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dmd-3, a *doublesex*-related gene regulated by *tra-1*, governs sex-specific morphogenesis in *C. elegans*

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Although sexual dimorphism is ubiquitous in animals, the means by which sex determination mechanisms trigger specific modifications to shared structures is not well understood. In *C. elegans,* tail tip morphology is highly dimorphic: whereas hermaphrodites have a whip-like, tapered tail tip, the male tail is blunt-ended and round. Here we show that the male-specific cell fusion and retraction that generate the adult tail are controlled by the previously undescribed *doublesex*-related DM gene *dmd-3*, with a secondary contribution from the paralogous gene *mab-3*. In *dmd-3* mutants, cell fusion and retraction in the male tail tip are severely defective, while in *mab-3; dmd-3* double mutants, these processes are completely absent. Conversely, expression of *dmd-3* in the hermaphrodite tail tip is sufficient to trigger fusion and retraction. The master sexual regulator *tra-1* normally represses *dmd-3* expression in the hermaphrodite tail tip, accounting for the sexual specificity of tail tip morphogenesis. Temporal cues control the timing of tail remodeling in males by regulating *dmd-3* expression, and Wnt signaling promotes this process by maintaining and enhancing *dmd-3* expression in the tail tip. Downstream, *dmd-3* and *mab-3* regulate effectors of morphogenesis including the cell fusion gene *eff-1*. Together, our results reveal a regulatory network for male tail morphogenesis in which *dmd-3* and *mab-3* together occupy the central node. These findings indicate that an important conserved function of DM genes is to link the general sex determination hierarchy to specific effectors of differentiation and morphogenesis.

KEY WORDS: Sexual dimorphism, Sex determination, Sexual differentiation, Sex differences, DM domain, DMRT, Cell fusion, Developmental timing, Selector gene

INTRODUCTION

The presence of two morphologically distinct sexes is a nearly universal characteristic of animal species. To bring about sex differences in form, the sex-determination mechanism must regulate specific changes in differentiation and morphogenesis. However, largely as a result of the enormous variation in animal sex-determination pathways, the mechanisms that integrate sexual information with other developmental pathways remain poorly described.

In the nematode *C. elegans*, one of the most prominent sexual dimorphisms is in tail tip morphology. Although the hermaphrodite tail tip is whip-like, the male tail is blunt-ended and harbors several copulatory structures (Sulston et al., 1980; Emmons, 2005). In larvae of both sexes, the tail tip comprises four nested larval cells, hyp8 through hyp11 (referred to here as hyp8-11). In hermaphrodites, this architecture remains static throughout development. By contrast, the male tail tip undergoes dramatic remodeling in the final (L4) larval stage (Sulston et al., 1980; Nguyen et al., 1999). During this morphogenesis, hyp8-11 fuse together and retract anteriorly to form a rounded tail tip. Subsequently, the distinct process of anterior tail retraction occurs, in which the entire tail is pulled anteriorly, leaving the elongated rays in its wake (Sulston et al., 1980; Nguyen et al., 1999).

Defects in male tail tip retraction result in a pointed or 'leptoderan' (Lep) tail tip phenotype (Nguyen et al., 1999). Previous work has shown that mutations in both Wnt signaling and the heterochronic (developmental timing) pathways result in Lep phenotypes. Mutations in the Wnt gene *lin-44* cause weak tail tip retraction defects (Zhao et al., 2002); additionally, loss of TLP-1, an Sp1-family Zn-finger factor that may act downstream of Wnt signaling, causes pronounced failure of tail tip morphogenesis (Zhao et al., 2002). In males carrying a gain-of-function allele of the heterochronic gene *lin-41* (Slack et al., 2000), the retraction program is delayed, resulting in a partially unretracted tail tip. By contrast, *lin-41(lf)* males have an over-retracted tail, with premature retraction initiating in L3 (Del Rio-Albrechtsen et al., 2006). However, the means by which Wnt signaling and developmental timing converge on tail morphogenesis are not clear. Moreover, the mechanism that brings sex-specificity to tail morphogenesis is unknown.

All sex differences in *C. elegans* ultimately arise from sex chromosome content: XX in hermaphrodites, X0 in males (Brenner, 1974; Madl and Herman, 1979). Downstream of this primary cue, a regulatory hierarchy controls the activity of the master sexual regulator TRA-1A, a Gli-family transcriptional repressor (Hodgkin, 1987; Zarkower and Hodgkin, 1992). *tra-1* activity is necessary and sufficient to generate essentially all somatic sexual dimorphism. Though TRA-1A is expressed in both sexes, it is fully active only in hermaphrodites, where it represses male-specific genes (Zarkower, 2006). Only three direct targets of TRA-1A in the soma are known: *mab-3* in the intestine (Yi et al., 2000), *egl-1* in the HSN neurons (Conradt and Horvitz, 1999) and *ceh-30* in the CEM neurons (Peden et al., 2007; Schwartz and Horvitz, 2007). However, these targets account for only a small subset of sex-specific development and control single-cell-level processes (yolk production and cell death). By contrast, it is not known how *tra-1* specifies sex-specific organogenesis, where sexual information must regulate cell fate, differentiation and morphogenesis.

Despite the great variety in sex-determination pathways of animal species, the conservation of DM family genes indicates that these mechanisms may derive from a common ancestor. The DM domain is an unusual DNA-binding Zn finger initially identified in the

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Drosophila sex-determination gene *doublesex* and the *C. elegans* sexual differentiation gene *mab-3* (Erdman and Burtis, 1993; Raymond et al., 1998). Genes of this family have since been implicated in sex-specific development across the animal kingdom. Interestingly, DM genes act at a variety of points in these pathways, from very early steps [e.g. *DMY* is the primary sex determining cue in Medaka (Matsuda et al., 2002; Matsuda et al., 2007)] to later sex-specific differentiation [e.g. *DMRT1* is necessary for differentiation of testes and the germline in mice (Kim et al., 2007a; Kim et al., 2007b)]. As a result, the nature of the ancestral conserved function of DM genes in sex determination and differentiation remains unclear.

In *C. elegans*, only two of the 11 DM genes predicted from genome sequence, *mab-3* and *mab-23*, have been characterized. As a direct target of *tra-1*, *mab-3* represses yolk production in the male intestine (Shen and Hodgkin, 1988; Yi et al., 2000). *mab-3* is also necessary for the male-specific expression of *lin-32*, a gene that triggers development of the male-specific sensory rays (Zhao and Emmons, 1995; Portman and Emmons, 2000), though this function seems to be indirectly regulated by *tra-1* (Ross et al., 2005). *mab-23* is also necessary for a variety of male-specific events, including ray sensory neuron patterning and male-specific muscle differentiation (Lints and Emmons, 2002). These sex-specific functions of *mab-23* also seem to be indirectly regulated by *tra-1.* Whether additional DM genes control other sex-specific characteristics in *C. elegans* is unknown, as is the extent to which DM genes act as the primary effectors of *tra-1* function.

Here, we find that a previously uncharacterized DM gene, *dmd-3*, is necessary for male-specific morphogenesis of the tail tip. Moreover, supplying *dmd-3* to the hermaphrodite tail is sufficient to bring about male-like morphogenesis. By coordinating sexual, temporal and spatial information, *dmd-3* occupies a crucial node in the regulatory network that coordinates tail remodeling. In addition, *mab-3* plays a secondary, partially redundant, role in tail tip morphogenesis. *dmd-3* and *mab-3* trigger at least two independent processes necessary for morphogenesis, including the male-specific expression of the cell fusogen EFF-1. Together, our studies identify a crucial role for two DM genes in a genetic mechanism that couples sex determination to the sex-specific modification of a set of shared cells.

MATERIALS AND METHODS

C. elegans genetics

Nematode culture was carried out as described (Brenner, 1974). The following mutations were used: *lin-44(n1792)*, *lin-41(bx42)*, *lin-41(bx37)*, *lin-41(ma104)*, *mab-3(e1240)*, *eff-1(ok1021)*, *tra-1(e1099)*, *pha-1(e2123)*, *tlp-1(bx85)*, *dmd-3(ok1327)*, *dmd-3(tm2863)*, *him-5(e1490)*, *mab-23(bx118)* and *ozDf2*. *lin-41(bx37)* and *lin-41(bx42)* were provided by D. Fitch (New York University) and *dmd-3(tm2863)* was provided by the National Bioresource Project (S. Mitani, Tokyo Women's Medical University). All other mutants were obtained from the Caenorhabditis Genetics Center. All strains except those carrying *tra-1(e1099)* contained *him-5(e1490)* to increase the frequency of spontaneous males.

