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Functional Requirement for Histone Deacetylase 1 in *Caenorhabditis elegans* Gonadogenesis

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Histone acetylation and deacetylation have been implicated in the regulation of gene expression. Molecular studies have shown that histone deacetylases (HDACs) function as transcriptional repressors. However, very little is known about their roles during development in multicellular organisms. We previously demonstrated that inhibition of maternal and zygotic expression of histone deacetylase 1 (HDA-1) causes embryonic lethality in *Caenorhabditis elegans*. Here, we report the identification of an *hda-1* genetic mutant which has also been called a *gon-10* mutant (for gonadogenesis defective 10) and show that loss of HDA-1 zygotic expression results in specific postembryonic defects in gonadogenesis and vulval development. We provide evidence that the *lag-2* gene, which plays a role in gonadogenesis and vulval development and encodes a Notch ligand, is derepressed in *gon-10* animals, suggesting that *lag-2* may be a target of HDA-1. Our findings reveal a novel and specific function for the ubiquitously expressed HDA-1 in *C. elegans* gonadogenesis and place *hda-1* in the Notch signaling pathway.

Histone acetylation and deacetylation play critical roles in transcriptional regulation. While histone acetylation facilitates chromatin opening and transcriptional activation, histone deacetylation contributes to chromatin condensation and gene silencing (reviewed in references 20, 28, 39, and 41). The histone deacetylation reaction is catalyzed by histone deacetylases (HDACs) that have been conserved and are found in organisms from *Saccharomyces cerevisiae* to humans. In humans, the list of histone deacetylase genes continues to grow. At the present time, there are 17 *hdac* genes (reviewed in reference 16) that can be grouped into three classes based on their similarity to the *S. cerevisiae* HDACs Rpd3p, Hda1p, and Sir2p (13, 19, 27, 33). While class I and II deacetylases share sequence features in the catalytic domain, the class II HDACs contain additional sequences that are absent in class I HDACs. Class II HDACs also display different tissue distributions, while expression of class I HDACs appears to be ubiquitous (13, 19). Class III deacetylases are exemplified by Sir2 which has a unique property in that it requires NAD as a cofactor for its enzymatic activity (27, 33, 44). On the basis of sequence homology, humans are predicted to have seven class III deacetylase genes (reviewed in reference 16). *Caenorhabditis elegans* has three class I, four class II, and four class III HDACs. Recently, HDA-1, a class I histone deacetylase, has been shown to collaborate with POP-1 and UNC-37, a *C. elegans* Groucho homolog, in the repression of *end-1*, an early E-cell fate-determining gene during *C. elegan*s embryogenesis (5).

The developmental roles of HDACs are just beginning to be identified. Generation of germ line clones of a strong hypomorphic allele of *Drosophila* Rpd3, a class I deacetylase, results in embryonic lethality, highlighting a specific role for RPD3 in segmentation control during embryogenesis (36). Furthermore, in *C. elegans*, RNA-mediated interference (RNAi) (12) of maternal and zygotic expression of the *C. elegans* homolog of Rpd3p, HDA-1, results in embryonic lethality. Although cells that form muscle, hypoderm, and intestine are present and appear to be terminally differentiated, the embryos nevertheless die prior to elongation (43; P. Dufourcq and Y. Shi, unpublished results). In addition to its role in embryogenesis, recent RNAi studies have suggested a possible postembryonic function for HDA-1 in *C. elegans* vulval development (35, 45).

Here, we report the identification and results of analyses of an *hda-1* genetic mutant. We provide genetic, molecular, and biochemical evidence that this *hda-1* mutant is the previously isolated *gon-10* mutant (named for gonadogenesis defective 10). Phenotypic analysis of *gon-10* animals revealed multiple developmental defects in gonadogenesis and vulval development. The gonadogenesis defects are characterized by the lack of organized somatic gonad structures, which suggests that these abnormalities may be due to defects in tissue morphogenesis. Hermaphrodite *gon-10* animals also display a protruding or everted vulva and often develop multivulvae as a result of hyperinduction of vulval cells. Since gonadogenesis and vulval development are regulated by *Notch* and *Ras*, respectively (reviewed in reference 26), our findings suggest that *hda-1* may be a component of these two signaling pathways. Consistent with this hypothesis, we have identified *lag-2*, a gene that encodes a homolog of the Notch ligand Delta, as a potential target for HDA-1. In summary, we provide compelling evidence that a ubiquitous histone deacetylase plays specific roles in a number of critical developmental decision processes in *C. elegans*.

MATERIALS AND METHODS

Strains and genetics. *C. elegans* was cultivated as described previously (3). The following mutant alleles and/or strains were used: *gon-10(e1795)/DnT1V*; YS40 *dpy-11(e224) gon-10(e1795)/unc-76(e911)V*; YS47 *gon-10(e1795)/unc-76(e911)*;

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dpy-11(e224)V; *unc-76(e911)V*; YS52 *dpy-11(e224) gon-10(e1795)/unc-76(e911)*; *bmEx1* [*phda-1* (wild-type *hda-1* gene) pPD97.93 (*myo-3::gfp*)]; *lin-15(n433)*; and *lin-15(n744)* (9). The presence of class A (*lin-15A*) or B (*lin-15B*) mutations in the context of the double mutant were confirmed by soaking larval stage 1 (L1) heterozygote *gon-10(e1795)/unc-76(e911); lin-15(n433)* or *gon-10(e1795)/unc-76(e911); lin-15(n744)* in *lin-15B RNAi* and *lin-15A RNAi,* respectively. The following genes were used in expression studies: *egl-26::gfp* (23); *lag-2::gfp* (14), *lim-7::gfp* (22), *flp-8::gfp* (K. Kim and C. Li, personal communication.), *egl-17::gfp* (4), *cdh-3::gfp* (40), *lip-1::gfp* (2), *myo-3::gfp* (pPD97.93) (A. Fire, S. Xu, J. Ahnn, and G. Seydoux, personal communication), *sur-5::gfp* (21), and *let-858::gfp* (29).

Isolation of *gon-10(e1795)*. A mutation induced by ethyl methanesulfonate, originally designated *gon-10(e1795),* was isolated while screening for abnormal sexual development and mapped to a location on linkage group V, between the *egl-41* and *unc-76* loci. Hermaphrodites homozygous for *e1795* were sterile and maintained as heterozygotes. *gon-10(e1795)* animals were outcrossed five times; the *gon-10(e1795)* gene was marked by *dpy-11(e224*) and balanced by *unc-76(e911)*. In order to score for progeny, larvae, or eggs, adult heterozygote hermaphrodites were allowed to lay eggs for a short period of time, and progeny were examined through time course analysis.

Transformation rescue of *gon-10(e1795)*. Nested PCR primers were used to isolate the *hda-1* gene using cosmid D1027 (canonical form of cosmid C53A5) as a template. The PCR primers were designed on the basis of the sequence information from cosmid C53A5. The outer primer pair is 3361S/6946R (TGCCTCAAA GAGCTTTCCTACG/CATCCAACATCAGATGAAGACAGAC), and the inner primer pair is 3881S/6799R (TTCAACATCGTGAGAGCGTGG/CGACATAAA CGATGTCAACTGC). Transgenic lines were established as described previously (37). To perform rescue experiments, we marked *gon-10(e1795)* with *dpy-11*. Since *dpy-11* is closely linked to *gon-10(e1795)*, we used the Dpy phenotype as an indicator for homozygosity of the *gon-10* locus; homozygote *dpy-11(e224) gon-10(e1795)* animals are phenotypically Dpy, characterized by short and fat physical appearance. If rescue is complete, the sterile homozygous *gon-10(e1795)* animals with the Dpy phenotype are expected to become fertile. Hermaphrodites were injected with a mixture of test DNA and a green fluorescence protein (GFP) marker pPD97.93 *myo-3::gfp* at 20 ng/μl. GFP was used to monitor the germ line transmission frequency of the *hda-1* transgene. For all transgenic lines, the transmission rate was approximately 60%.

