENs has become an essential tool for working with nematodes.

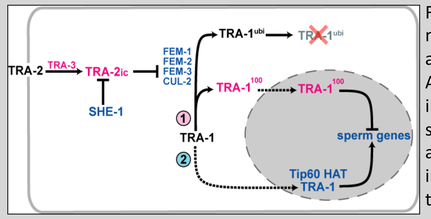


Our strategy is to use Cas9 RNA complexes for injections, if good Cas9 cleavage sites are available near the target site. However, the best targets for Cas9 end in a 3'-GGnGG sequence, which is infrequent in worms. Thus, we use TALENs in situations that require precise targets not cut by Cas9.



TRA-1 plays a central role in sex determination in *C. briggsae* germ cells

There are many parallels between the sex-determination pathways in the germ lines of *C. briggsae* and *C. elegans*. The core members of the pathway are conserved, and act in both the germ line and the soma. (For simplicity, only those genes downstream of *her-1* are shown here). However, some genes play unique roles in the germ lines of each species.



Factors promoting male fates are blue, and female fates red. Arrows show positive interactions, bars show negative ones, and dotted lines indicate import into the nucleus.

The central role is played by TRA-1, which appears to act as an activator with help from Tip60, and a repressor following cleavage of the carboxyl terminus. Thus, understanding how TRA-1 is regulated is critical.

C. elegans and *C. briggsae* WDR-5 mutants have opposite phenotypes

The WDR5 proteins are conserved components of a histone methyl-transferase complex normally associated with gene activation. Li and Kelly (2014) showed that *C. elegans* hermaphrodites lacking *wdr-5.1* and *wdr-5.2* at 25°C continue producing sperm during adulthood, rather than switching to oogenesis (a Mog phenotype). Furthermore, the WDR-5 proteins were required for TRA-1 to localize to chromatin and repress *fog-3* expression. Thus, we generated similar mutants in *C. briggsae*.

We made a *cbr-wdr-5.2* frame-shift mutation using TALENs, and then knocked down *cbr-wdr-5.1* by RNAi in the *cbr-wdr-5.2* background (null mutations of *wdr-5.1* are lethal). Surprisingly, **knocking down WDR-5 caused animals of both sexes to produce oocytes instead of sperm**.

Germline feminization by double knockdown of *cbr-wdr-5.1* and *cbr-wdr-5.2*

Genetic background	RNAi target	Sex	Sperm only	Sperm & Oocyte	Oocyte only	N
AF16		XX		132(100%)		132
AF16	*cbr-wdr-5.1*	XX		176(100%)		176
cbr-wdr-5.2(v341)		XX		189(100%)		189
cbr-wdr-5.2(v341)	*cbr-wdr-5.1*	XX		237(93%)	17(7%)	254
cbr-wdr-5.2(v341)		XO	52(100%)			52
cbr-wdr-5.2(v341)	*cbr-wdr-5.1*	XO	17(33%)	34(67%)		51

Furthermore, this phenotype was epistatic to the production of sperm and oocytes observed in the null mutant *cbr-tra-1(v181)* (below). These results indicate that the WDR-5 proteins have an effect on the sperm/oocyte switch in *C. briggsae* that is independent of TRA-1.

Finally, losing a single copy of *tra-1* also causes oogenesis in *wdr-5* mutants, so these mutations show synergistic effects.

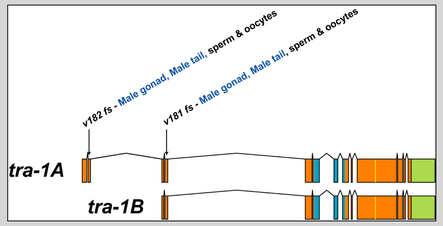
cbr-tra-1 and *cbr-wdr-5* genes act independently on germ cell fates

Genetic background	RNAi target	Sex	Sperm only	Sperm & Oocyte	Oocyte only	N
cbr-tra-1(v181); cbr-wdr-5.2(v341)		XX	3(5%)	59(95%)		62
cbr-tra-1(v181); cbr-wdr-5.2(v341)	*cbr-wdr-5.1*	XX			29(100%)	29
cbr-tra-1(v181)/+; cbr-wdr-5.1(v341)		XX		27(100%)		27
cbr-tra-1(v181)/+; cbr-wdr-5.1(v341)	*cbr-wdr-5.1*	XX			64(100%)	64

One possibility is that both TRA-1 and the WDR-5 proteins act on the same target promoter, such as *fog-3*. We do not know why the effects differ so dramatically between species.

