A CBP/p300 homolog specifies multiple differentiation pathways in *Caenorhabditis elegans*

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Mammalian p300 and CBP are related transcriptional cofactors that possess histone acetyltransferase activity. Inactivation of CBP/p300 is critical for adenovirus E1A to induce oncogenic transformation and to inhibit differentiation, suggesting that these proteins are likely to play a role in cell growth and differentiation. Here we show that a *Caenorhabditis elegans* gene closely related to CBP/p300, referred to as *cbp-1*, is required during early embryogenesis to specify several major differentiation pathways. Inhibition of *cbp-1* expression causes developmental arrest of *C. elegans* embryos with no evidence of body morphogenesis but with nearly twice the normal complement of embryonic cells. Mesodermal, endodermal, and hypodermal cells appear to be completely absent in most embryos, however, all of the embryos exhibit evidence of neuronal differentiation. Our analysis of this phenotype suggests a critical role for CBP-1 in promoting all non-neuronal pathways of somatic differentiation in the *C. elegans* embryo. In contrast, we show that *C. elegans* genes related to components of a conserved mammalian histone deacetylase, appear to have a role in repressing somatic differentiation. Our findings suggest a model in which CBP-1 may activate transcription and differentiation in *C. elegans* by directly or indirectly antagonizing a repressive effect of histone deacetylase.

[Key Words: p300; HDAC1; CBP-1; HDA-1; differentiation; *C. elegans*; histone acetyltransferase; histone diacetylase]

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Embryonic cells are exposed to numerous intrinsic and extrinsic signals that regulate differentiation. The precise control of these differentiation signals is required both to produce specific embryonic structures and to set aside cells for later differentiation events. Although a great deal is known about the genetic pathways that control embryonic patterning and select specific developmental fates, relatively little is known about factors that are involved in differentiation decisions in general.

In *Caenorhabditis elegans*, early embryonic cells exhibit distinct developmental potentials as early as the two-cell stage (for a recent review, see Schnabel and Priess 1997). Several of the key developmental regulators that specify cell fates in *C. elegans* embryos have been identified. These include two transcriptional activators, SKN-1 and PAL-1, which are localized to the posterior blastomere where they specify endodermal and mesodermal cell fates (Bowerman et al. 1992, 1993; Hunter and Kenyon 1996). A third posterior factor, PIE-1, specifies the germ-line fate and modulates the activities of SKN-1 and PAL-1, but appears to do so through a mechanism that involves transcriptional repression (Mello et al.

1992, 1996; Seydoux et al. 1996). Among anterior descendants, signaling through the Notch-related protein GLP-1 plays a major role in distinguishing cell fates (for review, see Schnabel and Priess 1997). In nearly all cases, these regulators play critical roles in selecting between alternative potential fates. In the present study, we describe the analysis of several *C. elegans* genes (introduced below) that appear to have general functions in regulating differentiation. Notably, one of these *C. elegans* genes, referred to here as *cbp-1* for *cbp*, p300-related gene, and its protein product as CBP-1, appears to be essential for the function of all of the currently identified activators of cell fate in the early *C. elegans* embryo. Strikingly, blocking the expression of CBP-1 is correlated with extra cell divisions and ectopic neuronal differentiation. Blocking the expression of a second set of *C. elegans* genes that encode proteins related to components of a conserved mammalian histone deacetylase causes nearly opposite phenotypic effects and can bypass partially the need for the differentiation promoting functions of both CBP-1 and of the SKN-1 transcription factor.

CBP-1 is a *C. elegans* protein that shares significant sequence homology with mammalian p300 and CBP proteins (Chrivia et al. 1993; Arany et al. 1994; Eckner et al.

