TRA-1 ChIP-seq reveals regulators of sexual differentiation and multilevel feedback in nematode sex determination

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How sexual regulators translate global sexual fate into appropriate local sexual differentiation events is perhaps the least understood aspect of sexual development. Here we have used ChIP followed by deep sequencing (ChIP-seq) to identify direct targets of the nematode global sexual regulator Transformer 1 (TRA-1), a transcription factor acting at the interface between organism-wide and cell-specific sexual regulation to control all sex-specific somatic differentiation events. We identified 184 TRA-1–binding sites in *Caenorhabditis elegans*, many with temporal- and/or tissue-specific TRA-1 association. We also identified 78 TRA-1–binding sites in the related nematode *Caenorhabditis briggsae*, 19 of which are conserved between the two species. Some DNA segments containing TRA-1–binding sites drive male-specific expression patterns, and RNAi depletion of some genes adjacent to TRA-1–binding sites results in defects in male sexual development. TRA-1 binds to sites adjacent to a number of heterochronic regulatory genes, some of which drive male-specific expression, suggesting that TRA-1 imposes sex specificity on developmental timing. We also found evidence for TRA-1 feedback regulation of the global sex-determination pathway: TRA-1 binds its own locus and those of multiple upstream masculinizing genes, and most of these associations are conserved in *C. briggsae*. Thus, TRA-1 coordinates sexual development by reinforcing the sex-determination decision and directing downstream sexual differentiation events.

Sexual development requires an initial sex-determination decision followed by sex-specific differentiation events that establish anatomical, physiological, and behavioral differences between the sexes. Although the sex-determination switch has been well studied in many animals, the molecular and genetic mechanisms by which sexual fate subsequently is manifested represent a major gap in our understanding of sexual development. Here we address this question in the nematode *Caenorhabditis elegans*.

Sex is determined genetically in *C. elegans*: Embryos with two X chromosomes (XX) develop as self-fertile hermaphrodites—females that transiently make sperm—whereas embryos with one X chromosome (XO) develop as males (1). The sex chromosome complement sets the activity of Transformer 1 (TRA-1), a transcription factor that directs sexual differentiation throughout the animal (Fig. 1) (2). High TRA-1 activity in XX animals promotes female differentiation, whereas low TRA-1 activity in XO animals allows male differentiation (3). Sexual dimorphism is extensive, with roughly a third of adult somatic cells showing obvious sex differences (4), and depends on *tra-1* activity. Null mutations in *tra-1* cause XX animals to develop as fertile pseudomales, whereas *tra-1* gain-of-function mutations cause XO animals to develop as fertile females (3). Indeed, stable strains have been constructed in which the genotype at the *tra-1* locus determines sex independent of the sex chromosomes (5). Genetic mosaic analysis showed that *tra-1* determines sex autonomously in most cells (6), although cell signaling does play a role in coordinating the sex determination decision among cells (7) and in local inductions of sexual cell fate (8).

tra-1 performs several distinct functions in sexual development. First, it directs female somatic development via transcriptional repression of genes that promote male differentiation. Examples include male abnormal 3 (*mab-3*) (9) and DM domain family 3 (*dmd-3*) (10), which jointly control specific aspects of male tail development, and *C. elegans* homeobox 30 (*ceh-30*) (11) and egg laying defective 1 (*egl-1*) (12), which regulate sex-specific cell deaths in the nervous system. Second, *tra-1* regulates germ-line sex determination by repressing feminization of germline 1 (*fog-1*) and *fog-3* to promote oogenesis (13, 14). Additionally, *tra-1* is required for proper cell organization in the early male somatic gonad (3) and modulates membrane synthesis in the female germ line to facilitate oogenesis (15), although the TRA-1 targets functioning in these processes remain unknown. Identifying the suite of TRA-1 target genes and determining how they collectively guide sexual differentiation in *C. elegans* will provide a paradigm for understanding how sexual regulators translate global sexual fate into specific differentiation events.

Results

TRA-1 Chromatin Association Exhibits Temporal and Tissue Specificity. To identify TRA-1–binding sites genome-wide, we performed ChIP-seq using an antibody that detects both major TRA-1

Significance

Sex-determining genes have been identified in many animals, but how they impose sex specificity on development is poorly understood. We ask how the nematode sex-determining transcription factor Transformer 1 (TRA-1) regulates sex by identifying where in the genome TRA-1 binds and which nearby genes may be affected by this binding. We find that TRA-1 promotes female development primarily by preventing the expression of genes involved in male development. Among the genes repressed by TRA-1 are a number that control the timing of developmental events and also several that function upstream of TRA-1 in the global sex-determination pathway. The suite of TRA-1 targets presented here provides a resource to continue uncovering the basis of sex-specific development.