Transgenes

The following integrated transgenic strains were used in this study: *syIs78* [*AJM-1*::*GFP, unc-119*(+)], *fsIs2* [*dmd-3*::*YFP, cc*::*GFP*], *fsIs3* [*dmd-3*::*YFP*, *cc*::*GFP*], *fsIs7* [pUR13 ($E(ht)_{\Delta TRA\text{-}1}$::*DMD-3*::*GFP*), *cc*::*GFP*], *fsIs9* [pUR12 (*E(ht)*::*DMD-3*::*GFP*), *cc*::*GFP*], *fsIs10* [pUR15 (*MAB-3*::*GFP)*, *cc*::*GFP*] and *fsIs12* [pUR14 ($E(ht)_{TRA\text{-}1\text{-}G\rightarrow A}$::*DMD-3*::*GFP*), cc::GFP]. The following extrachromosomal transgenic strains were used in the study: *fsEx110* [pUR18(*E(ht)*::nlsGFP, pBX1(*pha-1*+)], *fsEx114* [pUR4 (*E(ht)*::*GFP*), pBX1 (*pha-1(+)*)], *fsEx118* [pUR5 ($E(ht)_{\Delta TRA\text{-}1}$::*GFP*), pBX1(*pha-1(+)*)], *fsEx135* [pJDC41(*EFF-1*::*GFP* [translational]), pBX1(*pha-1(+)*)], *fsEx136* [pJE3(*eff-1*::*GFP* [transcriptional]), *fsEx154* [*DMD-3*::*YFP*, *cc*::*GFP*], *fsEx182* [pUR25($E(ht)_{\Delta dmd\text{-}3\Delta mab\text{-}3}$::nlsGFP, pBX1(*pha-1*+)], *fsEx183* [pUR17 (*EFF-1*::*GFP*::*mCherry*), pBX1(*pha-1*+)], *fsEx184* [pUR27 ($EFF\text{-}1_{\Delta dmd\text{-}3\Delta mab\text{-}3a\Delta mab\text{-}3b}$::*GFP*::*mCherry*), pBX1(*pha-1*+)]. pJDC41 and pJE3 were generously provided by W. Mohler (Mohler et al., 2002; del Campo et al., 2005). All conclusions drawn in the text were supported by additional independently derived transgenes.

dmd-3 alleles

The *dmd-3(ok1327)* deletion removes 926 bp, including the 3′ end of the final *dmd-3* exon, leaving a predicted mutant protein in which the 50 C-terminal residues are replaced with five novel amino acids. This deletion also removes much of the 3′ UTR of *dmd-3*. In *dmd-3(tm2863),* a 407 bp region that comprises much of exons 2 and 3 is replaced with a 10 bp insertion (Fig. 1A). This results in a predicted open reading frame encoding 55 N-terminal amino acids (including all but six amino acids of the first DM domain) followed by 12 novel amino acids and a stop codon. Thus, *dmd-3(tm2863)* is likely to be a molecular null allele. Both *dmd-3(ok1327)* and *dmd-3(tm2863)* are recessive, and have essentially identical male tail defects.

dmd-3 RNAi

Double-stranded *dmd-3* RNA was prepared as previously described (Fire et al., 1998) and injected into young adult hermaphrodites. F1 adult male and hermaphrodite offspring were examined for phenotypes.

DNA constructs

For the *dmd-3*::*YFP* transcriptional reporter, a genomic fragment from –4264 to +549 bp with respect to the *dmd-3* start codon was amplified. For the *DMD-3*::*YFP* translational reporter, a genomic fragment from –4264 to +4674 bp was amplified. These fragments were fused to *YFP*-coding sequence by overlap extension PCR (Boulin et al., 2006). A transcriptional *mab-3*::*GFP* reporter (pUR11) was generated by cloning a *mab-3* genomic fragment (–8786 to +12 bp) into pPD107.94. A translational *MAB-3*::*GFP* reporter (pUR15) was generated by inserting the *mab-3*-coding region (+13 to +3868 bp) into pUR11.

To generate *E(ht)*::*GFP*, the –2740 to –1595 region of the *dmd-3* promoter was first cloned into pPD107.94 to generate pUR18. The NLS was then removed by digesting with *Kpn*I and religating to generate *E(ht)*::*GFP* (pUR4).

To mutate the putative TRA-1A binding site within *E(ht)*, the 3′ end of the TRA-1A site was replaced with a *Sal*I site by cloning two PCR products into the *Sph*I and *Xba*I sites in pPD107.94 (WT TRA-1A site: TTTCTGTGTGGGTGTTC, mutant site: TTTCTGTGTGTCGACTC). The NLS was removed to generate $E(ht)_{\Delta TRA\text{-}1}$::*GFP* (pUR5). A point mutation in the TRA-1A site was generated with the QuickChangeII-XL Site-Directed Mutagenesis Kit (Stratagene) using complementary primers that changed the pUR5 TRA-1A site to TTTCTGTGTGAGTGTTC to generate $E(ht)_{TRA\text{-}1\text{-}G\rightarrow A}$::*GFP* (pUR10).

To express *dmd-3*(+) from *E(ht)*::*GFP*, the *dmd-3*-coding sequence (with GAAAAA added upstream of the start codon to aid translation) was cloned into pUR4 to generate *E(ht)*::*DMD-3*::*GFP* (pUR12). To put *DMD-3*::*GFP* downstream of the mutant TRA-1 sites, the wild-type *E(ht)* fragment was removed from pUR12 and replaced with an *Sph*I-*Xba*I fragment from pUR5 or pUR10 to generate $E(ht)_{\Delta TRA\text{-}1}$::*DMD-3*::*GFP* (pUR13) and $E(ht)_{TRA\text{-}1\text{-}G\rightarrow A}$::*DMD-3*::*GFP* (pUR14), respectively.

The putative DMD-3 and MAB-3 binding sites in *E(ht)*, at –2516 and –2465, respectively, were mutated using pUR18 as the starting template. The putative DMD-3 site was changed from TGTAACA to TGGATCC and the putative MAB-3 site was changed from CCCAACA to CTCGAGA to generate pUR25.

To generate an operon containing the *EFF-1*::*GFP* translational reporter followed by an *mCherry* transcriptional reporter, an outron and *mCherry* sequence were inserted into the *EFF-1*::*GFP* translational reporter 24 bp downstream of the GFP stop codon and 105 bp upstream of the *unc-54* 3′ UTR. To generate this construct, a *Not*I site was inserted at this position by mutating pJDC41 from CCGGTCGC to GCGGCCGC to generate pUR16. The outron and *mCherry*-coding sequence were amplified from pENTRY-*Srf*I-mCherry (a gift from J. White and E. Jorgensen) and cloned into the *Not*I site to generate pUR17.