Null alleles show that *Cbr*-TRA-1A is essential for female development

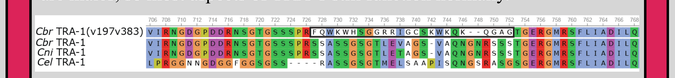
C. briggsae tra-1 produces two transcripts that encode very similar proteins (de Bono *et al.* 1996). The reference allele *Cbr-tra-1(nm2)* is a stop mutation located after the zinc fingers, and affects both transcripts. To learn if they have different functions, we used TALENs to make early frame-shift mutations in *Cbr-tra-1*. The *v181* allele disrupts both TRA-1A and TRA-1B, creating a true null mutant. By contrast, *v182* only eliminates TRA-1A. However, both mutants caused a complete transformation of *XX* animals into males that make sperm and then oocytes. Since this is also the null phenotype for *C. elegans,* **we conclude that both alleles are null mutations, and that *Cbr*-TRA-1A is essential for sex determination**.



TRA-2 might bind TRA-1 to block spermatogenesis

We generated the frameshift *cbr-tra-1(v197)*, which causes an early stop downstream of the zinc fingers. It encodes a C-terminally truncated TRA-1, that causes dominant feminization when the messages are stabilized by a *smg-5* mutation (right top). This result supports our model that full-length TRA-1 is an activator that promotes spermatogenesis, and cleaved TRA-1 is a repressor of male genes. Truncation mutants in *C. elegans* act similarly.

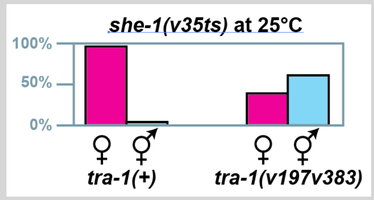
This site was adjacent to a conserved region that was likely to mediate interactions with TRA-2, based on yeast two-hybrid studies (Lum et al 2000; Wang and Kimble 2001). Thus, we used gene editing to introduce a second frameshift, which leaves 25 codons from the conserved region out of frame. In this mutant, *XX* animals are hermaphrodites and *XO* animals are males, so most aspects of TRA-1 still function normally.



(A) Yeast two hybrid studies show that *C. briggsae* TRA-1(v197v383) cannot bind TRA-2, whereas wildtype TRA-1 does bind TRA-2.

(B) *Cbr-tra-1(v197v383)* hermaphrodites produce many more sperm than the wild type. Four lines of evidence support this claim:

(1) This *tra-1* mutation suppresses the feminization of germ cells caused by a mutation in *cbr-she-1:*



(2) This *tra-1 m*utation causes a significant increase in brood size:

Strain	Brood Size	n
Wild type (AF16)	157	11
Cbr-tra-1(v197)/ +	182	13
Cbr-tra-1(v197v383)	**232**	13

(3) The switch from spermatogenesis to oogenesis is delayed. It is 0.4 hrs after the L4 molt for wild type, but 2.1 hrs later for *tra-1(v197 v383).*

(4) DAPI staining directly shows that *cbr-tra-1(v197 v383)* hermaphrodites make more sperm than AF16 or the parent *cbr-tra-1(v197)/dpy-18*.

We conclude that *v197v383* favors spermatogenesis, possibly by preventing TRA-2 from binding to TRA-1 activator. Studies of *tra-2; she-1 fem-3* triple mutants also suggest that TRA-2 binds TRA-1 to block spermatogenesis (Guo et al 2009).

The effects of the original *C. elegans tra-2(mx)* alleles, which cause oogenesis, remain confusing.