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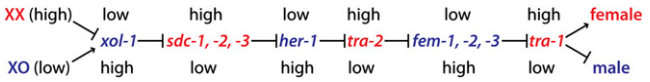

Fig. 1. Model for genetic control of *C. elegans* somatic sex determination. Minor genetic interactions and components have been omitted for simplicity.

isoforms and binds DNA-bound TRA-1 (Fig. S1) (15). We performed ChIP-seq in L2 larvae, L3 larvae, and defective spermatogenesis 11 (*spe-11*) young adults which fail to produce embryos because of nonfunctional sperm (16). We chose these time points, during which many TRA-1–dependent sex-specific differentiation events occur, in an effort to identify as many TRA-1–binding sites as possible. We also analyzed abnormal germ line proliferation 4 (*glp-4*) young adults deficient for germ cells (17) to examine tissue-specificity of TRA-1 binding. We then analyzed *tra-1(e1834)*–null mutant young adult pseudomales (3, 18) to identify sites enriched independently of TRA-1, which were excluded from further analysis. We identified a total of 184 sites that were specifically bound by TRA-1 in both biological replicates of at least one developmental condition (*SI Materials and Methods* and Dataset S1).

The 184 TRA-1–binding sites identified are fewer than typical for site-specific transcription factors. However, they likely include about half of the TRA-1–binding sites in *C. elegans,* because binding sites were identified near five of eight previously described TRA-1 targets, *dmd-3*, *mab-3*, XO lethal 1 (*xol-1*), *fog-1*, and *fog-3*. Binding was not observed near the previously described TRA-1 targets *egl-1*, *ceh-30*, or abnormal cell lineage 39 (*lin-39*). The five previously described targets that were bound are required for sex-specific development in a greater number of cells than the three targets not bound, suggesting that binding may be cell type specific and that whole-animal ChIP-seq may not identify targets bound only in a small number of cells. Alternatively, some targets may be bound at developmental time points we did not examine.

TRA-1 binds in vitro to a nonamer DNA motif very similar to that recognized by its mammalian homolog GLI (Fig. 2*A*) (19). The most significantly enriched motif at TRA-1–binding sites was a nearly exact match to this in vitro-defined motif (Fig. 2*A*), found at 169 of 184 (92%) TRA-1–binding sites, indicating that most, but not all, sites are bound through this motif. TRA-1 may bind other sites via more divergent motifs or indirect recruitment by other factors. Although most TRA-1–binding sites had this motif, only about 4% of the best matches to this motif in the *C. elegans* genome were bound by TRA-1 (Fig. 2*B*). In general, TRA-1 ChIP enrichment was stronger for sites with closer matches to the consensus motif, but many perfect matches were not detectably bound by TRA-1, and many bound sites had imperfect matches (Fig. 2*B*). Thus, like many transcription factors (20), TRA-1 binds DNA at most of its sites via the consensus binding sequence, but additional factors help determine whether TRA-1 binds this motif.

To examine how TRA-1 DNA occupancy changes during development, we assessed ChIP enrichment at each of the 184 TRA-1–binding sites at each of the developmental conditions tested, counting significant enrichment in either of two replicates as an indication of TRA-1 binding (Fig. 2*C*). By this criterion, 32 of 184 sites were bound by TRA-1 in L2, 105 in L3, and 164 in *spe-11* young adults, indicating an increase in TRA-1–occupied sites as development progresses. Thus, the previous suggestion that TRA-1 might bind its target sites at all times and in all tissues, causing sex-specific expression only in the cells in which nearby regulatory elements are active (21), appears unlikely, at least for many of the sites identified here. To ask whether TRA-1 associates tissue specifically with its binding sites, we compared TRA-1 binding in *glp-4* (severely reduced germ line) and *spe-11* (normal germ line but no fertilized embryos) young adults. Of the 164 sites with significant enrichment in *spe-11* animals, 45 were not bound in *glp-4* (*SI Materials and Methods* and Dataset S1), suggesting that TRA-1 binds to these sites primarily in the germ line. These germ line-specific TRA-1 targets provide candidates for future investigation of how TRA-1 regulates oogenic membrane organization (15).