RNAi. RNAi experiments were performed as described previously (12). yk109d9 (*hda-1* cDNA) served as a template for the production of double-stranded *hda-1* RNA using an in vitro transcription kit (Promega). Young adult animals were injected and allowed to lay eggs for 6 to 12 h. Five percent of the animals from eggs laid during the first 6 h survive through adulthood, while 100% of the animals from eggs laid during the second 6 h die during embryogenesis.

RT-PCR analysis. Reverse transcription-PCR (RT-PCR) analysis was performed to compare the mRNA levels corresponding to either the control *sc35* gene (34) or the *gfp* and *lag-2* genes in wild-type N2, *lag-2::gfp*, and *lag-2::gfp; gon-10* transgenic lines. Thirty hermaphrodites (L4 larvae) were collected in Trizol reagent (Gibco BRL Life Technologies). After three cycles of freeze-thawing, total RNA was isolated. RT was performed with 500 ng of total RNA using the Superscript II RNase H^- Reverse Transcriptase (Gibco BRL) and oligo(dT) primers (Gibco BRL) following the manufacturer's instructions. Three sets of primers were used for PCR as follows: sc35 F, 5′ CAATGGTCTAACT TCGCTG 3′; sc35 R, 5′ TATCTTGGAGATCTGGAGC 3′; GFP F, 5′ GTAA AGGAGAAGAACTTTTCACTGG 3′; GFP R, 5′ GTATAGTTCATCCATGC CATG 3′; lag-2 F, 5′ CGCTGTGACATCGGATGGATGG 3′; and lag-2 R, 5′ GATGGAGAAGATCACGAAGAGAGC 3′. The optimal number of cycles and amount of RT products used for the PCR were determined in preliminary experiments (not shown). Once the semiquantitative conditions were set up, the RT products were submitted to amplification with the different sets of primers. The samples were then subjected to analysis on an ethidium bromide-stained agarose gel.

Generation of HDA-1 polyclonal antibody and immunofluorescence. Rabbit polyclonal antiserum was raised against amino acids 374 to 460 of HDA-1 (now available at Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.). The purified antibody recognized a single band at the expected position of 50 kDa (data not shown). In addition, the antibody detected strong immunoreactive signals in wild-type embryos but not in *hda-1(RNAi)* embryos (not shown).

To synchronize larvae, 20 N2, YS40, or YS47 hermaphrodites were allowed to lay eggs for 4 h before being transferred to new plates; eggs and larvae were subsequently harvested at specific time points as needed. Adult animals were fixed as described previously (10). Anti-HDA-1 antibody was used at a 1/500-fold dilution, and the monoclonal antibody MH27, which recognizes adherens junctions (15), was used at a 1/2,000-fold dilution.

RESULTS

Animals in which zygotic *hda-1* expression is inhibited display phenotypes resembling those of *gon-10(e1795)* animals. Previous studies have shown that inhibition of both maternal and zygotic *hda-1* expression by *RNAi* results in highly penetrant embryonic lethality (43). Here, we present an investigation of the zygotic function of HDA-1. We focused on the progeny derived from the first batch of eggs laid from the worms injected with double-stranded *hda-1* RNA. These embryos contain maternally provided HDA-1 protein, therefore allowing us to analyze the consequence of selective inhibition of zygotic HDA-1 expression by *RNAi*. The resultant embryos hatched and reached adulthood but were sterile and displayed multiple defects in gonadogenesis. A prominent phenotype is the lack of organized somatic gonad structures. Using the *hda-1* zygotic phenotypes as a guide, we searched for an *hda-1* mutation candidate among previously isolated gonadogenesis-defective mutations that have been mapped to the region near the *hda-1* gene on chromosome V (*C. elegans* Data Base). This phenotype-based search identified *gon-10* (named for gonadogenesis defective 10) as a potential *hda-1* mutation. The *hda-1(RNAi)* and *gon-10(e1795)* homozygote mutant animals had similar defects in gonadogenesis and vulval development (detailed below). These phenotypic similarities suggest that the *gon-10(e1795)* mutant may be a *hda-1* mutant.

The *gon-10(e1795)* mutant is an *hda-1* mutant. To explore the hypothesis that the *gon-10(e1795)* mutant is an *hda-1* mutant, we sequenced the *hda-1* gene isolated from five independent *gon-10(e1795)* homozygote animals. We found that the *hda-1* gene carried a single base change in the coding region which converts a conserved glycine (G) residue to glutamic acid (E) within the catalytic domain of HDA-1 (Fig. 1). No mutations were detected in the promoter region (approximately 1.5 kb) or in the 3′ end of the gene (0.8 kb). This finding suggests the possibility that the *hda-1* mutation may underlie the defects observed in *gon-10(e1795)* animals.

Since the HDA-1^{G186E} mutation is within the predicted catalytic region of HDA-1 (11), it is possible that HDA-1 enzymatic activity is affected. To test this, we asked whether the same mutation would affect the activity of human HDAC1, the homolog of HDA-1, in mammalian cells. We introduced the mutation into human HDAC1 (HDAC1^{G182E}) and transfected the expression plasmid into HeLa cells. Surprisingly, we detected drastically reduced levels of the mutated protein by immunostaining or Western blotting. As shown in Fig. 2A, while the FLAG epitope-tagged wild-type HDAC1 is easily detectable by immunostaining, the mutant protein is detected at a significantly lower level (Fig. 2A, compare panels a and b). To identify transfected cells and to control for transfection efficiency, a plasmid encoding the GFP was cotransfected (Fig. 2A, panels c and d). In parallel, we compared the levels of the wild-type and mutant HDAC1^{G182E} proteins by Western blotting. FLAG-tagged p/CAF was cotransfected and used as a control. As indicated in Fig. 2B, the same result was obtained, i.e., the mutant HDAC1 protein is detected at a significantly reduced level (compare lanes 1 and 2).

We subsequently examined HDA-1 expression in both wild-type and *gon-10(e1795)* mutant animals. In the wild-type *C. elegans*, we found HDA-1 in the germ line, indicating that it is