C. briggsae TRA-1 mutants that make only repressor cause oogenesis

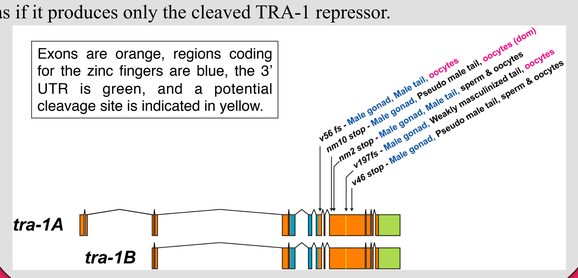
Several nonsense and frame-shifting mutations in *Cbr-tra-1* are located downstream of the zinc-finger region in the C-terminus. Surprisingly, the phenotypes of these mutants do not show any patterns that would reveal the functions of the truncated TRA-1 proteins. For example, *tra-1(nm2*stop*)* behaves like a null allele, whereas the mutations *tra-1(v56*fs*)* and *tra-1(nm10*stop*)* block spermatogenesis, even though they end closer to the amino-terminus. To resolve this problems we took two steps.

(1) We isolated additional *cbr-tra-1* deletion alleles.

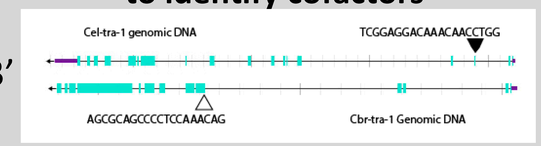
Using TALEN to greate *cbr-tra-1* mutation

Allele	XX phenotype	nt deletion
v197	male gonad, oocytes, weakly masculized tail	8 nt
v198	male gonad, oocytes, weakly masculized tail	20 nt
v199	hermaphrodites	9 nt
v200	hermaphrodites	6 nt
v201	hermaphrodites	6 nt
v202	hermaphrodites	3 nt
v360	hermaphrodites	6 nt

(2) We made a *smg*-5 mutation to stabilize mutant transcripts. Most importantly, *v197* appears to break upstream of the normal cleavage site. When combined with a *smg-5* mutation, it causes *dominant feminization*, as if it produces only the cleaved TRA-1 repressor.

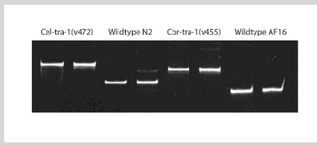


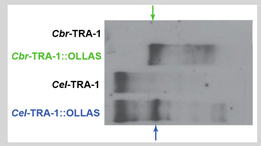
Using OLLAS-Tagged TRA-1 to identify cofactors



We used CRISPR/Cas9 to integrate a short OLLAS peptide into both *Cel*-TRA-1 and *Cbr*-TRA-1, near the N-terminus. The resulting OLLAS tags are located between the gain-of-function domain and the zinc finger domain.

In-frame OLLAS insertions were identified by single worm PCR and confirmed by sequencing. These *XX* OLLAS mutants *undergo normal hermaphrodite development*, indicating that TRA-1 function is not affected by the OLLAS epitope.





To identify the TRA-1 proteins, we picked 25-worm samples for lysis in SDS-PAGE buffer for Western Blotting. The result indicated the size of cleaved TRA-1was around 100 KD in both species, although slightly larger in *C. briggsae* than in *C. elegans*. To purify TRA-1, we performed a large liquid culture for *tra-1::*OLLAS strains of each species, floated the worms on 30% sucrose and snap-froze them in liquid nitrogen. After immunoprecipitation with anti-OLLAS antibodies, silver staining suggested that we have recovered TRA-1 and can use this method to isolate co-factors with which it interacts.

Our working model

We propose that full-length TRA-1 activates spermatogenesis genes like *fog-3,* and cleaved TRA-1 represses them. TRA-2 might bind full-length TRA-1 to block spermatogenesis. Potential co-factors like WDR-5 and the Tip60 HAT complex work with TRA-1 to regulate *fog-3* expression.