TRA-1 Target Genes Are Involved in Male-Specific Sexual Development. We performed a pilot screen to evaluate whether our list of 184 TRA-1–binding sites can be used to identify sexual regulators. We used reporter analysis to identify TRA-1–bound DNA segments capable of driving sex-specific expression (Fig. 3*A*) and RNAi to identify genes adjacent to TRA-1–binding sites that are required for sexual differentiation (Fig. 3*B*). We chose 35 sites to examine with reporter analysis (Dataset S1), based on the strength of TRA-1 ChIP enrichment, enrichment in multiple stages, and the presence of strong TRA-1 motifs. Six TRA-1–bound segments drove obvious male-specific expression when fused to a minimal promoter and GFP (Figs. 3*A* and 4*A*). One was adjacent to the zinc-finger transcription factor *ztf-6* and drove expression in male-specific tail lineages. Another was adjacent to *ptr-5*, which encodes a protein distantly paralogous to *Drosophila* PATCHED. A *ptr-5* reporter expressed male-specifically in body wall and stomatointestinal muscle as well as in sex muscles and some neurons of both sexes (Fig. 3*A*). Ablating the TRA-1–binding motif in this reporter activated expression in hermaphrodite body wall and stomatointestinal muscle cells (Fig. 3*A*). Another site was adjacent to *mab-23*, which encodes a DM-domain transcription factor that regulates male sexual differentiation (22). A short region containing this site drove male-specific reporter expression in the tail (Fig. 3*A*). Ablation of the TRA-1 motif in this reporter caused ectopic expression in the hermaphrodite tail, but the expression was weak and variable,

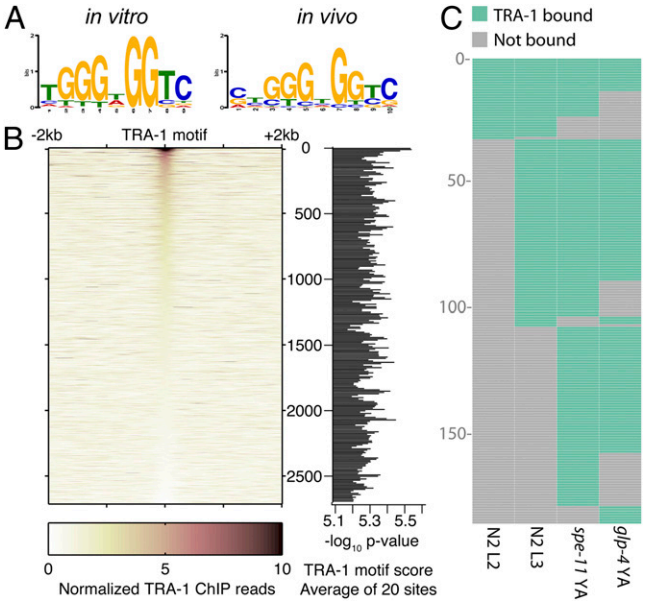

Fig. 2. Analysis of TRA-1–binding sites in the *C. elegans* genome. (*A*) Comparison of in vitro- and in vivo-derived TRA-1–binding motifs. (*B*) Analysis of TRA-1 occupancy at the 2,713 closest matches to the TRA-1 motif found in the *C. elegans* genome. Motifs are ordered by level of TRA-1 ChIP enrichment at the L3 stage. (*Left*) Normalized TRA-1 ChIP reads within a 4-kb window centered on each TRA-1 motif. (*Right*) Strength of match to TRA-1–binding site for TRA-1 motifs. Each bar represents an average score ($-\log_{10}$ of *P* value) for 20 motifs, sliding every 10 motifs. (*C*) Analysis of stage- and tissue-specific TRA-1 occupancy at 184 in vivo TRA-1–binding sites. Green boxes indicate a site was significantly bound ($P < 10^{-5}$; fold enrichment > 4) in at least one of two replicates performed for a given developmental condition.

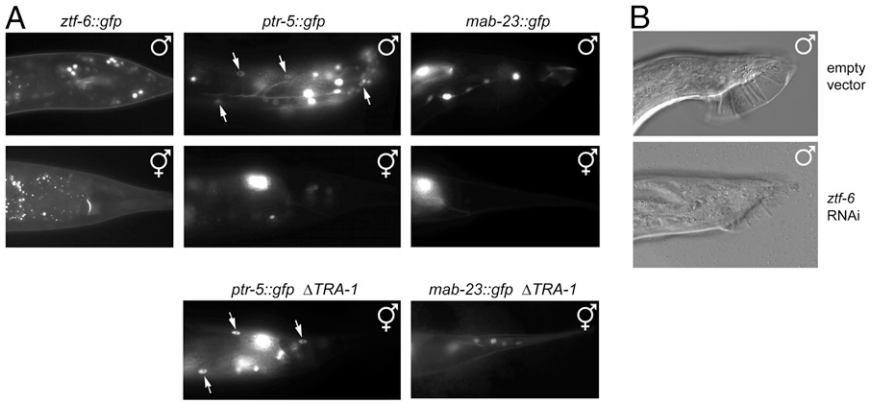

Fig. 3. Newly identified TRA-1 target genes exhibit male-specific expression patterns and reduction-of-function phenotypes. (*A*) Genomic intervals containing TRA-1–binding sites drive male-specific reporter gene expression. (*Left*) *ztf-6::gfp* is expressed male specifically in the developing tail. (*Center*) *ptr-5::gfp* is expressed male specifically in body wall (arrows) and stomatointestinal muscle. The sex specificity of this expression is dependent on the presence of a TRA-1 motif. (*Right*) *mab-23::gfp* is expressed male specifically in the tail, and the sex specificity of this expression is dependent on the presence of a TRA-1 motif. (*B*) RNAi of *ztf-6* results in defects in male tail development, including fused rays and a reduced fan.