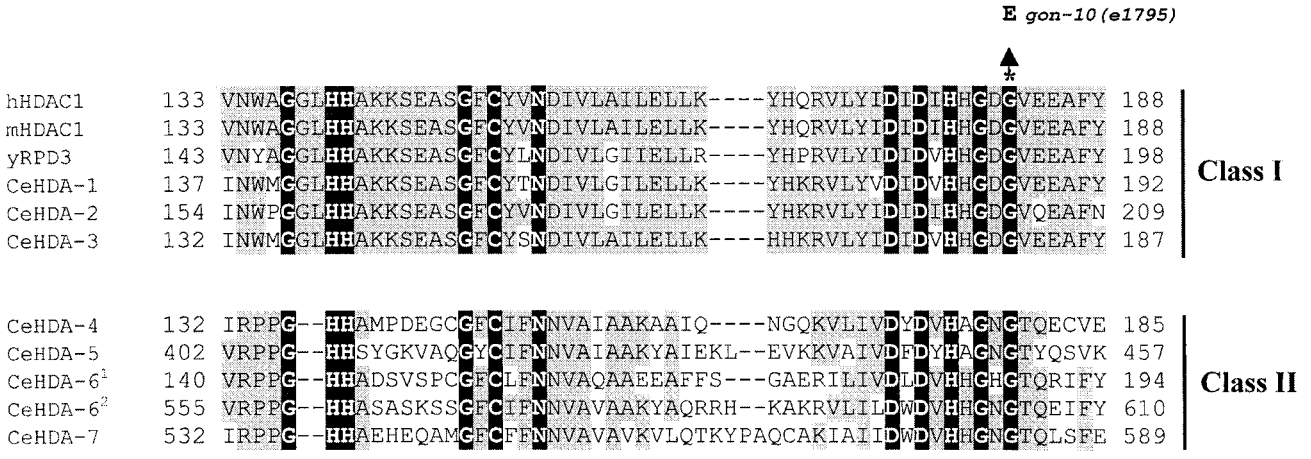


FIG. 1. A point mutation in the *hda-1* gene from *gon-10(e1795)* mutant animals is located in the conserved region of the protein. The sequences of the predicted *C. elegans* HDA-1 to HDA-7 proteins (CeHDA-1 to CeHDA-7) and HDA-1 homologs in humans (hHDAC1), mice (mHDAC1), and yeast (yRPD3) were aligned using the ClustalX program (47). Amino acids conserved in all species and *C. elegans* class I and class II histone deacetylases are shown on a black background. In each class of histone deacetylases, any amino acid that is conserved in more than three deacetylases is shown on a gray background. CeHDA-6[1] and CeHDA-6[2] refer to the first and second catalytic domain of HDA-6, respectively. The amino acid positions of the first and last amino acids of the catalytic domain are given to the sides of the sequences. The position of the G186E point mutation found in the *gon-10(e1795)* mutant and introduced into the human HDAC1 is indicated by the asterisk. Dashes represent gaps introduced to maximize sequence alignment. The HDAs and the open reading frames are as follows: CeHDA-1, C53A5.3; CeHDA-2, C08B11.2; CeHDA-3, R06C1.1; CeHDA-4, Y51H1A.5; CeHDA-5, F43G6.4; CeHDA-6[1], F41H10.6A; CeHDA-6[2], F41H10.6B; CeHDA-7, C10E2.3.

a maternally provided protein (data not shown). We also found HDA-1 expressed ubiquitously throughout embryonic and postembryonic development (Fig. 2C, panel b; also data not shown). HDA-1 immunoreactivity was found in virtually all cell types except sperm (Fig. 2C, panel b). HDA-1 was localized in the nucleus with the exception of the germ line including the oocytes where the signal was also detected in the cytoplasm. Unlike wild-type animals, HDA-1 was virtually undetectable in the *gon-10* homozygous mutants (Fig. 2C, panel d). Therefore, the same G-to-E mutation causes a drastically reduced level of HDA-1/HDAC1 expression in both *C. elegans* and mammalian cells. This finding is consistent with the hypothesis that a severe reduction of HDA-1 protein may be the molecular basis for the phenotypes observed for *gon-10(e1795)* animals.

We predicted that if *gon-10(e1795)* is an *hda-1* mutant, a wild-type copy of the *hda-1* gene would rescue the gonad and sterile phenotype. To accomplish this, three independent transgenic lines were generated using a 3.3-kb *hda-1* gene fragment which includes 1.8 kb of the promoter region, the entire coding region, and 0.54 kb of the 3′ sequence. The *gon-10* mutation was marked with the recessive marker *dpy-11* (see Materials and Methods). We asked whether the transgenic F_2 Dpy animals were fertile. In the absence of the *hda-1* transgene, 6% (22 of 324) of the Dpy animals laid eggs as a result of recombination between the *dpy-11* and *gon-10* loci. However, in the presence of the *hda-1* transgene, the fertility of Dpy animals rose to 54% (97 of 181). This increase is in line with the germ line transmission frequency of the *hda-1* transgene (60%). Significantly, from the *gon-10(e1795)* homozygous but fertile animals that carry the *hda-1* transgene (*dpy-11 gon-10; bmEx1* [*phda-1* (wild-type *hda-1* gene) pPD97.93 (*myo-3::gfp*)]), we were able to establish three independent lines that are viable and fertile for at least three generations. These results show that a wild-type copy of the *hda-1* gene can rescue *gon-10(e1795)* defects. Interestingly, HDA-1 with a point mutation (HDA-1^{H145F}) predicted to abrogate the catalytic activity (24) failed to rescue *gon-10*, suggesting that the enzymatic activity of HDA-1 is important for its biological function.

Taken together, our findings show that HDA-1 protein is present at nearly undetectable levels in the *gon-10(e1795)* animals due to a point mutation in the *hda-1* gene. This suggests that the *gon-10(e1795)* mutation causes a severe loss of function. In support of this interpretation, heterozygote animals carrying a copy of the *gon-10* mutation in *trans* with a deficiency covering the *hda-1* locus display phenotypes identical to those of the *gon-10* homozygotes or animals in which zygotic *hda-1* expression is selectively inhibited by RNAi. Thus, the result is consistent with the hypothesis that *gon-10(e1795)* is likely a genetic null *hda-1* mutation.

The lack of zygotically expressed *gon-10* HDA-1 causes defects in gonadogenesis and vulval development. To investigate the function of HDA-1 in *C. elegans* development, we analyzed the development of mutant hermaphrodites. Surprisingly, the *gon-10(e1795)* mutant exhibited a restricted role for HDA-1 in specific developmental processes. *gon-10(e1795)* homozygote animals complete embryogenesis and mature to adulthood presumably due to the maternal supply of the HDA-1 protein. However, adult animals are sterile with a number of interesting gonadal and vulval phenotypes. The main phenotypes are summarized in Table 1 and described below.

We found that gonadogenesis is severely affected in the *gon-10(e1795)* homozygote animals (Fig. 3). Although cells necessary for the formation of the somatic gonad appear to be present and differentiated, the corresponding tissues are nevertheless severely disorganized (Fig. 4 and data not shown).