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1994; Lundblad et al. 1995), which are related transcriptional cofactors that function to integrate transcriptional and signaling events in cells (for review, see Eckner 1996; Janknecht and Hunter 1996; Goldman et al. 1997; Shikama et al. 1997). p300 was first identified in coimmunoprecipitation experiments as one of the adenovirus E1A-associated polypeptides (Yee and Branton 1985; Harlow et al. 1986), whereas CBP was first characterized as a cAMP response element-binding protein (CREB) that potentiates the transcriptional activity of CREB (Chrivia et al. 1993; Arias et al. 1994; Kwok et al. 1994). The biological functions of p300 and CBP were first inferred from studies of the E1A oncoprotein, which can induce oncogenic transformation of primary rodent cells in cooperation with a second oncogene such as adenovirus E1B (van der Elsen et al. 1982, 1983; Ruley 1983). This growth-promoting and transforming potential of E1A is correlated, at least in part, with its ability to regulate the activity of the p300 family members (Whyte et al. 1989; Wang et al. 1995). Consistent with a role of p300 in growth regulation, overexpression of p300 suppresses E1A-induced transformation (Smits et al. 1996). More recently, p300 mutations have been identified in certain cancers (Muraoka et al. 1996), suggesting a potential connection between p300 and tumorigenesis. The ability of E1A to interfere with CBP/p300 function is also critical for E1A to inhibit differentiation (Mymryk et al. 1992; Kirshenbaum and Schneider 1995). Collectively, these observations suggest an essential role for CBP/p300 in controlling cell growth and differentiation.

Consistent with the hypothesis that CBP/p300 is important for differentiation, recent studies have shown that differentiation of myoblast cells *in vitro* can be inhibited by microinjection of α -p300 antibodies (Eckner et al. 1996; Puri et al. 1997). The role of CBP/p300 in development is further supported by the finding that a *Drosophila* homolog is important for pattern formation during embryogenesis (Akimaru et al. 1997). CBP/p300 are targeted to promoters through interactions with multiple sequence-specific DNA-binding transcription factors (for review, see Eckner 1996; Janknecht and Hunter 1996; Goldman et al. 1997; Shikama et al. 1997). Several mechanisms have been proposed to account for the transcriptional activity of CBP/p300, including their ability to interact with basal transcription factors (Kwok et al. 1994; Lee et al. 1996; Swope et al. 1996) or with the RNA polymerase II complex (Nakajima et al. 1997a,b), or both. Recently, CBP/p300 have been shown to possess histone acetyltransferase (HAT) activity (Bannister and Kouzarides 1996; Ogryzko et al. 1996), and to associate with other HATs (X.-J. Yang et al. 1996; Chen et al. 1997). This raises an additional possibility that they may activate transcription by modifying histones and possibly other factors that are involved in transcription (Gu and Roeder 1997; Imhof et al. 1997).

Hyperacetylation of histones has been correlated with increased transcription, whereas hypoacetylation has been correlated with transcriptional repression in general (for review, see Brownell and Allis 1996; Roth 1996; Wolffe 1996; Grunstein 1997; Pazin and Kadonaga 1997;

Wade and Wolffe 1997). The level of histone acetylation is determined by the action of both HATs and histone deacetylases. The fact that CBP/p300 possess HAT activity suggests that CBP/p300, as transcriptional regulators, may promote transcription by overcoming a repressive function of histone deacetylases.

The first biochemically characterized histone deacetylase in mammalian cells is composed of two major components, HDAC1 and RbAp48 (Taunton et al. 1996; Hassig et al. 1997). HDAC1 is the mammalian homolog of the yeast RPD3 protein, which was first identified as a global transcriptional regulator that participates in both repression and activation of transcription (Nasmyth et al. 1987; Vidal et al. 1990; Vidal and Gaber 1991; Stillman et al. 1994), and has also been demonstrated to be part of a histone deacetylase complex (Rundlett et al. 1996). HDAC proteins can repress transcription when targeted to the promoters, and the repression is, at least in part, dependent on the histone deacetylase activity (X.-J. Yang et al. 1996; Alland et al. 1997; Hassig et al. 1997; Heinzel et al. 1997; Kadosh and Struhl 1997; Laherty et al. 1997; Nagy et al. 1997). On the other hand, RbAp48 was first identified as an Rb-interacting protein (Qian et al. 1993), and since has been shown to participate in a number of protein complexes including the histone deacetylase (Taunton et al. 1996), the chromatin assembly factor 1 (Smith and Stillman 1989; Kamakaka et al. 1996), and the chromatin remodeling NURF complexes (Martinez-Balbas et al. 1998). The precise function of RbAp48 in these complexes is currently unclear.