suggesting that TRA-1 may act redundantly to repress *mab-23* or that hermaphrodites lack an activator required for robust expression. *mab-23* is one of three DM-domain transcription factors (along with *mab-3* and *dmd-3*) implicated in male sexual differentiation and directly regulated by TRA-1. Thus, transcriptional regulation of this protein family, whose members also regulate sex in *Drosophila* and in vertebrates (23), is a major mechanism by which TRA-1 regulates somatic sexual differentiation.

Because previously described TRA-1–binding sites generally lie within or adjacent to the gene that is being regulated, we speculate that most of the 184 TRA-1–binding sites will regulate an overlapping gene or one of the two immediately adjacent nonoverlapping genes. We identified 444 genes either containing or adjacent to a TRA-1–binding site and chose 34 to examine by RNAi (Dataset S1), based on their proximity to strong TRA-1–binding sites and predicted molecular functions consistent with a role in sexual differentiation. RNAi depletion of one of these genes, *ztf-6,* caused male tail defects (Fig. 3*B*), including fused rays and a reduced fan. Thus, *ztf-6*, *ptr-5*, and *mab-23* are likely direct TRA-1 target genes with roles in male sexual differentiation. Because this pilot screen examined expression patterns for only 20% of the 184 TRA-1–binding sites and RNAi phenotypes for only 8% of the 444 genes near these sites, it is highly likely that the ChIP data presented above can be used to find additional regulators of male differentiation.

TRA-1 Binds to and Regulates the Expression of Genes That Control Developmental Timing. Developmental timing in *C. elegans* is controlled by a complex network of heterochronic regulatory genes (24). Heterochronic mutations cause defects in multiple aspects of male sexual development, probably by disrupting the timing of critical cell divisions (25). Strikingly, among the roughly two dozen heterochronic genes, TRA-1 bound adjacent to six, abnormal dauer formation 12 (*daf-12*), protein kinase 20 (*kin-20*), *lin-4*, *lin-14*, *lin-28*, and *lin-42* (Fig. 4*A* and Dataset S1). DNA segments containing three of these sites, those adjacent to *lin-42*, *kin-20*, and *lin-4*, drove strong expression in lineages that undergo male-specific developmental programs, particularly the P10.p and B lineages of the developing tail (Fig. 4*B*). A *lin-28* reporter showed male-specific expression in body wall muscle, a sexually specialized cell type found in both sexes (Fig. 4*C*), and ablating the TRA-1–binding motif activated expression in hermaphrodite body wall muscle (Fig. 4*C*). These results collectively indicate that TRA-1 directly represses transcription of heterochronic genes in hermaphrodites.

TRA-1 Feeds Back onto the Sex Determination Pathway at Multiple Levels. We also identified TRA-1–binding sites representing two forms of feedback regulation on the sex determination pathway. First, the *tra-1* locus contained three TRA-1–binding sites (Fig. 5*A*). Genetic evidence supports *tra-1* autoregulation: The feminizing effect of dominant *tra-1* gain-of-function alleles can be reduced by adding wild-type copies of the gene (18), and many recessive hypomorphic *tra-1* alleles show equivalent masculinization when either homozygous or hemizygous (26). Second, we observed apparent feedback on upstream sex determination genes. TRA-1 can bind to a site in the *xol-1* promoter in vitro and regulates *xol-1* reporter transgenes via this site (27). ChIP-seq confirmed in vivo TRA-1 binding to this site (Fig. 5*A*) and also identified TRA-1–binding sites adjacent to feminization 3 (*fem-3*) and suppressor 26 (*sup-26*) (28), suggesting more extensive upstream feedback (Fig. 5 and Dataset S1; also see Fig. 7). A site near hermaphrodization 1 (*her-1*) also showed TRA-1 binding, but below the significance cutoff applied. All these upstream targets normally promote male development, suggesting that TRA-1 transcriptional repression provides multilevel reinforcement of the feminizing mode of the sex determination switch.