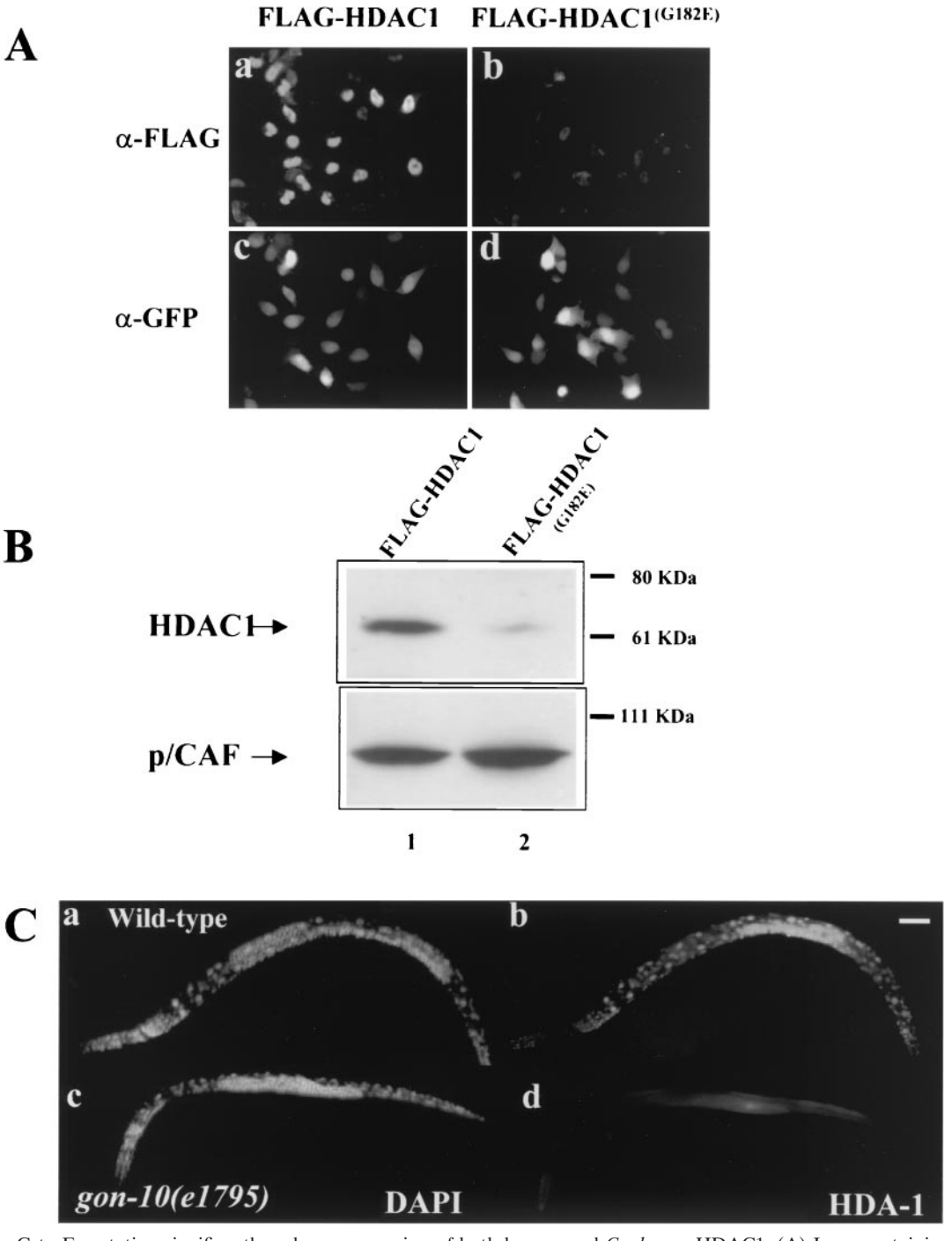


FIG. 2. The G-to-E mutation significantly reduces expression of both human and *C. elegans* HDAC1. (A) Immunostaining of wild-type and mutant human HDAC1 proteins in transfected HeLa cells. The wild-type and mutant HDAC1^{G182E} proteins were tagged with FLAG at the C terminus, cotransfected with a GFP-expressing plasmid into HeLa cells, and visualized by using an anti-FLAG antibody (α-FLAG). The transfected cells were identified by a GFP polyclonal antibody that recognizes the cotransfected GFP (α-GFP). (B) Western blot analysis of wild-type and mutated human HDAC1 proteins in transfected HeLa cells. Protein extracts from HeLa cells expressing the wild-type (lane 1) or mutated version of human HDAC1 (lane 2) were probed with an anti-FLAG antibody. Cotransfected, FLAG-tagged p/CAF protein was used as controls. The FLAG-tagged HDAC1 and p/CAF proteins can be distinguished by their molecular masses. The positions of molecular mass markers are indicated to the right of the blots. (C) Expression of *C. elegans* HDA-1 in the wild type and *gon-10(e1795)* mutants. Wild-type and mutant L4 worms were stained with the HDA-1 polyclonal antibody (b and d) and costained with 4′,6′-diamidino-2-phenylindole (DAPI) (a and c). For a control for immunostaining, the same animals were found to be stained positive for the monoclonal antibody MH27 (data not shown). Bar, 50 μm.

TABLE 1. Summary of developmental defects in *gon-10(e1795)* animals

Tissue	Defect	% of animals with defects[a]
Gonad		
Somatic	Shorter proximal gonad arm[b]	99 (163/164)
	Shorter distal gonad arm[c]	100 (164/164)
	No spermatheca	100 (164/164)
Germ line	Fewer oocytes	82 (56/68)
	No oogenesis	16 (11/68)
Vulva	Protruding vulva	100 (>300/>300)
	Multivulvae	20 (16/82)

[a] The number of animals with defects/total number of animals studied is shown in parentheses.
[b] Abnormal early turn of gonadal arms.
[c] Abnormal distal parts of the gonadal arms not properly elongated.

This is best illustrated through our analysis of GFP reporter expression in somatic gonad tissues. The *lim-7::gfp* reporter marks 16 of the 20 terminally differentiated sheath cells in a wild-type gonad. We found that all 16 sheath cells were present in the *gon-10(e1795)* homozygotes. However, instead of being located along the sheath, as in the wild-type animals, they remain located centrally in the region of the vulva (Fig. 4, compare panels B and D). Similarly, improper localization of the spermatheca cells, as seen with reporter *egl-26::gfp*, was observed (Fig. 4, compare panels F and H). Compared with the somatic gonad tissues, germ cell development does not appear to be as grossly affected. We detected both mitotic and meiotic populations of the germ cells in *gon-10(e1795)* animals (data not shown). However, the number of germ cells was reduced by a third (380 versus 500; $n = 3$), fertilized eggs were rarely found (for eggs with fewer than 100 cells, data not shown), and none of the homozygous mutants were fertile ($n > 500$). It is unclear whether the germ cell defects are direct or indirect consequences of the *gon-10(e1795)* mutation.

***gon-10(e1795)* affects vulval induction.** In addition to gonadogenesis defects, vulval development is affected by the *gon-10(e1795)* mutation. For instance, 20% of *gon-10(e1795)* animals display a multivulva (Muv) phenotype (Table 1). In wild-type animals, vulva formation is induced by the anchor cell. Thus, one possible explanation for the observed Muv phenotype could be the presence of supernumerary anchor cells. However, this does not appear to be the case, as the wild-type number of anchor cells (1 cell, $n = 50$) is present in the mutant (1 cell, $n = 68$), as observed using the anchor cell reporter *cdh-3::gfp* (Fig. 4J and L).

The vulva is derived from three of six potential vulval precursor cells (VPCs) (P5.p to P7.p cells) through a series of stereotypically oriented cell divisions. Whereas these VPCs undergo two longitudinal divisions, the third division is either longitudinal or transversal; 2 of the 12 cells resulting from the second division do not divide further (42). All *gon-10(e1795)* hermaphrodites display what appears to be a protruding vulva (Fig. 3B). Lineage analyses in *gon-10(e1795)* revealed that the first two rounds of cell division are oriented longitudinally, as in the wild type. However, cells that divide transversally during the third division in the wild type sometimes divide instead along the longitudinal axis in the mutant (Fig. 5C and D). Therefore, HDA-1 activity appears to be specifically required either for preventing the longitudinal division or promoting the transverse division during the final round of vulval cell division. In *gon-10(e1795)* animals, the abnormal division orientation is often accompanied by ectopic invagination in the region of the descendants of P5.p to P7.p cells (Fig. 5A and E).

In addition to the lineage defect, approximately 20% of the *gon-10(e1795)* animals have multivulvae, suggesting a role for HDA-1 in vulval induction. Vulval development in *C. elegans* includes positive regulation by *Ras* signaling and negative regulation by a set of genes termed the synMuv genes (for synthetic multivulva) (8). Worms carrying double mutations of a synMuvA gene and a synMuvB gene result in synthetic Muv phenotypes (9). Previous studies using RNAi suggested that HDA-1 is a member of the synMuv family, either solely as a synMuvB gene or as both a synMuvA and synMuvB gene (35, 45). We tested the role of HDA-1 in the synMuv pathway by constructing double mutants carrying *gon-10(e1795)* and either a synMuvA *lin-15A(n433)* or synMuvB *lin-15B(n744)* gene. As indicated in Table 2, we failed to observe an increase in the induction of VPCs in the double mutants over that for *gon-10(e1795)* single mutants. This observation together with the finding that 20% of the *gon-10(e1795)* animals already displayed a Muv phenotype suggest that *hda-1* is not a classical synMuv gene.