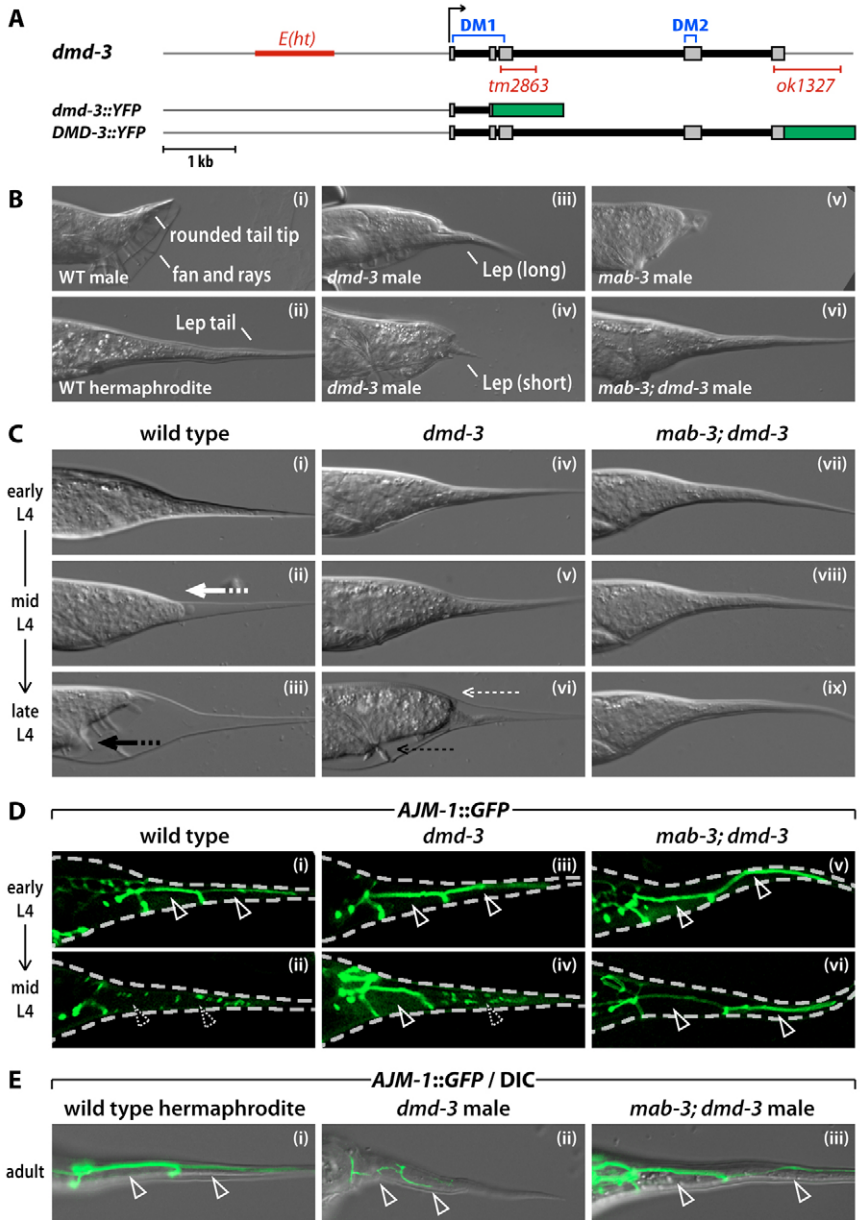

Fig. 1. *dmd-3* acts with *mab-3* to direct cell fusion and retraction of the male tail. (**A**) The *dmd-3* locus and *dmd-3* reporter genes. Thin gray lines indicate the *dmd-3* promoter and 3′ UTR; gray boxes mark exons. The two DM domains, the *E(ht)* enhancer element and the deletions in the *dmd-3(tm2863*) and *dmd-3(ok1327)* alleles are indicated. (**B**) DIC images of the tails of a wild-type adult male (i), wild-type adult hermaphrodite (ii), *dmd-3(ok1327)* adult male (iii,iv), *mab-3(e1240)* adult male (v) and *mab-3(e1240); dmd-3(ok1327)* adult male (vi). Representative examples of the two classes (short and long) of Lep tails observed in *dmd-3* males are shown (iii,iv). (**C**) Individual wild type (i-iii), *dmd-3* mutant (iv-vi) and *mab-3; dmd-3* double mutant (vii-ix) L4 males were observed periodically by DIC microscopy. Tail-tip retraction is indicated by a bold white arrow; anterior tail retraction is indicated by a bold black arrow. Thin white and black arrows indicate the partial tail tip retraction and partial anterior tail retraction, respectively, seen in *dmd-3* larvae. (**D**) Confocal images of wild-type (i,ii), *dmd-3* mutant (iii,iv) and *mab-3; dmd-3* mutant L4 males (v,vi) expressing the apical junction marker *AJM-1*::*GFP*. The broken line indicates the larval cuticle. Solid arrowheads indicate intact cell boundaries; broken arrowheads indicate cell fusions. (**E**) *AJM-1*::*GFP*/DIC images of the unretracted tail tips of an adult hermaphrodite (i), an adult *dmd-3* male (ii) and an adult *mab-3; dmd-3* male (iii). Arrowheads indicate intact cell boundaries.

The putative DMD-3 binding site (at position –4121 in the *eff-1* promoter) and the two putative MAB-3 binding sites (–5501 and –3201) were mutated using pUR17 as the starting template. The putative DMD-3 site was changed from TGCAACA to TGCATGC and the putative MAB-3 sites were changed from CGCAACA to CGGATCC to generate pUR27. Unexpectedly, pUR27 also contained a 11 bp deletion (–3892 to –3882) that does not appear to affect *EFF-1*::*GFP* expression.

RT-PCR and RACE

The sequence of the *dmd-3* cDNA was determined from RT-PCR products generated using Superscript III One-step RT-PCR w/ Platinum Taq (Invitrogen). The 5′ end of the *dmd-3* cDNA was determined by sequencing PCR products generated using the 5′RLM-RACE protocol from the First Choice RLM-Race Kit (Ambion).

Microscopy

Images were obtained using a Zeiss Axioplan 2 with epifluorescence illumination and ApoTome structured illumination (Carl Zeiss Microimaging) or by confocal microscopy using a Leica TCS NT. Digital images were processed using Adobe Photoshop. L4 larvae were staged according to linker cell migration, tail tip retraction and anterior tail retraction. In early L4, the linker cell has just completed migrating to the ventral side and the tail tip cells hyp8-11 are unfused and unretracted. In early mid-L4, the linker cell has progressed roughly halfway from its ventral turn to the hindgut, and the tail tip is unfused and unretracted. By mid-L4, the linker cell has migrated completely to the hindgut and the tail tip is undergoing cell fusion but not retraction. In late mid-L4, the tail tip is fully fused and retraction is under way. In early late L4, the tail tip is fully retracted but anterior retraction has not yet begun. In late L4, anterior retraction is under way, generating elongated rays and the fan.

MH27 antibody staining

Mid-L4 *fsIs7; him-5* and *fsIs9; him-5* larvae were permeabilized by freeze-cracking (Hurd and Kemphues, 2003) and fixed with methanol (Miller and Shakes, 1995). Larvae were stained with the anti-AJM-1 antibody MH27 (Developmental Studies Hybridoma Bank, University of Iowa) (Francis and Waterston, 1991; Koppen et al., 2001) followed by Texas Red-labeled goat anti-mouse IgG (Jackson ImmunoResearch).

Table 1. Tail tip retraction defects in adult males*

Genotype†	*n*	Wild type	Mab non-Lep	Lep (short)	Lep (long)	Unretracted
Wild type	51	100	0	0	0	0
dmd-3(ok1327)	136	0	0	38	62	0
dmd-3(tm2863)	59	0	0	44	56	0
dmd-3(ok1327); fsIs9‡	35	86	14	0	0	0
tra-1(e1099)§	31	100	0	0	0	0
tra-1(e1099); dmd-3(ok1327)§	102	0	0	7	93	0
mab-3(e1240)	26	0	92	8	0	0
mab-3(e1240); dmd-3(ok1327)	60	0	0	0	0	100
mab-3(e1240); dmd-3(ok1327); fsIs10¶	52	0	0	0	88	12

*Numbers indicate the percentage of animals that fell into each classification. *n*, number of animals scored. Mab non-Lep indicates animals that did not display tail tip retraction defects but that did have other 'male abnormal' (Mab) tail phenotypes. Lep (short) indicates animals with a short partially retracted tail tip (e.g. Fig. 1B, part iv). Lep (long) indicates animals with a long partially retracted tail tip (e.g. Fig. 1B, part iii). Unretracted indicates animals with a completely unretracted tail tip (e.g. Fig. 1B, part vi).
†All strains except those carrying *tra-1(e1099)* also included the mutation *him-5(e1490)*.
‡*fsIs9* is *E(ht)*::*DMD-3*::*GFP*.
§Animals carrying *tra-1(e1099)* are XX pseudomales.
¶*fsIs10* is *MAB-3*::*GFP*.

RESULTS

dmd-3 and *mab-3* are necessary for male-specific tail tip morphogenesis

We identified the previously uncharacterized *C. elegans* gene Y43F8C.10 in microarray studies of gene expression in the male tail sensory rays (Portman and Emmons, 2004). Y43F8C.10 is predicted to encode a 251-amino acid protein with two DM domains (Fig. 1A). We renamed this gene *dmd-3*, as the third characterized *C. elegans* DM-domain gene (Raymond et al., 1998; Lints and Emmons, 2002). Because DM domains have been implicated in sex-specific development, we examined hermaphrodites and males carrying the *dmd-3* deletion alleles *dmd-3(ok1327)* and *dmd-3(tm2863)* (Fig. 1A). *dmd-3* mutant hermaphrodites appeared morphologically wild type. By contrast, *dmd-3(ok1327)*, *dmd-3(tm2863)* and *dmd-3(RNAi)* males displayed marked abnormalities in tail morphology. Most notably, they possessed partially unretracted Lep tail tips reminiscent of those of adult hermaphrodites (Fig. 1B, Table 1; data not shown). This phenotype resulted from a developmental defect in tail tip retraction: the hypodermal cells hyp8-11 failed to pull back completely from the larval cuticle (Fig. 1C). In addition, the anterior region of the tail failed to retract completely in *dmd-3* mutants (Fig. 1B,C). We observed similar partial retraction defects in *dmd-3(ok1327)*, *dmd-3(tm2863)*, *dmd-3(RNAi)* and hemizygous *dmd-3(ok1327)*/*ozDf2* males (Table 1; data not shown), indicating that this is probably the null phenotype.