To explore feedback regulation further, we focused on *fem-3*. Regulation of *fem-3* in the XX germ line involves posttranscriptional repression (29), but expression of FEM-3 in somatic tissues has not been described. A FEM-3::GFP fosmid reporter was expressed in the vas deferens region of the male somatic gonad but was undetectable in the hermaphrodite gonad (Fig. 6*A*). Ablating the TRA-1 motif activated expression in the somatic gonad of the hermaphrodite, with robust expression restricted to the spermatheca (Fig. 6*A*), although this ectopic FEM-3 expression did not result in any obvious developmental defects. We also examined endogenous FEM-3 expression by immunofluorescence, which confirmed stronger expression in male than hermaphrodite soma (Fig. 6*B*). We conclude that somatic expression of *fem-3* involves transcriptional feedback regulation by TRA-1, which potentially functions to reinforce the global sex determination decision.

TRA-1 Targets Are Evolutionarily Conserved. TRA-1 homologs determine sex in other nematode species including *Pristionchus pacificus* (30) and *Caenorhabditis briggsae*, which are diverged from *C. elegans* by about 200 million years and 100 million years, respectively. Despite the evolutionary stability of the TRA-1 sex-determining function, it is unknown whether any genes regulated by TRA-1 in *C. elegans* are also regulated in other species, although sequence analysis has shown that TRA-1–binding motifs are present near the *C. briggsae* orthologs of some identified *C. elegans* TRA-1 targets, including *mab-3* and *fog-3* (9, 31). The TRA-1 antibody recognizes *C. briggsae* TRA-1 (15), so we performed ChIP-seq in *C. briggsae* L3 larvae and identified 78 CBR-TRA-1–binding sites (Dataset S1). CBR-TRA-1–binding sites were highly enriched for the TRA-1 motif, which appears to be identical in the two species and was found at 54 of 78 (69%) CBR-TRA-1–binding sites. For 64 of 78 CBR-TRA-1–binding sites, syntenic

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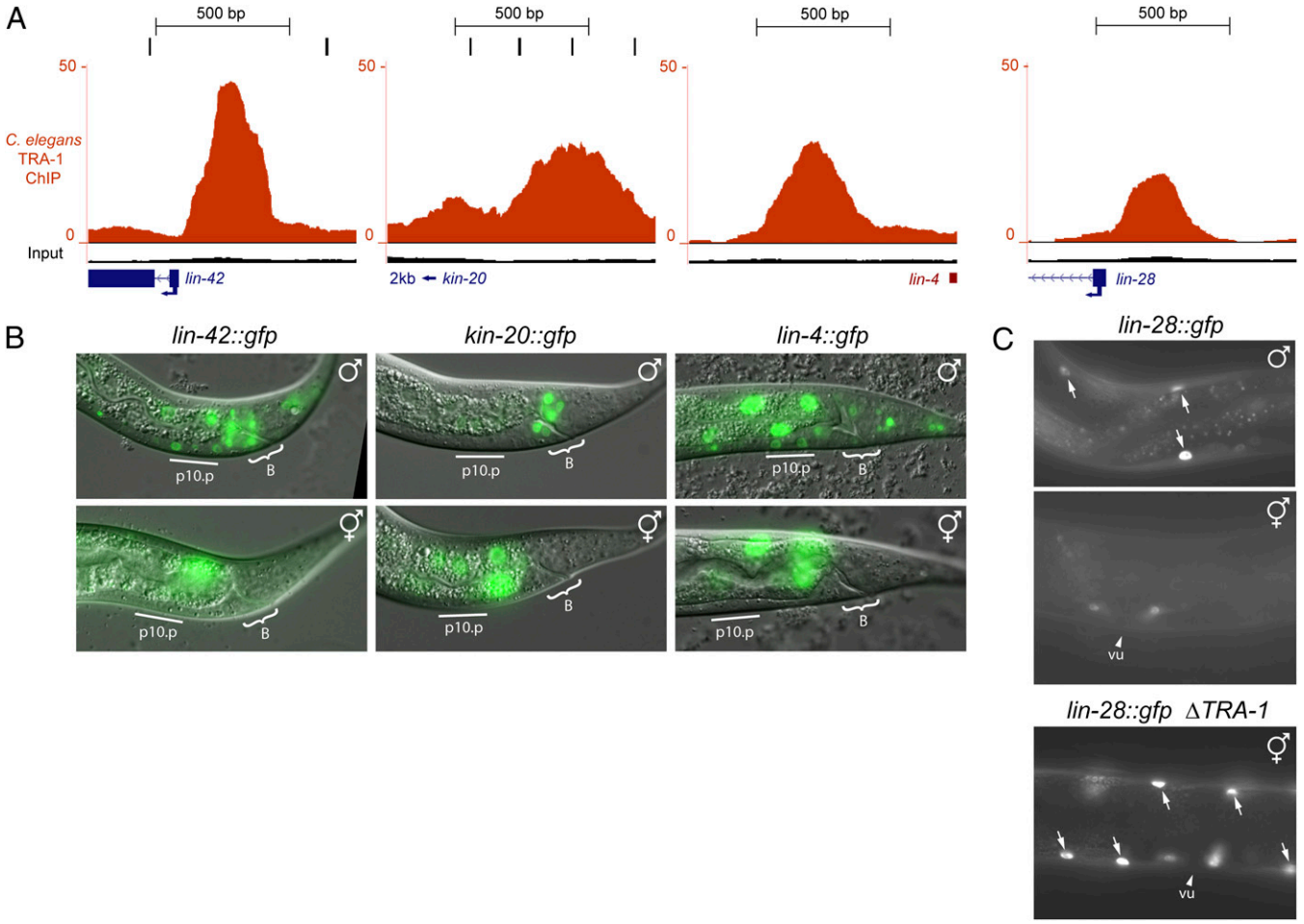