The *in vivo* biological functions of CBP/p300 and the underlying mechanisms through which these factors control transcription, cell growth, and differentiation are by and large undefined. We decided to turn to *C. elegans*, in which these issues can be addressed using genetic means. Our findings suggest that CBP-1 plays a central role in promoting several major differentiation events during *C. elegans* embryonic development, whereas HDAC1- and RbAp48-related proteins appear to have antagonistic, repressive functions.

Results

CBP-1 appears to promote differentiation pathways in early embryos

A search of the *C. elegans* database identified three sequences related to CBP/p300, R10E11.1, K03H1.10, and F42F12.7. The R10E11.1 locus encodes a functional protein, which we refer to here as CBP-1, that has been reported to be related to mammalian CBP and p300 (Arany et al. 1994; Lundblad et al. 1995). A multiple sequence alignment of human CBP, p300, and CBP-1 is shown in Figure 1, which illustrates the extensive sequence conservation throughout the whole protein between CBP/p300 and CBP-1. The largest block of a highly conserved region between human CBP/p300 and CBP-1 spans amino acids 861–1757 of CBP-1. This region is encoded by a single exon (the eighth exon) of CBP-1 and contains several important functional domains de-

p300 and differentiation in *C. elegans* early embryos

Figure 1. Sequence comparison of CBP-1 with human CBP and p300. The amino acid sequences of human CBP, p300, and *C. elegans* CBP-1 (R10E11.1) were aligned using the PIMA program (Human Genome Center, Baylor College of Medicine, Houston, TX). The regions of amino acid identity are boxed using the SeqVu program, Garvan Institute of Medical Research, Sydney, Australia).

fined in CBP/p300. These include the HAT domain that possesses HAT activity (Bannister and Kouzarides 1996; Ogryzko et al. 1996), the region that is critical for binding to E1A and the HAT, P/CAF (Eckner et al. 1994; Yang et al. 1996), as well as the bromodomain and the C/H 2 domain (Eckner et al. 1994) that mediate p300 interaction with ATF-2 (Kawasaki et al. 1998). Between CBP and CBP-1, sequence comparison shows a 51% amino acid identity in the HAT domain (CBP, amino acids 1174–1850; CBP-1, amino acids 953–1653) and 70% in the region important for E1A and P/CAF interactions (CBP, amino acids 1801–1850; CBP-1, amino acids 1586–1635). The bromodomain and the C/H2 domain (Eckner et al. 1994) show 61% and 50% amino acid identity, respectively. Another functional domain that is highly conserved is the KIX domain of CBP (amino acids 586–679) that binds transcription factor CREB (Parker et al. 1996), and it shares 70% amino acid identity with CBP-1.

In contrast, K03H1.10 and F42F12.7 are two probable pseudogenes that are each associated with large direct repeats flanking a reverse transcriptase gene and are predicted to encode only truncated proteins (D. Lawson, pers. comm.). Therefore, the fact that CBP/p300 and CBP-1 share sequence homology throughout the whole protein, and the fact that all the functional domains are highly conserved, strongly suggest that CBP-1 is an evolutionarily conserved *C. elegans* homolog of mammalian CBP/p300.

We used a reverse genetic assay, termed RNAi (Rocheleau et al. 1997), to determine the phenotypic consequences of blocking CBP-1 expression. Several recent studies have shown that this RNA injection procedure induces phenotypes that appear identical to those that are known to result from strong or null genetic mutations in the genes tested (Guo and Kumphues 1995, 1996; Lin et al. 1995; Mello et al. 1996; Powell-Coffman et al. 1996; Guedes and Priess 1997; Rocheleau et al. 1997; Thorpe et al. 1997). Where examined, these RNA-induced phenotypes have been correlated with a lack of protein expression from the targeted genes (Lin et al. 1995; Powell-Coffman et al. 1996). We will refer to specific experiments and to embryos thus treated with RNAi by listing the gene name followed by *RNAi*.