Because null mutations in the DM gene *mab-3* have been reported to cause minor low-penetrance tail-tip retraction defects (Shen and Hodgkin, 1988), we constructed *mab-3; dmd-3* double mutants (Fig. 1B,C; Table 1). Strikingly, the *mab-3; dmd-3* adult male tail was almost identical to that of a hermaphrodite, with a long, smoothly tapered tail tip and no fan or rays. No tail tip or anterior retraction movements were seen in *mab-3; dmd-3* L4 males. Thus, *dmd-3* and *mab-3* act in a partially redundant fashion to bring about male tail tip morphogenesis, with *dmd-3* playing the primary role. We also examined *mab-23; dmd-3* double mutants, but found no indication that *mab-23* also acts redundantly in tail tip morphogenesis (data not shown). Interestingly, the consensus DNA binding site for DMD-3, as determined by in vitro site-selection experiments (M. Murphy and D. Zarkower, personal communication) closely matches that of MAB-3 (Yi and Zarkower, 1999), suggesting that the partial functional redundancy between *dmd-3* and *mab-3* arises from an ability to regulate common downstream targets.

Tail tip retraction is preceded by the male-specific fusion of hyp8-11 (Nguyen et al., 1999). In wild type, 100% of mid-L4 males (*n*=28) showed hyp8-11 fusion, compared with 0% of hermaphrodites (*n*=41). However, in *dmd-3* single mutant males, we observed cell fusion in only 44% of mid-L4 males (*n*=50), and these fusions usually occurred only between hyp9 and hyp10 (Fig. 1D). Again, the loss of *mab-3* enhanced this phenotype, such that no hyp8-11 fusions were detectable in mid-L4 *mab-3; dmd-3* males (0% of animals showed cell fusion; *n*=35) (Fig. 1D). By contrast, cell fusion occurred normally in *mab-3* single mutants (100%; *n*=40). In both *dmd-3* and *mab-3*; *dmd-3* males, cell boundaries often persisted into adulthood (Fig. 1E). Thus, *dmd-3* acts with *mab-3* to coordinate both tail tip cell fusion and retraction.

dmd-3 and *mab-3* are expressed male specifically in the tail tip coincident with retraction

To better understand how *dmd-3* mediates tail tip morphogenesis, we constructed transcriptional (*dmd-3*::*YFP*) and translational (*DMD-3*::*YFP*) reporter genes (Fig. 1A). These reporters exhibited essentially identical cellular expression patterns, and *DMD-3*::*YFP* was able to rescue the *dmd-3* tail phenotype (data not shown). In males, we found that *dmd-3*::*YFP* was expressed in a number of sexually dimorphic or sex-specific cells, including the tail tip, hindgut, B lineage, ray RnA neurons and somatic gonad (Fig. 2A,C). By contrast, hermaphrodites exhibited strong *dmd-3*::*YFP* expression only in the anchor cell (not shown), a hermaphrodite-specific somatic gonad cell that induces development of the vulva (Kimble, 1981). Non-sex-specific expression of these reporters was weak, occurring primarily in the body hypodermis. In addition, expression in phasmid neurons of both sexes was sometimes seen during L3 and L4 (not shown).

In hyp8-11, *dmd-3*::*YFP* expression was male specific and coincided with morphogenesis (Fig. 2B,C; see Materials and methods for a description of L4 sub-stages). Tail tip expression initiated in early-mid L4 males, first in hyp8, hyp9 and hyp11, and shortly thereafter in hyp10 (Fig. 2C). We occasionally observed weak expression in hyp9 in late L3 males (see Fig. 4B). Expression levels peaked during tail tip retraction and decreased rapidly upon its completion. *dmd-3* was also expressed in hyp13, a male-specific, bi-nucleated hypodermal cell thought not to have a role in tail tip morphogenesis (D. H. A. Fitch, personal communication) (Nguyen et al., 1999). Importantly, *dmd-3*::*YFP*

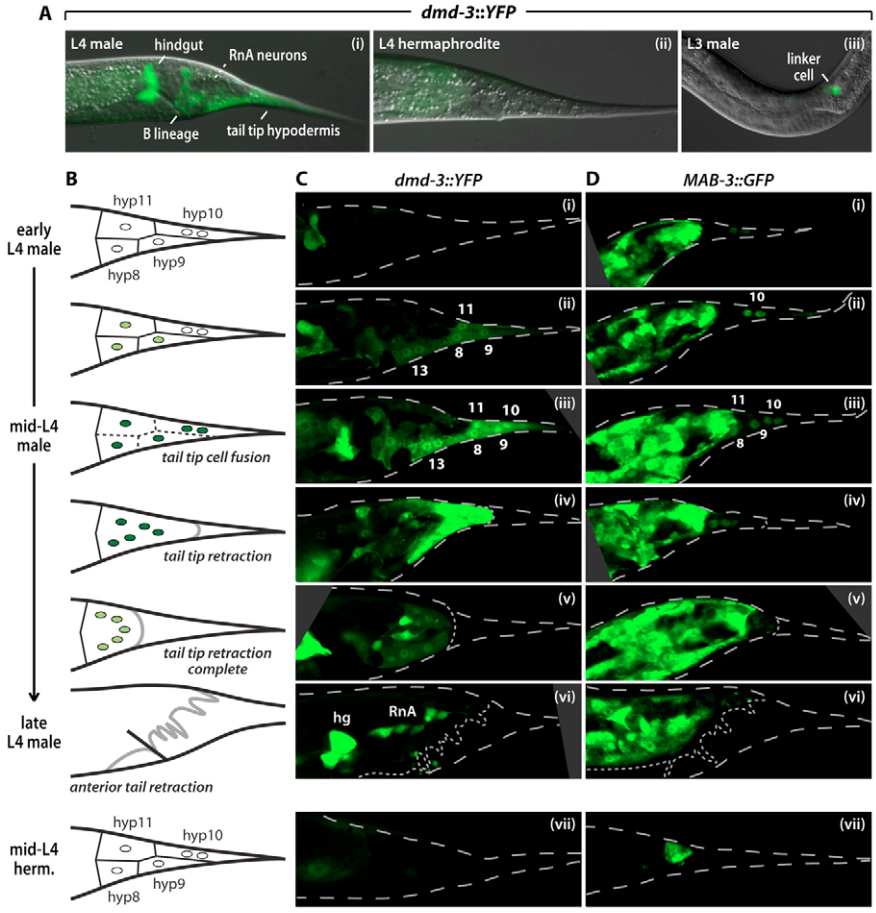

Fig. 2. *dmd-3* and *mab-3* reporters are sex-specifically expressed in the tail tip hypodermis. (**A**) *dmd-3*::*YFP* (*fsIs2*) expression in an L4 male (i) demonstrating expression in the hindgut, B lineage, tail tip (hyp8-11) and hyp13. The future position of the *dmd-3*-expressing RnA neurons is marked with a broken white line. An L4 hermaphrodite carrying *dmd-3*::*YFP* (ii) demonstrates the male-specificity of expression. In late-L3 (iii) and L4 males, *dmd-3*::*YFP* is expressed in the linker cell of the developing male gonad. (**B**) Schematic diagrams of the progression of tail tip morphogenesis. Circles indicate tail tip hypodermal cells; green shading reflects the intensity of *dmd-3* expression. Solid lines between hypodermal cells indicate intact boundaries and broken lines indicate cell fusion events. The thick black line represents the L4 cuticle, whereas the thick gray line indicates the newly formed adult cuticle. See Materials and methods for a description of L4 staging criteria. (**C**,**D**) Confocal microscopy images of L4 males (i-vi) and hermaphrodites (vii) carrying *dmd-3*::*YFP (fsIs2)* (C) or *MAB-3*::*GFP (fsIs10)* (D). Numbers indicate tail tip hypodermal cells. hg and RnA indicate the hindgut and ray RnA neurons, respectively. The larval cuticle is outlined with dashed lines; dotted lines indicate the developing adult cuticle.

was not expressed in hermaphrodite hyp8-11 at any stage (Fig. 2C, part vii). Thus *dmd-3* expression parallels tail tip remodeling, consistent with a cell-autonomous, instructive role for *dmd-3* in the control of morphogenesis.

By contrast, an existing *MAB-3*::*GFP* translational reporter (Yi et al., 2000) was not reported to be expressed in the tail tip, nor could it rescue the tail tip retraction defects of *mab-3; dmd-3* males (data not shown). We therefore generated a *MAB-3*::*GFP* translational reporter carrying 7.3 kb of additional upstream regulatory sequence. This transgene was able to rescue the tail tip retraction defects of *dmd-3; mab-3* males to a *dmd-3*-like phenotype (Table 1). *MAB-3*::*GFP* was expressed in numerous cells of the male tail, including weak expression in hyp8-11 (Fig. 2D). In early L4 males, *MAB-3*::*GFP* was predominantly found only in hyp10, although hyp9 expression was occasionally observed. By mid-L4, we observed expression in all tail tip hypodermal cells. As with *dmd-3*, no *MAB-3*::*GFP* expression was detected in hyp8-11 in hermaphrodites at any stage (Fig. 2D, part vii). Together, these results suggest that *dmd-3* and *mab-3* act in hyp8-11 to bring about male-specific morphogenesis.

dmd-3 is likely to be a direct *tra-1* target

The male-specific expression of *dmd-3* in two sets of cells present in both sexes – the tail tip hypodermis and the hindgut – indicated that *dmd-3* could be a direct target of repression by TRA-1A in hermaphrodites. Consistent with this, we found that *dmd-3* is both expressed during and is necessary for the tail tip retraction that occurs in *tra-1(e1099)* XX pseudomales (Fig. 3A; Table 1) (Hodgkin, 1987). Interestingly, the severity of the *dmd-3* phenotype is slightly exaggerated in *tra-1* pseudomales, though the reasons for this are unclear. Nevertheless, these data show that *dmd-3* lies genetically and molecularly downstream of *tra-1*.