Fig. 4. TRA-1–binding sites near four heterochronic regulatory genes drive male-specific expression. (*A*) TRA-1 associates with chromatin near *lin-42*, *kin-20*, *lin-4*, and *lin-28*. TRA-1-ChIP (red) and input DNA tracks (black) are shown at same scale. Close matches to the TRA-1 consensus binding motif are indicated by vertical black bars above the ChIP trace. (*B*) Regions surrounding TRA-1–binding sites near *lin-42*, *kin-20*, and *lin-4* drive reporter gene expression in male-specific cell lineages in the tail, including the P10.p lineage (bar) and B lineage (bracket). (*C*) The region surrounding the TRA-1–binding site near *lin-28* drives male-specific expression in body wall muscle (arrows), and the sex specificity of this expression is dependent on the presence of a TRA-1 motif. Arrowheads indicate the position of the hermaphrodite vulva (vu).

alignments unambiguously identified a single region in the *C. elegans* genome corresponding to the bound region in *C. briggsae*. Of these 64 syntenic regions, 19 coincided with one of the 184 *C. elegans* TRA-1–binding sites (Dataset S1), suggesting substantial conservation of TRA-1 regulation through nematode evolution. Notably, sites representing feedback regulation of the

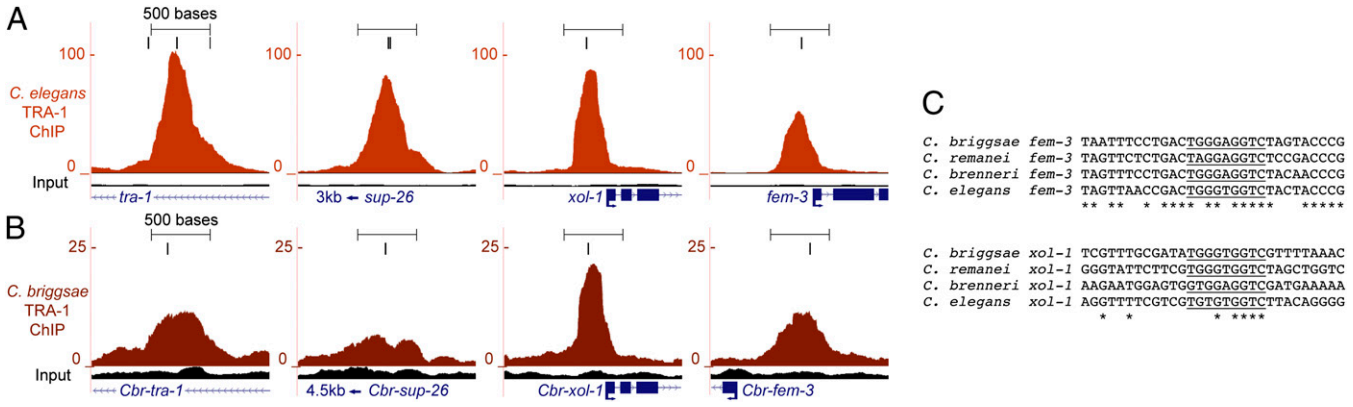

Fig. 5. TRA-1 feeds back on the sex determination pathway at multiple levels. (*A*) *C. elegans* TRA-1 binds near the *tra-1*, *xol-1*, *sup-26*, and *fem-3* loci. (*B*) *C. briggsae* TRA-1 binds near the *Cbr-tra-1*, *Cbr-xol-1*, and *Cbr-fem-3* loci. A site near *Cbr-sup-26* showed occupancy by CBR-TRA-1 but below the threshold we applied. (*C*) Alignments of TRA-1 motifs (underlined) identified near the *xol-1* and *fem-3* loci in *C. elegans*, *C. briggsae*, *C. brenneri*, and *C. remanei*.

sex determination pathway, including those adjacent to *tra-1*, *fem-3*, and *xol-1*, showed TRA-1 occupancy in both species (Fig. 5*B*). A site near *Cbr-sup-26* also showed weak CBR-TRA-1 binding, but this was below the significance cutoff. Likewise, TRA-1 binding adjacent to the heterochronic genes *daf-12*, *kin-20*, and *lin-14* also is conserved, suggesting that these binding events likely mediate biologically relevant regulation.