***lag-2* is derepressed in *gon-10(e1795)* homozygotes.** To begin to address the molecular mechanisms underlying the *gon-10(e1795)* phenotypes, we screened for potential target genes of HDA-1 by looking for the ectopic activation of various cell type-specific promoters fused to *gfp* reporter transgenes in the *gon-10(e1795)* background. The expression of the majority of these reporter transgenes was not detected outside the cells normally expressing the transgenes (data not shown). A notable exception is the *lag-2* promoter which is misregulated in the mutant background (Fig. 6). *lag-2* encodes a Delta/Serrate homologue, a ligand of the Notch receptor family which has previously been implicated in somatic gonad cell fate decisions (49) and in germ cell (25, 46) and vulval development (31) in *C. elegans*. As shown in Fig. 6A, in wild-type animals the expression of the *lag-2* promoter-driven GFP is restricted to the distal tip cells and a few cells of the vulva. However, this promoter was globally derepressed in *gon-10(e1795)* animals (Fig. 6A, right panel). Derepression of the *lag-2::gfp* gene was observed using three independent lines of transgenic animals where the reporter gene was either integrated on different chromosomes (two lines) or not integrated.

Using RT-PCR, we also compared the levels of *gfp* transcripts in *lag-2::gfp* transgenic animals with and without the mutation. As expected, *gfp* expression was significantly increased in mutant animals (Fig. 6B, compare lanes 2 and 3), which is consistent with the results for GFP activity in the *lag2::gfp* transgenic animals. We next asked whether the endogenous *lag-2* promoter was similarly regulated by HDA-1 by comparing the levels of *lag-2* transcripts in wild-type and *gon-10(e1795)* animals. As shown in the top panel of Fig. 6B, a low level of endogenous *lag-2* was detected in wild-type N2 animals (lane 1) and in the *lag2::gfp* transgenic animals (lane 2), probably due to the limited number of cells expressing *lag-2*. However, the level of *lag-2* transcripts was

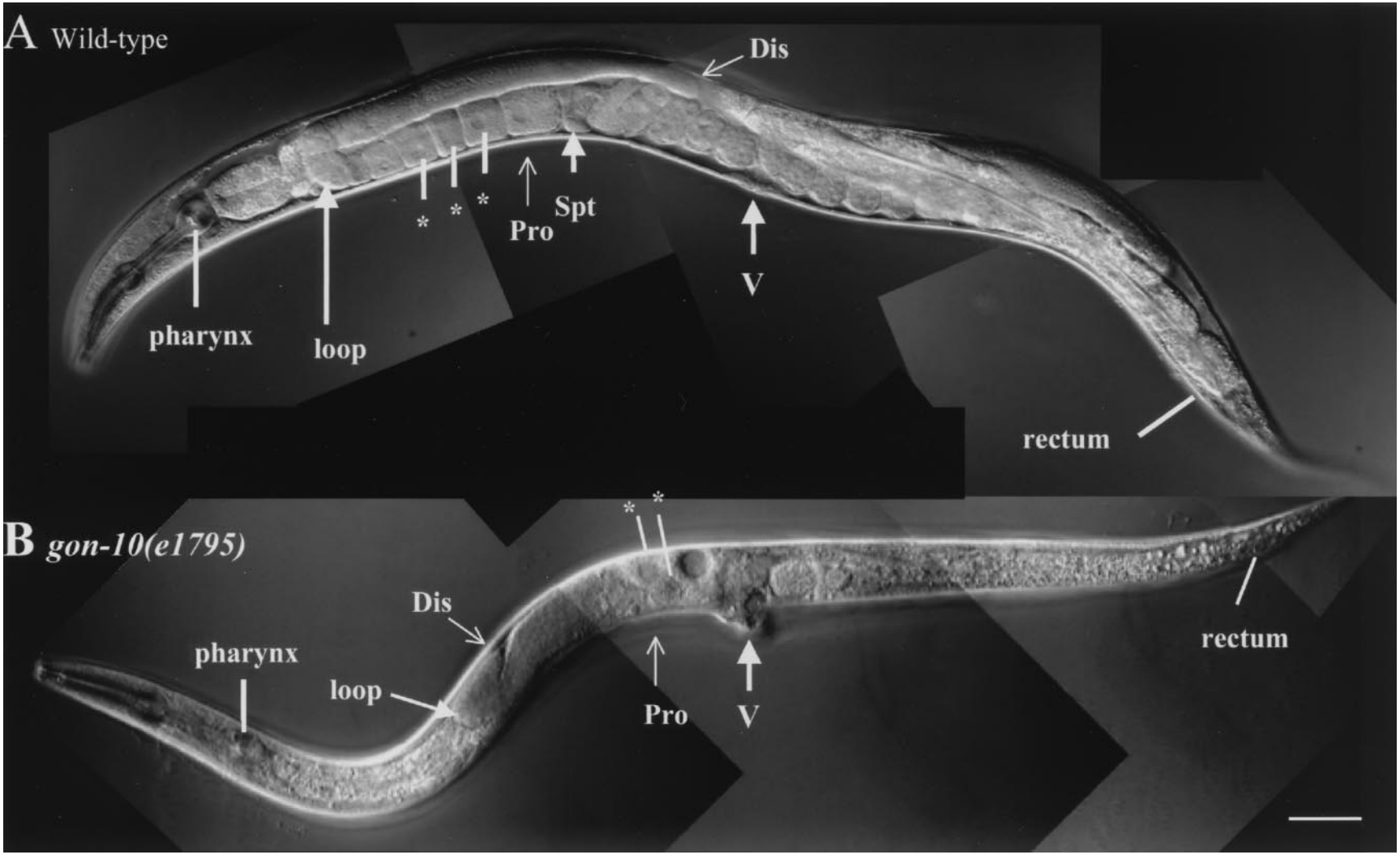


FIG. 3. HDA-1 is necessary for hermaphrodite gonadogenesis and vulval development. Differential interference-contrast Nomarski images of wild-type and *gon-10(e1795)* adult hermaphrodites. The distal (Dis) and proximal (Pro) parts of the gonad, oocytes in the proximal half of the gonad (asterisks), spermatheca (Spt), and vulva (V) are indicated. The distal part of the gonad is not fully elongated and fewer developing oocytes are located in the region of the vulva in the *gon-10* (*e1795*) animals compared to the wild-type animals. No visible gonadal somatic structure, such as the spermatheca and uterus, are detected in the mutant animals. Note the protruding vulva in the *gon-10(e1795)* animal. The anterior part of the animal is on the left, while the posterior part is on the right. Bar, 50 μm.