To ask how *tra-1* regulates *dmd-3*, we first identified a ~1.1 kb region ~1.6 kb upstream of the *dmd-3* start codon, *E(ht)*, that was both necessary and sufficient to direct male-specific expression in the hindgut and tail tip (Fig. 1A, Fig. 3C; data not shown) when placed upstream of the basal promoter Δ*pes-10* (Seydoux and Fire, 1994). Expression of DMD-3 under the control of *E(ht)*::Δ*pes-10* was sufficient to rescue the *dmd-3* tail morphology defect (Table 1). Thus *E(ht)* contains cis-acting elements that direct the sexual and temporal regulation of *dmd-3* in the tail tip hypodermis and hindgut.

E(ht) contains a nearly exact match to the TRA-1A consensus binding site (Zarkower and Hodgkin, 1993; Yi et al., 2000), varying at only one nucleotide (Fig. 3B). To disrupt this site, we replaced the central GGTGT with TCGAC to create *E(ht)*$_{\Delta TRA\text{-}1}$::*DMD-3*::*GFP*. Strikingly, this change led to clear *DMD-3*::*GFP* expression in the L4 hermaphrodite tail tip (93%; *n*=67 compared with 0%; *n*=51 for *E(ht)*::*DMD-3*::*GFP*) and hindgut (Fig. 3C, part xii). In addition, a single point mutation (TGGG→TGAG) in the putative TRA-1A site led to similar expression in the L4 hermaphrodite tail tip (100%; *n*=40) and hindgut (data not shown). This specific G→A change has also been demonstrated to disrupt TRA-1A binding in the context of the *egl-1* and *ceh-30* promoters (Conradt and Horvitz, 1999; Schwartz and Horvitz, 2007). We cannot rule out the possibility that this site indirectly mediates sexual regulation of *dmd-3* by TRA-1A. However, together with the finding that TRA-1A can bind to nearly identical sites in vitro (Zarkower and Hodgkin,

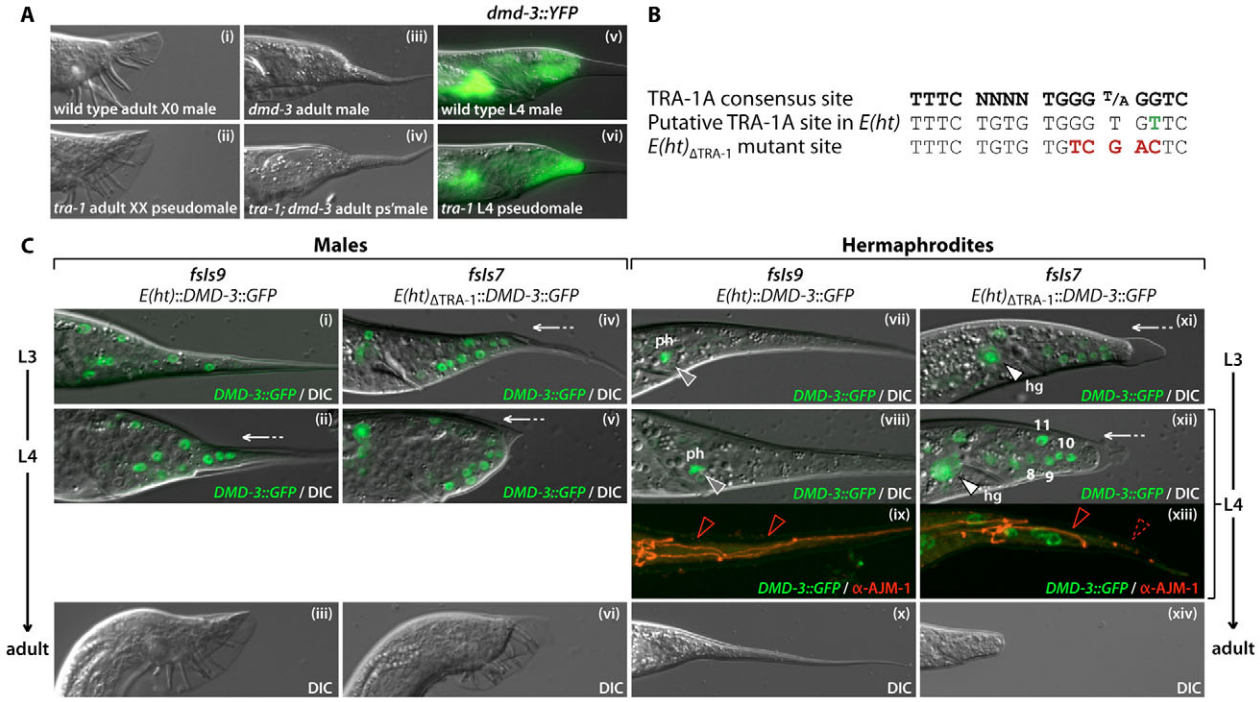

Fig. 3. *dmd-3* is likely to be a direct target of TRA-1A and is sufficient to trigger hermaphrodite tail tip retraction. (**A**) DIC images of an adult wild type X0 male (i), an adult *tra-1(e1099)* XX pseudomale (ii), an adult *dmd-3(ok1327)* X0 male (iii) and an adult *tra-1(e1099); dmd-3(ok1327)* XX pseudomale (iv). Overlaid *dmd-3::YFP*/DIC images of a wild-type late mid-L4 X0 male (v) and a *tra-1(e1099)* late mid-L4 XX pseudomale (vi) expressing *dmd-3::YFP* (*fsIs2*). (**B**) Sequences of the TRA-1A consensus binding site (Zarkower and Hodgkin, 1993; Yi et al., 2000), the putative TRA-1A binding site in the *dmd-3* promoter and the ΔTRA-1 mutation. The single nucleotide difference between the consensus site and the *dmd-3* site is shown in green; nucleotides mutated in ΔTRA-1 are shown in red. (**C**) *DMD-3*::*GFP*/DIC (i, ii, iv, v, vii, viii, xi, xii), *DMD-3*::*GFP*/MH27 antibody staining (red) (ix, xiii) and DIC (iii, vi, x, xiv) images of late L3 (i, iv, vii and xi), mid-L4 (ii, v, viii, ix, xii, and xiii) and adult (iii, vi, x, xiv) males (i-vi) and hermaphrodites (vii-xiv) carrying *E(ht)*::*DMD-3*::*GFP* (*fsIs9*) (i-iii and vii-x) or *E(ht)*$_{\Delta TRA-1}$::*DMD-3*::*GFP* (*fsIs7*) (iv-vi and xi-xiv). Expression of *E(ht)*$_{\Delta TRA-1}$::*DMD-3*::*GFP* in hermaphrodite tail tip hypodermal cells (numbers in xii) and hindgut (hg) (white arrowheads in xi and xii) is indicated. Gray arrowheads (vii and viii) indicate expression in phasmid neurons (ph). Solid red arrowheads (ix and xiii) mark intact hyp8-11 boundaries; the dashed red arrowhead (xiii) indicates cell fusion. White arrows (ii, iv, v, xi and xiii) mark hyp8-11 retraction.

1993; Conradt and Horvitz, 1999; Yi et al., 2000), our results indicate that *dmd-3* is very likely to be a direct target of repression by TRA-1A in the hermaphrodite tail tip and hindgut.

dmd-3 can bring about male-like tail tip morphogenesis in hermaphrodites

We next took advantage of the expression of *E(ht)*$_{\Delta TRA-1}$ in both sexes to ask whether providing *DMD-3* to the hermaphrodite would be sufficient to masculinize the tail tip. Although all adult hermaphrodites carrying the wild-type *E(ht)*::*DMD-3*::*GFP* transgene *fsIs9* displayed normal whip-like tail tips (*n*=88), the mutant *E(ht)*$_{\Delta TRA-1}$::*DMD-3*::*GFP* transgene *fsIs7* produced a male-like rounded tail tip in 94% of adult hermaphrodites (*n*=101) (Fig. 3C, part xiv). Consistent with this, the tail tip hypodermal cells of L4 hermaphrodites carrying *fsIs7* exhibited clear retraction-like movements and some cell fusion (Fig. 3C, parts xii, xiii). Thus, sexually dimorphic *dmd-3* expression determines the sexual specificity of tail tip morphogenesis.