Discussion

Here we have used ChIP-seq to identify 184 binding sites of the nematode global sexual regulator TRA-1. A pilot analysis of genes adjacent to these sites identified seven TRA-1 target genes that appear to promote male sexual differentiation based on male-specific expression patterns and/or male-specific RNAi phenotypes. However, none of the targets we examined showed a hermaphrodite-specific reduction-of-function phenotype or expression pattern. Likewise, ChIP-seq indicated that TRA-1 feedback onto the upstream sex determination pathway occurs exclusively on male-promoting genes (Fig. 7), consistent with their repression by TRA-1 in hermaphrodites. Additionally, 12 of 256 (4.7%) coding genes (Dataset S1) adjacent to sites bound by TRA-1 at L2 or L3 showed male-biased expression in published L4 RNA-seq analysis (32), compared with 577 of 19,313 (3.0%) total coding genes with male-biased expression. In contrast, none of these 256 genes showed hermaphrodite-biased expression. We cannot exclude the possibility that TRA-1 can activate as well as repress transcription as do some other GLI proteins. However, our data support the view that TRA-1 functions primarily as a transcriptional repressor, generating sexually dimorphic gene expression by restricting the expression of its targets in the hermaphrodite.

Our data and previous genetic analyses (26, 33) indicate that TRA-1 has two distinct roles in regulating sexual development: It controls sex-specific cell and tissue differentiation by regulating

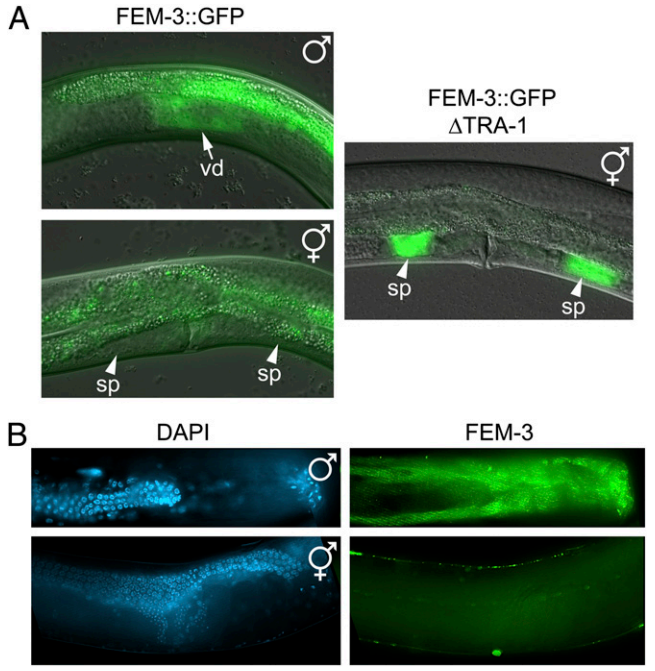

Fig. 6. TRA-1 regulates *fem-3* expression. (*A*) A FEM-3::GFP reporter is expressed in the vas deferens (vd) (arrow) of the male somatic gonad but shows no expression in the hermaphrodite somatic gonad. FEM-3::GFP ΔTRA-1 is expressed in the spermatheca (sp) (arrowheads) of the hermaphrodite somatic gonad. (*B*) FEM-3 antibody staining shows somatic expression of FEM-3 is normally restricted to males.

Fig. 7. Model for TRA-1 regulation of nematode sexual development. TRA-1 represses sex-determination genes that globally promote male development, including *xol-1*, *sup-26*, *fem-3*, and possibly *her-1*, as well as sexual differentiation genes that promote tissue-specific male sexual development, including *lin-28 and ptr-5*.