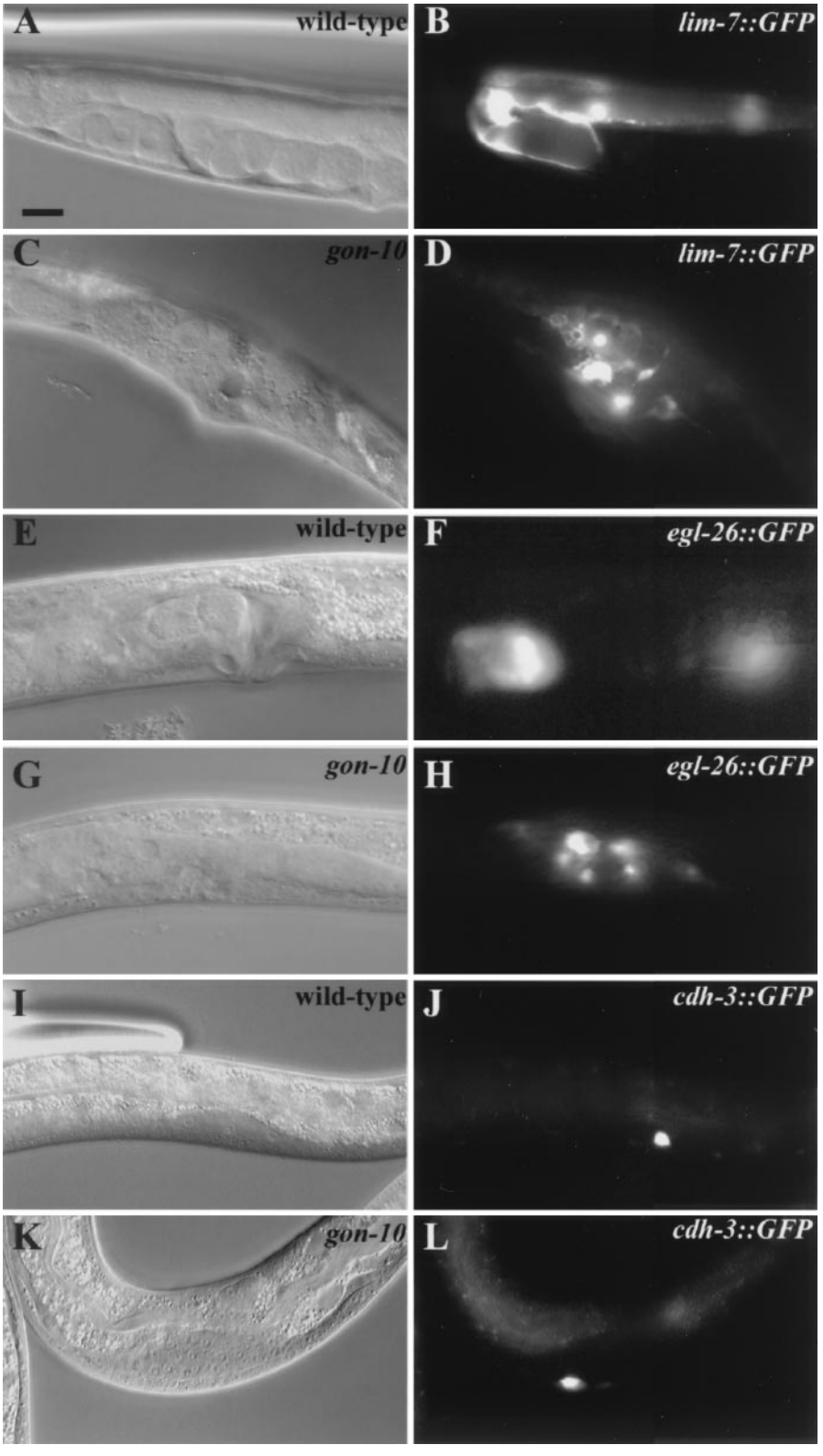
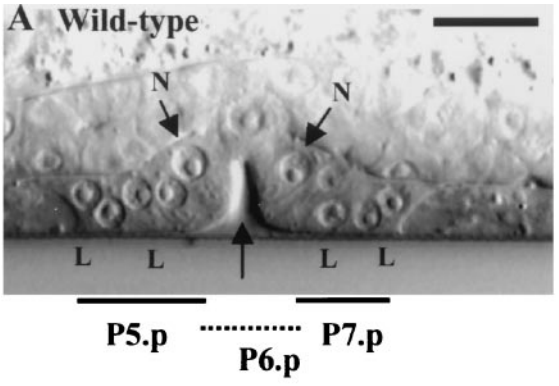
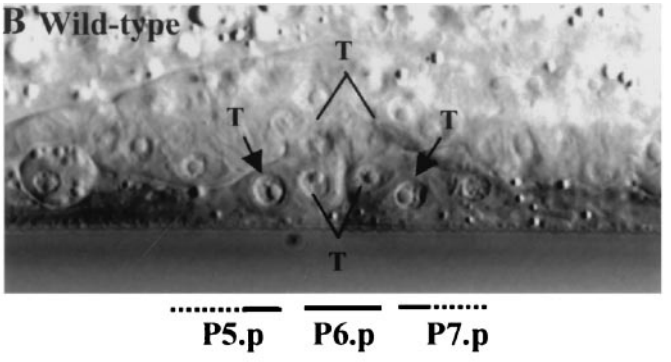
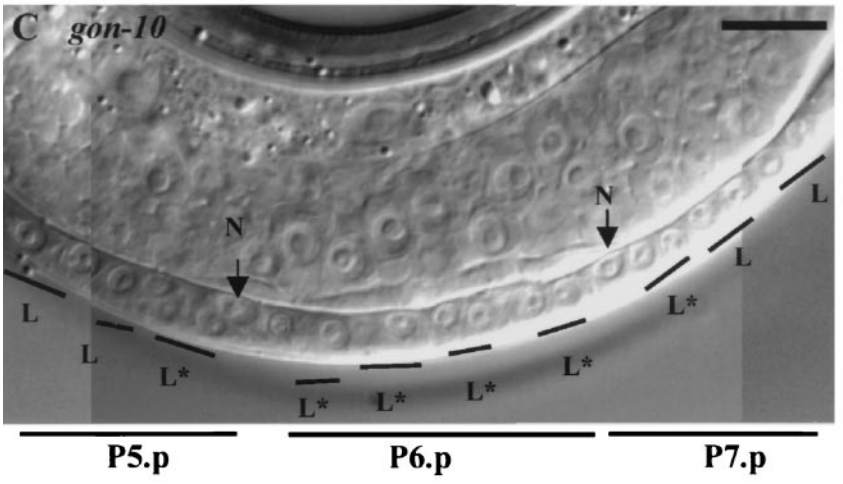


FIG. 4. Abnormal somatic tissue organization in *gon-10(e1795)* animals. Paired differential interference-contrast Nomarski and fluorescence images of the wild-type and *gon-10(e1795)* mutant animals expressing GFP reporter transgenes are shown. *lim-7:: GFP* was used as a sheath cell marker (A and B versus C and D), *egl-26:: GFP* was used as a marker of the spermatheca (E and F versus G and H), and *cdh-3:: GFP* was used to visualize the anchor cell (I and J versus K and L). Animals are oriented so that the anterior part is to the left. Bar, 10 μm.