Unexpectedly, these mutations in *E(ht)* also disrupted the timing of *dmd-3* expression. In both sexes, the expression of *DMD-3*::*GFP* from *E(ht)*$_{\Delta TRA-1}$ and *E(ht)*$_{TRA-1-G\rightarrow A}$ initiated prematurely in L2 and L3 larvae (Fig. 3C; data not shown), suggesting that the regions mediating sexual and temporal input overlap in the *dmd-3* promoter. By contrast, only minor premature expression was seen in L3 and early L4 males carrying the wild-type transgene; this effect probably results from increased positive autoregulation caused by *DMD-3* overexpression (see below).

The premature expression of these transgenes also demonstrated that *dmd-3* activity was sufficient to trigger retraction at an inappropriate time. *fsIs7* induced precocious tail tip retraction in both male and hermaphrodite L3 larvae, such that essentially all *fsIs7* L4 males and many L4 hermaphrodites had clearly pre-retracted tail tips (Fig. 3C), a phenotype not seen in *fsIs9* L4 males. Furthermore, adult *fsIs7* males exhibited a clear 'over-retraction' phenotype (Del Rio-Albrechtsen et al., 2006) (Fig. 3C, part vi). Thus, *dmd-3* expression in the tail tip can provide an instructive cue for morphogenesis regardless of sex or developmental stage.

Wnt signaling and heterochronic genes regulate tail tip morphogenesis through *dmd-3*

Both Wnt and heterochronic genes are necessary for normal male tail tip morphogenesis (Zhao et al., 2002; Del Rio-Albrechtsen et al., 2006). However, the mechanisms underlying these functions are unknown. We therefore tested the possibility that these phenotypes result from misregulation of *dmd-3*. To determine whether the heterochronic gene *lin-41* regulates *dmd-3*, we examined *dmd-3*::*YFP* in *lin-41* mutants. Temporally delayed *lin-41(bx42gf)* males have a Lep phenotype (Del Rio-Albrechtsen et al., 2006). We found

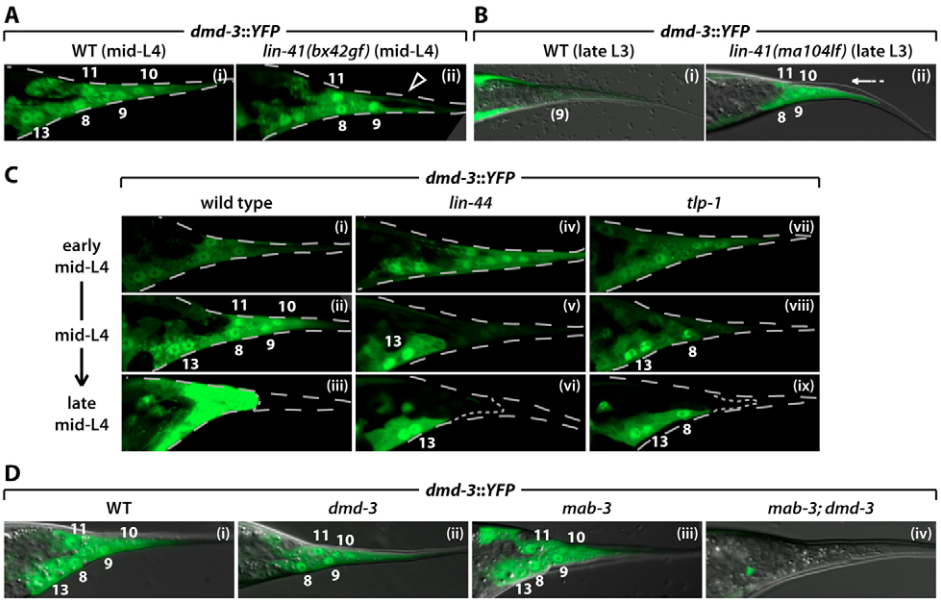

Fig. 4. *dmd-3* expression in the tail tip is regulated by *lin-41*, Wnt signaling and a positive-feedback loop. (**A**) *dmd-3*::*YFP* (*fsIs3*) expression in wild-type (i) and *lin-41(bx42gf)* (ii) mid-L4 males. The arrowhead in ii indicates the absence of *dmd-3*::*YFP* expression in hyp10 in *lin-41(bx42)* males. (**B**) *dmd-3*::*YFP* (*fsIs3*) expression in wild type (i) and *lin-41(ma104lf)* (ii) late L3 males. The arrow in ii highlights the precocious tail tip retraction in *lin-41(ma104)* males. (**C**) *dmd-3*::*YFP* expression in wild type (i-iii), *lin-44(n1792)* (iv-vi) and *tlp-1(bx85)* (vii-ix) L4 males of the indicated stages. (**D**) *dmd-3*::*YFP* (*fsIs2*) expression in wild type (i), *dmd-3(ok1327)* (ii), *mab-3(e1240)* (iii) and *mab-3(e1240); dmd-3(ok1327)* (iv) mid-L4 males. In A,C, the larval cuticle is shown with dashed lines. Dotted lines indicate the developing adult cuticle.

that *dmd-3*::*YFP* expression in these mutants initiated at the correct time in hyp8, hyp9 and hyp11, but was frequently absent from hyp10 even into late L4 (Fig. 4A). We observed this hyp10-specific expression defect in 89% of *lin-41(bx42)* mid-L4 males (*n*=27) and 33% of *lin-41(bx37)* mid-L4 males (*n*=48), but never in wild-type mid-L4 males (*n*=35). Interestingly, hyp10 is generally the only cell that fails to fuse in *lin-41(gf)* mutants (Nguyen et al., 1999; Del Rio-Albrechtsen et al., 2006). As the lack of *dmd-3* expression specifically in hyp10 is characteristic of early-mid L4 wild-type males (Fig. 2C, part ii), we interpret the *lin-41(gf)* phenotype to be a defect in the maturation of *dmd-3* expression. Conversely, we observed strong expression of *dmd-3*::*YFP* in the pre-retracting tail tips of *lin-41(ma104lf)* (Slack et al., 2000) L3 males (87%; *n*=15, compared with 0%; *n*=22 for wild type) (Fig. 4B). This indicates that the early retraction in these animals (Del Rio-Albrechtsen et al., 2006) probably results from premature *dmd-3* expression. Consistent with this, we found that the premature-retraction phenotype of *lin-41(lf)* males (95% L4 pre-retracted tail; *n*=80) was suppressed in *lin-41; dmd-3* (13%; *n*=82) and *lin-41; mab-3; dmd-3* (2%; *n*=54) mutants. Thus, *lin-41* controls the stage specificity of tail tip morphogenesis by regulating *dmd-3* expression.

We also examined the effects of a mutation in the Wnt ligand *lin-44* (Herman et al., 1995) on *dmd-3*::*YFP* expression. In these animals, tail tip *dmd-3*::*YFP* expression initiated at the correct time, but was not sustained (Fig. 4C), such that we observed defects in *dmd-3*::*YFP* expression in some or all tail tip cells in 98% of mid-L4 *lin-44* males (*n*=40), compared with 0% in wild type (*n*=36). However, the Lep defect of *lin-44* mutants is subtle, and we found that its severity was enhanced by a mutation in *mab-3* (data not shown), suggesting that *mab-3* can compensate for the reduction of *dmd-3* expression in *lin-44* males. Males carrying a mutation in the putative Wnt effector *tlp-1* (Zhao et al., 2002) displayed a *dmd-3*::*YFP* expression defect similar to that of *lin-44* males: initial expression was normal but it was not properly maintained in most mid-L4 males (75%, *n*=72) (Fig. 4C). These results indicate that tail tip *dmd-3* expression is regulated in two distinct phases: an initial, Wnt-independent induction of *dmd-3* expression, followed by Wnt-dependent maintenance and amplification.