For the RNAi assay, in vitro transcribed RNA from a *cbp-1* cDNA clone (yk6f6; kind gift of Y. Kohara, National Institute of Genetics, Mishima, Japan) was injected into wild-type adult hermaphrodites. Beginning a few hours after injection, wild-type hermaphrodites that received *cbp-1* RNA produced exclusively inviable embryos (see Materials and Methods). Strikingly, the terminal *cbp-1(RNAi)* embryos arrest development with nearly twice the normal complement of cells, and most embryos completely lack evidence of muscle, intestinal, and hypodermal differentiation, as judged by their morphology in the light microscope and by immunostaining with tissue-specific antibodies (Fig. 2, cf. a with b; Table 1; data not shown). In contrast, the germ cells seem unaffected and appear normal morphologically (indicated by arrowheads in Fig. 2b) and show proper localizations of the germ-line-specific P-granules (data not shown;

Figure 2. Expression and phenotypic analysis of CBP-1. Wild-type and *cbp-1(RNAi)* embryos are shown on the left and the right columns, respectively. (a,b) Nomarski images ~12 hr after fertilization. The wild-type embryo in a is a threefold embryo ready to hatch. The *cbp-1(RNAi)* embryo in b exhibits numerous small cells and shows no evidence of mesodermal, endodermal, or hypodermal tissue organization. Arrowheads indicate the two germ-line cells that appear normal morphologically. (c–h) Neuronal differentiation. Expression of UNC-86 protein (c,d); expression of MEC-7 (e,f), one of the four cells that expresses MEC-7 in wild-type embryos is shown in e; expression of HLH2::GFP (g,h). Two-cell embryos stained with affinity purified anti-human CBP antibodies, CBP 451 (i,j). Note that the nuclear staining is eliminated in the *cbp-1(RNAi)* embryo (j); however, apparently nonspecific cytoplasmic and membrane staining is not eliminated (i,j). Under similar exposure conditions, the levels of apparent nonspecific cytoplasmic staining in both wild-type and *cbp-1(RNAi)* embryos are comparable.

Strome and Wood 1982). Furthermore, it appears that normal programmed cell death occurred in 100% of the *cbp-1(RNAi)* embryos (data not shown).

The numerous small cells produced in these embryos most closely resemble neurons, which led us to hypoth-

Table 1. Tissue differentiation in embryos lacking *Cbp-1* expression

Tissue	Percent positive	No. of embryos scored
Muscle	8	224
Intestine	9	131
Pharynx	0	294

C. elegans cbp-1(RNAi) embryos were analyzed with antibodies recognizing proteins that are specific for muscle, gut, and pharynx tissues. Muscle tissue was scored by mAb 5.6, intestinal cells with mAb 2CB7, and pharyngeal tissue with mAb 921 (see Materials and Methods for details).

esize that many of the cells that would have differentiated into other tissues in *cbp-1(+)* embryos have instead adopted neuronal fates. Consistent with this hypothesis, we found that *cbp-1(RNAi)* embryos contain approximately twice the normal complement of cells expressing two neuronal differentiation specific proteins, UNC-86 (Finney and Ruvkun 1990) and MEC-7 (Hamelin et al. 1992). In normal development, the UNC-86 transcription factor is expressed in 47 neuronal precursors (Finney and Ruvkun 1990; Fig. 2c). After inhibition of CBP-1 expression, ~100 cells were positively stained with α -UNC-86 antibodies (Fig. 2d). The *mec-7* gene encodes a β -tubulin specific to the touch receptor neurons, and is a marker for terminally differentiated neurons (Hamelin et al. 1992). Using an α -MEC-7 antibody (gift of G. Ruvkun, Massachusetts General Hospital, Boston, MA), we found MEC-7 expression in all four touch receptor neurons in wild-type embryos where it is detectable in both the cell body and axonal extensions. One such cell is shown in Figure 2e (the other three positive cells are in different focal planes). Five to 10 MEC-7-positive cells were found in terminal stage embryos lacking CBP-1 expression and these cells showed evidence of axon outgrowth consistent with neuronal differentiation (Fig. 2f). Finally, we found that a GFP reporter construct, HLH-2::GFP, which is expressed predominantly in neurons late in *C. elegans* embryogenesis (Krause et al. 1997; Fig. 2g), is expressed in numerous cells in *cbp-1(RNAi)* embryos (Fig. 2h), and many of these cells exhibit what appear to be axonal extensions (data not shown). These observations suggest that CBP-1 is required for mesodermal, endodermal, and hypodermal differentiation in *C. elegans* and that extra cell divisions and neuronal differentiation may represent default developmental pathways in its absence.