D

genotype	P5.p	P6.p	P7.p
Wild-type	LLTN	TTTT	NTLL
	LLLN	LLLL	NLLL
	LNLN	TLLT	NLLL
gon-10	LLLN	LTTL	NLLL
	LLLN	TTTL	NLLL
	LLLN	LLLT	NTLL

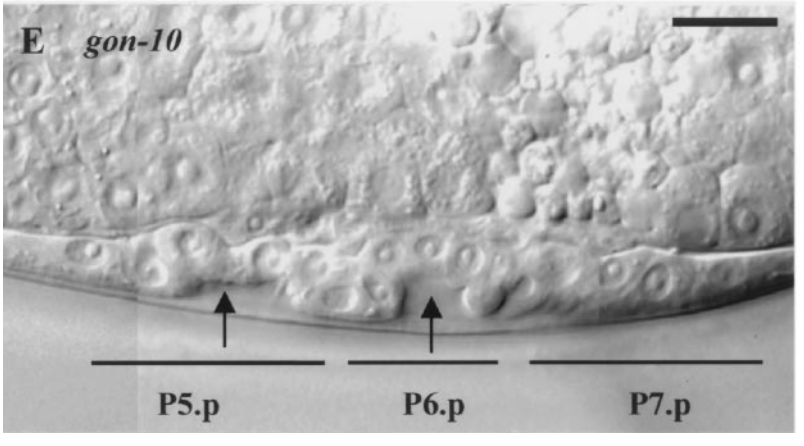


FIG. 5. Abnormal orientation of cell division during vulval development in *gon-10(e1795)* animals. The orientation of the division is longitudinal (L) or transversal (T); cells which do not divide (N) are indicated. (A and B) Stereotypical divisions of P5.p to P7.p cells result in a three-dimensional vulval structure with cells in two focal planes in wild-type animals. The unlabeled, black arrow in panel A points to an invagination. (C) Defective transverse divisions in *gon-10(e1795)* animals leave all descendants of P5.p to P7.p cells in the same focal plane. Abnormal transverse divisions are indicated by asterisks. (D) Orientation of the third division of P5.p to P7.p cells in wild-type and *gon-10(e1795)* animals. (E) Abnormal invagination in *gon-10(e1795)*. Two invaginations are indicated by the unlabeled, black arrows. Bar, 10 μm.

significantly elevated in *gon-10(e1795)* animals (lane 3), suggesting that this gene is derepressed in the absence of HDA-1 function. For a control, *sc35*, a ubiquitously expressed constitutive splicing factor (34), was found to be expressed at comparable levels in both wild-type and mutant animals (Fig. 6B). The finding that *lag-2* is derepressed in *gon-10(e1795)* suggests that *lag-2* may be a direct target of HDA-1 repression. Furthermore, these results implicate deregulation of Notch/Delta signaling in the gonad phenotype of *gon-10(e1795)* mutant animals.

TABLE 2. Vulval induction phenotype caused by *gon-10(e1795)* and genetic interactions between *gon-10* (*e1795*) and synMuv mutant[a]

Genotype	No. of induced VPCs	No. of animals examined
Wild-type	3.0	15
lin-15A(n433)	3.0	15
lin-15B(n744)	3.0	15
gon-10(e1795)[b]	3.2	39
gon-10(e1795); lin-15A(n433)[b]	3.2	28
gon-10(e1795); lin-15B(n744)[b]	3.2	24

[a] The average number of induced VPCs for each genotype is shown. The state of morphogenesis was assessed by counting the number of induced VPCs (normally three in the wild type) at the L4 stage.

[b] Homozygote *gon-10(e1795)* animals are easily distinguished from heterozygotes by their abnormal gonad.

DISCUSSION

HDA-1 has previously been shown to play a critical role during early development, as animals depleted of the maternal and zygotic HDA-1 die as embryos (43). In this study, we provide evidence that zygotic HDA-1 is required during gonadogenesis and vulval development. We have identified a mutation in the *hda-1* gene as the cause of the gonadogenesis and vulval defects of the previously isolated *C. elegans* mutant *gon-10(e1795)* animals. This conclusion is based on the following observations. (i) *gon-10(e1795)* worms carry an *hda-1* gene with a point mutation that results in a change of a conserved amino acid within the catalytic domain of HDA-1. (ii) This point mutation is correlated with nearly undetectable levels of HDA-1 expression in *gon-10(e1795)* homozygotes. (iii) A wild-type copy of the *hda-1* gene can fully

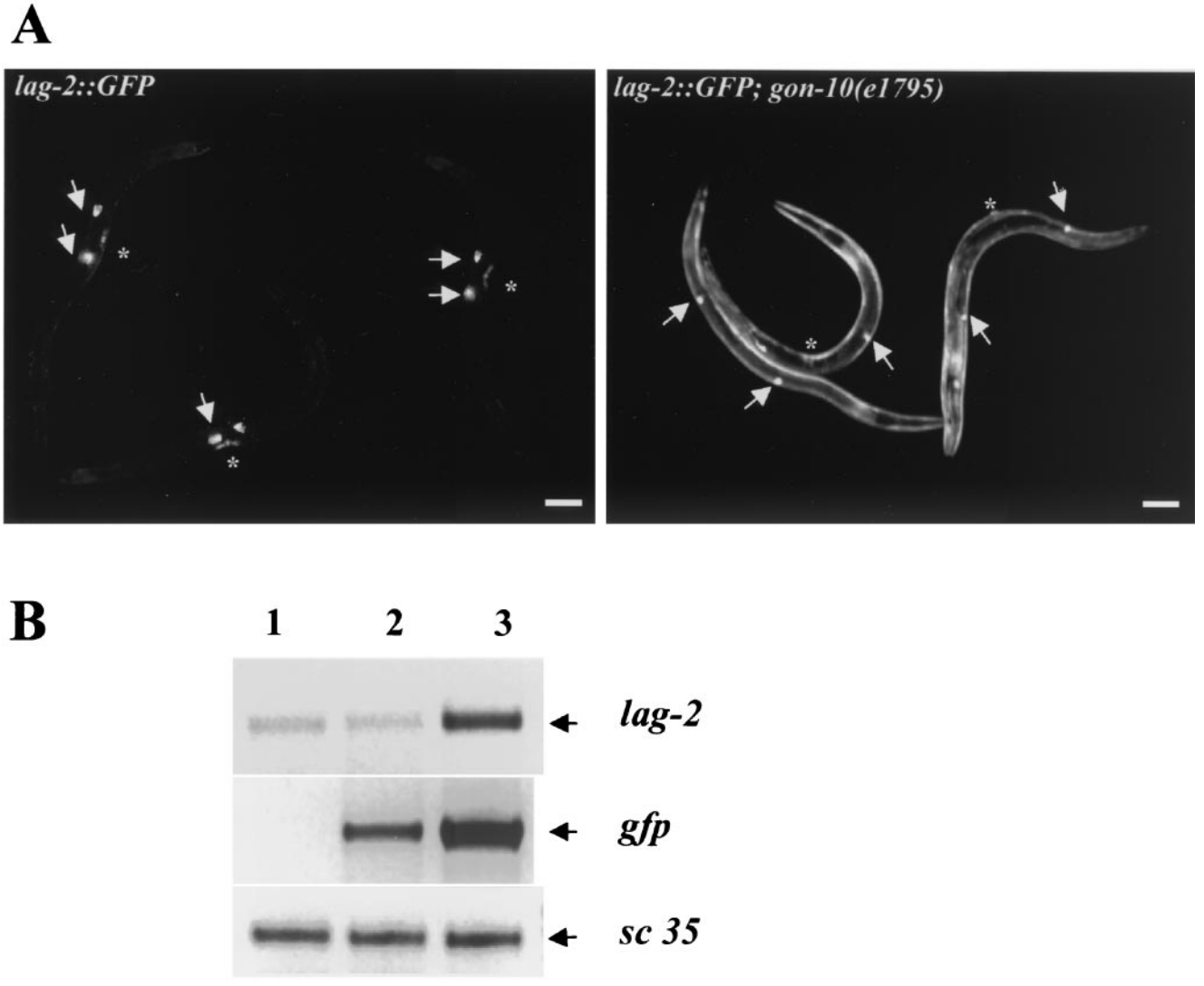


FIG. 6. *lag-2* is derepressed in the *gon-10(e1795)* mutant. (A) The *lag-2:: GFP* transgene is derepressed in *gon-10* mutants. *lag-2:: GFP* transgene expression in wild-type animals (left panel) and *gon-10(e1795)* animals (right panel). Arrows indicate the locations of the distal tip cell, and the asterisks mark the position of the vulva. Bar, 50 μm. (B) The endogenous *lag-2* promoter is derepressed in *gon-10* animals. Total RNA was isolated from wild-type N2, *lag-2::gfp*, and *lag-2::gfp; gon-10(e1795)* transgenic lines. RT-PCR experiments were performed to determine the levels of the endogenous *lag-2* mRNA and *gfp* mRNA in the wild-type N2 (lane 1), *lag-2::gfp* (lane 2), and *lag-2::gfp; gon-10* transgenic (lane 3) animals. Both the endogenous and ectopic *lag-2* promoters were derepressed (compare lanes 2 and 3). The expression of *sc35*, a ubiquitous mRNA, was used as an internal control.