a suite of downstream effector genes, and it feeds back at multiple upstream steps to affect the primary sex determination decision (Fig. 7). TRA-1 binding near *fem-3* and *xol-1* has been conserved between *C. elegans* and *C. briggsae*, and sequences containing TRA-1–binding motifs also are conserved near these genes in *Caenorhabditis brenneri* and *Caenorhabditis remanei* (Fig. 5*C*). Reporter analysis and immunofluorescence confirmed the role of TRA-1 as a repressor of *fem-3* transcription in the hermaphrodite soma. Multicopy transgenes do not normally express in the germ line, so we have not yet examined whether this transcriptional feedback also occurs in germ cells; however, the mutually exclusive germ-line distribution of nuclear TRA-1 and FEM-3 (15) suggests that it may. Somatic FEM-3 expression has not been shown previously (34) but is supported by genetic data: Somatic feminization of *tra-1(gf)* males is suppressed by gain-of-function mutations in the *fem-3* 3′ UTR that do not affect *fem-3* transcript levels (26). Thus, FEM-3 activity is regulated through at least three separate means: The transmembrane receptor protein TRA-2 represses FEM-3 protein activity via physical interaction (35), *fem*-3 mRNA is negatively regulated via the 3′ UTR (36), and we find that *fem-3* also is transcriptionally repressed by TRA-1. Therefore *fem-3* appears to be an important node for control of the sex determination pathway. Feedback from TRA-1 may serve at least two purposes. First, we speculate that it renders the global sex-determination decision more robust and helps ensure the coordinated execution of downstream sexual differentiation events. Second, feedback loops might provide additional inputs to control the temporary switch from female to male sex determination that occurs in hermaphroditic species during germ-line development.

The TRA-1–binding sites we have identified provide a resource for identifying and functionally defining the suite of genes that control sexual differentiation in nematodes. The restricted male-specific expression patterns and limited male-specific developmental defects for putative target genes are consistent with the view that TRA-1 controls sexual development by regulating many minor players in male development rather than a few major ones. To evaluate fully the roles these candidate target genes may play in sexual development, it will be important to examine possible functional overlaps, such as those that occur between *mab-3* and *dmd-3* (10), as well as to perform phenotypic analysis in sensitized backgrounds and at finer scale.

Sex-determination pathways impose sex specificity on the spatial and temporal control of development in animals with diverse body plans, behaviors, and physiologies. The conservation of TRA-1–binding between *C. elegans* and *C. briggsae* suggests substantial evolutionary conservation of sexual development within nematodes. However, TRA-1 is among the fastest evolving genes in Caenorhabditids (37), and its orthologs do not appear to regulate sex determination outside nematodes. *DMRT* genes (orthologs of the TRA-1 targets *mab-3*, *mab-23*, and *dmd-3*) determine sex in many other animals including insects and vertebrates (38). Comparing how TRA-1 and *DMRT* genes overlay sex specificity on development therefore may shed considerable light on the evolution of sexual development in metazoans.

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Materials and Methods

Worm Strains and Culture. The following strains and alleles were obtained from the *Caenorhabditis* Genetics Center: wild-type *C. elegans* (N2), wild-type *C. briggsae* (AF16), LG I: *spe-11(hc77)*, *glp-4(bn2)*, LG II: *rrf-3(pk1426)*, LG III: *tra-1(e1834)*, LG IV: *him-8(e1489)*, hT2[*bli-4(e937)* let-?(*q782*) qIs48] (I;III). Animals were maintained in nematode growth medium plates with added bacteria (*Escherichia coli* strain OP50) at 22 °C, unless otherwise indicated.

ChIP-seq and Bioinformatics Analysis. Detailed description is provided in *SI Materials and Methods*. Raw sequencing reads and processed data for genome browser visualization are available at the Gene Expression Omnibus (GEO) website, www.ncbi.nlm.nih.gov/geo, under accession ID GSE48917.

Expression Analysis. The 500- to 1,000-bp regions surrounding TRA-1–binding sites were cloned into pPD107.94 (39). ΔTRA-1 reporters were generated by replacing the invariant GG in the gggtGGtc consensus binding motif with AA, using the Agilent QuikChange XL site-directed mutagenesis kit. FEM-3::GFP was generated by recombineering fosmid WRM067dB02 according to ref. 40, with the *gfp* coding sequence replacing the *fem-3* stop codon. FEM-3::GFP ΔTRA-1 also was generated by recombineering according to ref. 40, making the same change described above. Reporter constructs were injected with *str-1::gfp* as a coinjection marker (5–20 ng/μL reporter, 20 ng/μL *str-1::gfp*, and sheared N2 genomic DNA to 100 ng/μL total DNA) into *him-8(e1489)* animals. Stable transgenic lines were assayed for *gfp* expression.

RNAi. *him-8(e1489)*; *rrf-3(pk1426)* animals were used for all RNAi experiments, which were performed according to the standard *C. elegans* feeding RNAi protocol (wormbook.org). L4 hermaphrodite animals were placed on standard RNAi plates, and their progeny were scored by differential interference contrast microscopy for defects in sexual development.

FEM-3 Antibody Staining. Antibody staining was as described in ref. 41. Wild-type animals were analyzed as young adults. FEM-3, ref. 15, antibody was used at a 1:200 dilution.

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