CBP-1 is required for early events in mesoderm and endoderm development

To determine when in development CBP-1 protein is expressed, we stained wild-type embryos with affinity-purified antibodies that recognize CBP-1 (CBP 451, Santa Cruz Biotech, CA). As shown in Figure 2i, CBP-1 protein is first detectable in nuclei beginning at the two-cell stage, and this ubiquitous nuclear staining persists at least through the 100-cell stage of embryogenesis (data

not shown). This nuclear staining is completely absent in *cbp-1(RNAi)* embryos (Fig. 2j; data not shown), suggesting a correlation between the lack of CBP-1 expression and the phenotypes described above. The fact that CBP-1 protein is present at the two-cell stage, before the earliest detected zygotic transcription, suggests that CBP-1 is likely to be a maternally expressed protein in *C. elegans*.

The phenotype of *cbp-1(RNAi)* embryos and the observation that CBP-1 protein is present during the early cleavage stages raise the possibility that CBP-1 functions in the early embryo along with maternally provided cell-fate determining sequence-specific DNA-binding transcription factors such as SKN-1 and PAL-1. The SKN-1 and PAL-1 proteins are localized to posterior lineages beginning at the two-cell stage where they are required to specify endodermal, mesodermal, and certain hypodermal cell fates (Bowerman et al. 1992a; Hunter and Kenyon 1996). To address the question of when in development CBP-1 activity is needed, we examined *cbp-1(RNAi)* embryos for early events in the differentiation of mesoderm and endoderm.

The first evidence of mesodermal-specific development is the expression of the *C. elegans* MyoD homolog *hlh-1* in muscle precursors in the early embryo (Krause et al. 1990), which is downstream of SKN-1 (Mello et al. 1992) and PAL-1 (Hunter and Kenyon 1996). Consistent with an early role for CBP-1 in muscle specification, we found that GFP expression driven by the *Ce-MyoD* promoter (kind gift of M. Krause) was absent in nearly all *cbp-1(RNAi)* embryos examined (Fig. 3, cf. a with b). The PAL-1 transcription factor activates muscle differentiation pathways in the posterior of the embryo and directly or indirectly activates MyoD expression. In *cbp-1(RNAi)* embryos, maternal PAL-1 protein showed wild-type localization and normal levels of expression (data not shown). This finding, together with the fact that *pal-1* and *cbp-1(RNAi)* embryos both show a lack of MyoD expression and muscle differentiation, suggests that CBP-1 is likely to function either along with PAL-1 (as its cofactor) or downstream of PAL-1 in the early embryonic events, leading to the activation of MyoD and the specification of mesodermal precursors.