rescue the developmental defects associated with the *gon-10* mutant. Although *gon-10(e1795)* animals lack zygotic HDA-1, they express the two other highly related class I HDACs, HDA-2 and HDA-3 (unpublished result). Interestingly, the presence of HDA-2 and -3 in the reproductive system does not substitute for the loss of HDA-1, indicating that zygotic HDA-1 plays a highly specific role in gonadogenesis. We also provide evidence that HDA-1 plays a role in the control of cell division orientation of the vulval precursor cells during vulval development. Taken together, these findings identify a surprisingly specific developmental requirement for an ubiquitously expressed histone deacetylase.

HDA-1 and *C. elegans* gonadogenesis. Gonadogenesis in *C. elegans* involves the development of both germ cells and somatic gonad tissues. Somatic gonadogenesis involves two morphogenic processes, the extension of tissue buds that elongate and form the *C. elegans* bilobal gonad long arms and the formation of complex, differentiated epithelial tubes composed of distinct modular units, i.e., the uterus, spermatheca, and sheaths in hermaphrodites and the vesicle and vas deferens in males (30). One salient feature of the gonadogenesis phenotype in *gon-10(e1795)* animals is the lack of organized somatic gonad tissues. Using cell type-specific promoter-driven GFP genes as markers, we were able to visualize the presence of the various differentiated cells necessary for the formation of these tissues. Thus, the lack of organized somatic tissue is probably due to defects in tissue morphogenesis. Taken together, these findings suggest that genes whose products are important for cell-cell communication and cell polarity may be targets of HDA-1. It will be interesting to identify HDA-1 target genes during gonadogenesis to test this hypothesis.

Compared with the somatic gonad tissue defects, the germ line development appeared to be affected to a lesser extent in *gon-10(e1795)* mutants. Both mitosis and meiosis appear to take place, and as a result we could identify sperm and oocytes by Nomarski analysis. However, a reduction in the number of meiotic cells in the mutants was observed. At present, it is unclear whether germ line defects are direct or indirect consequences of the mutation. In our rescue experiments, we introduced the wild-type gene into *gon-10(e1795)* animals as simple tandem arrays, which are susceptible to germ line silencing (29). We were nonetheless able to achieve efficient rescue in which the rescued lines were stable for several generations. Since a low level of germ line expression might be sufficient for rescue, these findings do not rule out the possibility that the germ line defects may also be a direct consequence of the *gon-10(e1795)* mutation.

HDA-1 and *C. elegans* vulval development. Our analysis of *gon-10(e1795)* indicates that HDA-1 plays a crucial role in vulval development, a process in *C. elegans* which is known to be regulated positively by *Ras* signaling and negatively by the synMuv genes (named for synthetic multivulva) (8). Worms carrying combinations of two mutations of the synMuvA and synMuvB genes result in the synthetic multivulval phenotype (9). A number of transcription factors have been identified as synMuv genes, including the *C. elegans* homologs of E2F, DP1, and Rb (6, 35). Previous studies suggested that *hda-1* acts as a synMuv gene (35, 45). However, we were unable to observe an increase in vulval cell induction when *gon-10(e1795)* was placed either in a synMuvA or synMuvB background. This discrepancy could be due to the fact that previous studies used RNAi, while this study analyzed a genetic mutant. We also cannot rule out the presence of a persisting maternal HDA-1 component or other histone deacetylase activities in the *gon-10(e1795)* mutant which might mask a synMuv phenotype. Interestingly, the lineage defect or morphogenic phenotype observed for *gon-10(e1795)* animals has also been reported for *lin-40* metastasis-associated factor 1 (MTA) (7), which has been identified along with HDAC1 as a member of the NURD complex (32, 38). Further analyses are necessary to understand the precise molecular role of HDA-1 in vulval development.

Vulval development in *C. elegans* is also regulated by the LIN-12/Notch signaling pathways (reviewed in references 17 and 31). Through a mechanism of lateral inhibition, Notch signaling prevents certain VPCs from adopting the primary vulval cell fate. We show here that transcription of one of the Notch ligand-encoding genes, *lag-2*, is derepressed in *gon-10(e1795)* mutants, resulting in widespread expression of LAG-2. It is interesting to speculate that the abnormal expression of LAG-2 may contribute to the Muv phenotypes seen in *gon-10(e1795)* animals. It is possible that derepression of the expression of LAG-2 leads to overactivation of LIN-12/Notch signaling and mimics gain-of-function alleles of *lin-12* which have already been shown to result in multivulval phenotypes (17, 18).

Corepressor complexes and development of the reproductive systems. In mammals, class I HDACs such as HDAC1 and HDAC2 (which are both homologs of HDA-1) are components of multiple corepressor complexes. HDAC1 and -2 have been found to be present in at least two distinct biochemical complexes, i.e., the SIN3 and NURD/Mi-2 complexes (32, 38). Members of the NURD/Mi-2 complex, with the exception of MBD3, are conserved in *C. elegans*. Interestingly, in addition to HDA-1, two other members of the NURD/Mi-2 complex, the *C. elegans* MTA1 homolog LIN-40, and Mi-2 homologs LET-418 and CHD-3 have recently been shown to play a role in vulval development (7, 45, 48). The *C. elegans* SIN3 complex is less well understood, but at least two components of this complex, SIN3 (encoded by open reading frame F02E9.4) and SAP18 (encoded by open reading frame C16C10.4), are present in *C. elegans* (1). Preliminary experiments suggest that inhibition of SIN3 expression results in sterile animals (unpublished result) and therefore may play a role in either one of these two processes.

In addition, HDAC1 and -2 also interact with Rb and Groucho, both of which are corepressors and can be targeted to promoters via interactions with DNA-binding transcription factors (reviewed in reference 32). The *C. elegans* Rb homolog LIN-35 has been shown to play a role in vulval development (35): the role of the *C. elegans* Groucho homolog UNC-37 in gonadogenesis and vulval development is unknown. However, on the basis of biochemical results, we predict that the SIN3 complex and UNC-37 probably play a role in either one or both of these processes. Since HDA-1 is a component of a number of the different corepressor complexes discussed above, it is not surprising that the *hda-1* mutation can affect both gonadogenesis and vulval development in postembryonic *C. elegans*, perhaps acting through distinct corepressor complexes.

In summary, we have provided compelling evidence that the *gon-10(e1795)* mutant is an *hda-1* mutant. We have shown that mutation in this ubiquitous histone deacetylase causes surprisingly specific defects during *C. elegans* development, compro-

mising the development of somatic gonad tissues (germ cells and the vulva). Our findings highlight the essential and specific roles ubiquitously expressed histone deacetylases play in a multicellular organisms and suggest possible important functions for HDAC-containing corepressor complexes in the development of reproductive systems of other organisms as well.

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