An autoregulatory loop is important for tail tip morphogenesis

To determine whether *dmd-3* autoregulation contributes to the maintenance phase of *dmd-3* expression, we examined *dmd-3*::*YFP* in *dmd-3*, *mab-3* and *dmd-3*; *mab-3* mutants (Fig. 4D). We observed a subtle decrease in *dmd-3*::*YFP* expression in hyp8-11 in *dmd-3* males. By contrast, *mab-3* mutant males exhibited wild-type levels of *dmd-3*::*YFP* tail tip expression. More clearly, *dmd-3*::*YFP* expression was essentially abolished in hyp8-11 of *mab-3; dmd-3* mid-L4 males. Therefore, *dmd-3* and *mab-3* are necessary for strong *dmd-3* expression, and, at least in *mab-3* mutants, *dmd-3* has a positive autoregulatory function. To determine whether this autoregulation might occur through direct activation by DMD-3 itself, we identified and disrupted two candidate DMD-3/MAB-3 binding sites in the *E(ht)* region. However, mutating these sites did not result in a loss of hyp8-11 expression (not shown). Thus, *dmd-3*-dependent expression of *dmd-3* may be mediated through intermediate regulators. As maintenance-phase expression of *dmd-3* requires *lin-44* and *tlp-1*, it is possible that *dmd-3* activates a Wnt signal that then directly promotes *dmd-3* expression.

dmd-3 and *mab-3* activate sex-specific expression of the cell fusogen EFF-1

Though tail tip morphogenesis involves both cell fusion and retraction, it is unclear whether these two steps occur independently or, alternatively, if retraction is simply a consequence of cell fusion. To investigate this, we asked whether *eff-1*, the primary regulator of cell fusion in *C. elegans* (Mohler et al., 2002; Shemer et al., 2004; Podbilewicz et al., 2006), was necessary for hyp8-11 fusion and retraction. Consistent with previous findings (Mohler et al., 2002; Shemer and Podbilewicz, 2003), we detected no hyp8-11 fusion in males carrying the putative null allele *eff-1(ok1021)* (Fig. 5A). However, retraction proceeded with only subtle abnormalities in these animals, resulting in a non-Lep, blunt-ended tail (Fig. 5A). Thus, the fusion of hyp8-11 is not a prerequisite for retraction, indicating that these two events are regulated in parallel.

Consistent with the requirement of *eff-1* for hyp8-11 fusion, we found that an *EFF-1*::*GFP* translational reporter (del Campo et al., 2005) was expressed in the male tail tip in a pattern that correlated

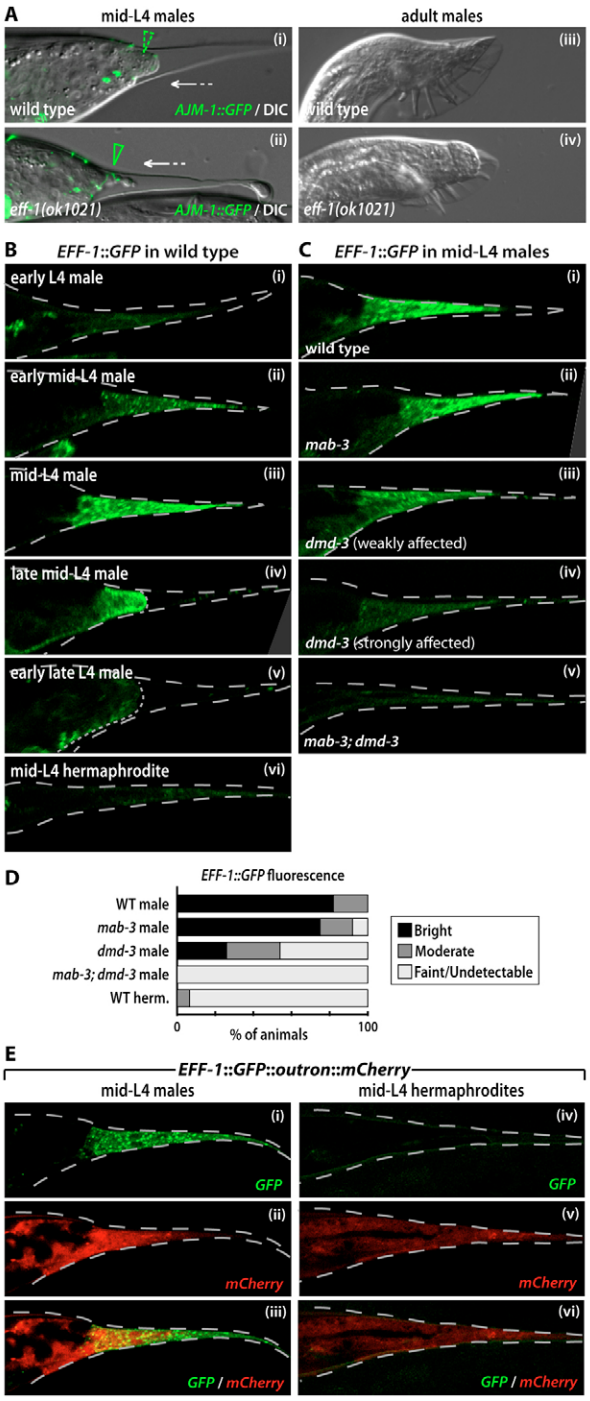

Fig. 5. EFF-1 is regulated by *dmd-3* and *mab-3*. (**A**) *AJM-1*::*GFP (syIs78)*/DIC and DIC images of wild-type and *eff-1(ok1021)* L4 (i,ii) and adult (iii,iv) males. The broken green arrowhead indicates cell fusion; the solid green arrowhead marks intact cell boundaries. The white arrows indicate tail tip retraction. (**B**) Tail tip expression of an *EFF-1*::*GFP* translational reporter (*fsEx135*) in male (i-v) and hermaphrodite (vi) larvae. (**C**) Expression of *EFF-1*::*GFP* in wild type (i), *mab-3* (ii), *dmd-3* (iii,iv) and *dmd-3; mab-3* (v) mid-L4 males. The examples in iii and iv represent the range of *EFF-1*::*GFP* expression levels seen in *dmd-3* mutants. (**D**) Categorization of *EFF-1*::*GFP* fluorescence intensity in wild-type (*n*=39), *mab-3* (*n*=24), *dmd-3* (*n*=61) and *mab-3; dmd-3* (*n*=33) mid-L4 males and wild-type mid-L4 hermaphrodites (*n*=31) based on confocal images. (**E**) Expression of *EFF-1*::*GFP*::*outron*::*mCherry* in a wild-type mid-L4 male (i-iii) and hermaphrodite (iv-vi). GFP (i,iv), mCherry (ii,v) and GFP/mCherry (iii,vi) overlays are shown.

closely with *dmd-3*::*YFP* expression. *EFF-1*::*GFP* was transiently expressed in hyp8-11 in mid-L4 males, peaking around the time of cell fusion, but was expressed only very weakly in the tail tip of L4 hermaphrodites (Fig. 5B,D). Interestingly, a transcriptional *eff-1*::*GFP* reporter lacking *eff-1* coding sequence (Mohler et al., 2002) displayed only limited sex differences in expression (not shown). To further explore the mechanisms of *eff-1* regulation, we generated a construct in which the *eff-1* promoter drove expression of an artificial operon (Blumenthal, 2005; White et al., 2007) containing *EFF-1*::*GFP*-coding sequence followed by an artificial 'outron' and *mCherry* coding sequence (*EFF-1*::*GFP*::*outron*::*mCherry*). In this construct, mCherry fluorescence should reflect transcriptional regulation by the *eff-1* promoter, while GFP fluorescence reveals the net influence of transcriptional and post-transcriptional controls on *eff-1* expression. We observed mCherry expression in hyp8-11 of both sexes, but at lower levels in hermaphrodites than in males. By contrast, *EFF-1*::*GFP* expression was barely detectable in the hermaphrodite (Fig. 5E). Together, these results indicate that the male-specificity of tail tip syncytium formation arises from the regulation of *EFF-1*, and that this regulation probably occurs through both transcriptional and post-transcriptional mechanisms.

We next asked whether *dmd-3* and *mab-3* were necessary for the sexual dimorphism in *EFF-1*::*GFP* expression (Fig. 5C,D). We found that *EFF-1*::*GFP* levels were often reduced in the tail tips of mid-L4 *dmd-3* males, consistent with their partial cell fusion defects. In *mab-3; dmd-3* double mutants, *EFF-1*::*GFP* was always faint or absent, similar to the pattern observed in wild-type L4 hermaphrodites. However, in *mab-3* single mutants, *EFF-1*::*GFP* was expressed at essentially wild-type levels, in agreement with the observation that hyp8-11 fusion is unaffected in these mutants.

To ask whether the transcriptional regulation of *eff-1* by *dmd-3* and *mab-3* might be direct, we searched the *eff-1* promoter for candidate DMD-3- and MAB-3-binding sites. We identified three putative DMD-3/MAB-3 elements and disrupted them in the context of the *EFF-1*::*GFP*::*outron*::*mCherry* reporter. However, this resulted in no detectable change in GFP or mCherry fluorescence in mid-L4 larvae of either sex (data not shown), indicating that the transcriptional activation of *eff-1* by DMD-3 and MAB-3 may not be direct.

DISCUSSION

We have found that *dmd-3*, a previously undescribed member of the DM family, is both necessary and sufficient for male tail tip morphogenesis, a process that generates one of the most prominent sexual dimorphisms in the *C. elegans* soma. We also find a secondary partially redundant role of the related gene *mab-3* in this process. Together, our findings lead to a model in which *dmd-3* instructively specifies tail tip morphogenesis by integrating multiple developmental signals and regulating at least two downstream events (Fig. 6). The temporal control of *dmd-3* is specified by the heterochronic pathway through the regulator *lin-41*. [Mutations in the *lin-41* regulator *let-7* also cause Lep phenotypes (Del Rio-Albrechtsen et al., 2006), indicating that *let-7* also acts in this pathway, though we have not tested this possibility directly.] Positional cues regulate *dmd-3* through a Wnt pathway that includes the ligand LIN-44 and its downstream target *tlp-1*. Interestingly, this cue seems to be most important for the maintenance and amplification of *dmd-3* expression; the initial positional or cell-type activator of *dmd-3* remains unknown. Finally, the male-specificity of *dmd-3* expression arises through regulation by the master sexual regulator TRA-1A.

The phenotype of *mab-3; dmd-3* double mutants indicates that the functions of these two genes partially overlap. Though only a small percentage of *mab-3* males have Lep defects, *mab-3* enhances the

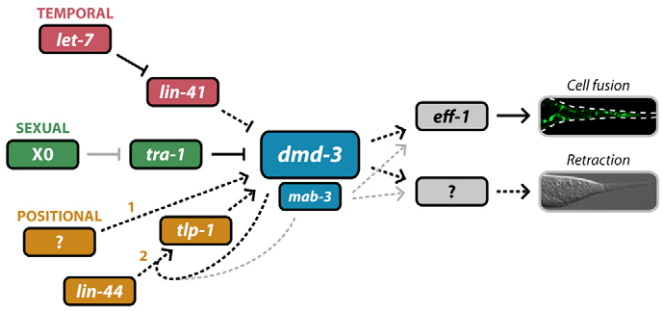

Fig. 6. *dmd-3* and *mab-3* occupy the central node of the tail tip retraction network. Our results support a model in which multiple upstream regulatory pathways converge on *dmd-3* and *mab-3* to regulate the temporal (red), sexual (green) and cell-type (yellow) specificity of tail tip morphogenesis. Temporal specificity is imparted by the heterochronic pathway via *let-7* and *lin-41*. Sexual specificity arises by the regulation of *dmd-3* by *tra-1*, which is likely to be direct. At least two pathways can be thought of as cell-type determinants: the yellow '1' depicts the induction phase of *dmd-3* expression in hyp8-11, while the yellow '2' indicates the maintenance and amplification phase. Downstream of *dmd-3* and *mab-3* lie multiple effectors of morphogenesis, including *eff-1*. The targets that mediate hyp8-11 retraction are unknown, as is the upstream regulatory pathway that initiates *dmd-3* expression (1) in the tail tip. Black arrows and bars indicate regulatory events that are likely to be direct. The solid gray bar indicates indirect regulation. Broken gray and black arrows and bars indicate steps for which the molecular mechanism is unknown. Thin gray arrows indicate that the function of *mab-3* in tail tip morphogenesis is secondary to that of *dmd-3*.

phenotype of every animal in a *dmd-3* background (Table 1). In addition, overexpression of *mab-3(+)* in a *mab-3; dmd-3* double mutant can sometimes rescue animals to a nearly wild-type phenotype (not shown). As the in vitro selected binding site for MAB-3 closely resembles that of MAB-3 (M. Murphy and D. Zarkower, personal communication) (Yi and Zarkower, 1999), we interpret this redundancy to reflect a partial overlap in the set of target genes that DMD-3 and MAB-3 can regulate.

The clear loss of sex specificity in the hindgut and tail tip upon disruption of the putative TRA-1A site in *dmd-3* provides strong evidence that *dmd-3* is a direct target of TRA-1A. This site is a close match to the consensus site for binding in vitro, differing at only the +15 position (Zarkower and Hodgkin, 1993; Yi et al., 2000). We note that this +15 position is likely to be important for TRA-1A binding in vitro and in the TRA-1A target *egl-1* (Zarkower and Hodgkin, 1993; Conradt and Horvitz, 1999). However, in contrast to *egl-1*, the TRA-1A site in *dmd-3* matches the TRA-1A consensus at every other position, making it possible that the +15 site is less important in the context of the *dmd-3* promoter. The *C. briggsae dmd-3* ortholog, *Cbr-dmd-3*, also contains a strong match to the TRA-1A consensus site within a 50 bp conserved region of upstream sequence (not shown). Although we cannot rule out the possibility that sexual regulation of *dmd-3* occurs indirectly through a site closely resembling that of TRA-1A, we consider this to be unlikely.

We believe that the sex-determination and heterochronic pathways probably converge on a common *cis*-element in *dmd-3*, as disruption of its TRA-1A site altered both sexual and temporal specificity of *dmd-3* expression. These results support the short-range repression model proposed for TRA-1A function (Conradt and Horvitz, 1999; Yi et al., 2000; Zarkower, 2001), in which this factor acts locally to impart local sex specificity to a single enhancer rather than to the entire locus. An alternative possibility, that sexual and temporal regulation are both mediated by TRA-1A, is unlikely, as precocious tail tip retraction is not observed in *tra-1* XX pseudomales (Fig. 3A). Interestingly, a similar phenomenon has been observed in the Hox cluster gene *egl-5:* disruption of a putative upstream TRA-1A-binding site was found to alter the sexual, temporal and spatial specificity of *egl-5* expression in seam cells (Teng et al., 2004). Thus, the overlap of TRA-1A sites with other regulatory elements may be a common property of sexually regulated genes.

In contrast to sexual regulation, our results indicate that the regulation of *dmd-3* by *lin-41* may be indirect. Previous work has indicated that *lin-41* controls its targets post-transcriptionally (Slack et al., 2000). However, as mutating the *E(ht)* promoter fragment altered the timing of its expression, *dmd-3* temporal control is likely to be mediated transcriptionally. Although other known effects of *lin-41* on developmental timing are mediated through the transcription factor LIN-29 (Slack et al., 2000), *lin-29* mutant males do not exhibit an unretracted tail tip (Euling et al., 1999). Thus, it seems likely that an unidentified target of *lin-41* (Del Rio-Albrechtsen et al., 2006) regulates *dmd-3*.

The phenotypes of *lin-44* and *tlp-1* mutants indicate that Wnt signaling is important for *dmd-3* maintenance and amplification, but not for its initial expression. Mutation of *tlp-1* leads to a defect in maintenance of *dmd-3* expression and a pronounced Lep phenotype. By contrast, though loss of *lin-44* leads to a similar *dmd-3* expression defect (Fig. 4C), the tail tip retraction phenotypes of these animals are relatively subtle. This could indicate that residual *dmd-3* expression in *lin-44* mutants is still able to exert a significant level of function. Our finding that *mab-3* can enhance the *lin-44* phenotype indicates that while *dmd-3* is regulated primarily through *lin-44*, a different Wnt ligand might act preferentially on *mab-3*. Both of these Wnt signals would probably act primarily through *tlp-1*.

Because of its relatively simplicity, tail tip retraction serves as an excellent model to explore the links between developmental signals and morphogenesis. We have found that *dmd-3* and *mab-3* trigger tail tip cell fusion by promoting expression of the fusogen EFF-1, probably indirectly, through both transcriptional and post-transcriptional mechanisms. As cell fusion and retraction can vary independently in related nematode species (Fitch, 1997), and *eff-1* mutant males clearly undergo retraction, *dmd-3* and *mab-3* must activate additional unknown effectors of morphogenesis. Furthermore, yet other genes are likely to mediate the effects of *dmd-3* and *mab-3* on the genetically separable process of anterior tail retraction.

How do these findings inform our understanding of the role of DM genes in sexual development? As discussed above, the surprising diversity in the nature of the sex-specific functions of DM factors has made it difficult to understand the basis for their conservation in these processes. Interestingly, *dmd-3*, *mab-3* and *dsx* all function at the interface between the general sex determination hierarchy and the regulation of specific developmental events. Thus, we suggest that the ancestral role of DM genes was to act as cell-autonomous determinants of sexual information, directly linking sex to the modulation of differentiation and morphogenesis. In a primitive system, the differential expression of these genes could have allowed them to act as 'selector' genes of sexual information (Mann and Carroll, 2002), much as Hox cluster genes specify positional information. Once this crucial function became fixed, upstream regulatory hierarchies could have evolved to allow more complex mechanisms of interpreting the primary sex-determining cue (Wilkins, 1995), giving rise to the present day roles of *dmd-3*, *mab-3* and *dsx*. The selective expression of DM genes in one sex may also have allowed their functions to be captured

in further downstream steps. Further exploration of this unique gene family will undoubtedly shed light onto the intersection of sex determination and developmental patterning.

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