The earliest evidence for differentiation of the endoderm in *C. elegans* occurs when gastrulation begins at the 28-cell stage, at which time two endodermal precursors (the E-cells) migrate into the interior of the embryo and are covered by surrounding blastomeres. The E-cells divide again only after gastrulation is complete, resulting in a delay of ~20 min in division timing in the E-lineage relative to other blastomeres in the embryo (Fig. 2c,d). This delay in division timing and the gastrulation process itself are both dependent on transcription (Powell-Coffman et al. 1996) and require SKN-1 activity (Bowerman et al. 1992a, 1993). Consistent with an early block in endoderm differentiation in *cbp-1(RNAi)* embryos, we found that the E-cells showed accelerated division timing (2-min delay in the mutant vs. 26 min in the wild-type embryos) and failed to gastrulate in all five lineages examined (cf. Fig. 3, e and f, with the wild type shown in

Figure 3. Early development and CBP-1 activity. Embryos from wild-type animals (*top*) are compared to similarly staged *cbp-1(RNAi)* embryos (*bottom*). (*a–b*) MyoD::GFP expression in eight wild-type embryos (*a*), and the lack of expression in all but one cell of nine *cbp-1(RNAi)* embryos (*b*). (*c–f*) Early division patterns. Relevant cells are labeled and the arrows that parallel the mitotic apparatus indicate the direction of cleavage. Division of the MS sister cells, MSa and MSp, occurs with similar relative timing in wild-type (*c*) and *cbp-1(RNAi)* embryos (*e*). Division of E-sister cells occurs inside the embryo with a left–right orientation, 26 min after the MS division in wild-type embryos (*d*). In *cbp-1(RNAi)* embryos these cells divide on the ventral surface of the embryo, only 2 min after the MS division (*f*).

Fig. 2, c and d; data not shown). These observations suggest that CBP-1 acts before the 28-cell stage in *C. elegans* and may function along with SKN-1 in endoderm specification.

Identification of multiple *C. elegans* genes related to components of a conserved mammalian histone deacetylase

Both p300 and CBP are HATs, and one possible mechanism by which they activate transcription and differentiation is by modifying histones and factors involved in transcription (Bannister and Kouzarides 1996; Ogryzko et al. 1996; Gu and Roeder 1997; Imhof et al. 1997). Therefore, we wished to investigate the genetic relationship between CBP-1 and *C. elegans* homologs of HDAC1 and RbAp48, components of a conserved mammalian histone deacetylase (Taunton et al. 1996), in differentiation.

We identified at least six *C. elegans* genes that share varying degrees of sequence similarity with HDAC1 (data not shown). Three of these genes (C53A5.3, C08B11.2, and an open reading frame in the cosmid R06C1) share significant homology with HDAC1 and yeast RPD3 (Fig. 4), and we have named them *hda-1*, *hda-2*, and *hda-3* (for HDAC1-related gene), respectively. Among them, *hda-1* shares the highest sequence homology with human HDAC1 (The GCG GAP program identifies 74% amino acid similarity and 64% amino acid identity between the two proteins). Significantly, the histidine residues located at position amino acids 150, 151, and 188 in yeast RPD3, which have been shown to be important for the histone deacetylase activity (Kadosh and Struhl 1998), are conserved in all three putative *C. elegans* histone deacetylases (Fig. 4).

We also identified two open reading frames, K07A1.11 and K07A1.12, that share extensive sequence homology with RbAp48, RbAp46, an RbAp48-related protein in mammalian cells (Qian and Lee 1995), and with p55, the RbAp48 homolog in *Drosophila* (Tyler et al. 1996). We refer to these two *C. elegans* genes as *rba-1* and *rba-2*, for RbAp48-related genes. Using the GCG GAP program, we find that RBA-1 shares 53% amino acid identity and 63%

amino acid similarity with RbAp48, 52% and 63% with RbAp46, and 53% and 62% with *Drosophila* p55. RBA-2 shares 72% amino acid identity and 79% amino acid similarity with RbAp48, 71% and 80% with RbAp46, and 72% and 81% with *Drosophila* p55. A multiple sequence alignment between RbAp48, the *Drosophila* p55, RBA-1, and RBA-2 using the PIMA program is shown in Figure 5.

We examined the phenotypic consequences of inhibiting expression of HDAC1- and RbAp48-related proteins in *C. elegans* using the RNAi assay. We found that inhibiting expression of *hda-1* or either of the *rba* genes gives rise to embryos that arrest development at the one-fold stage (Fig. 6a; data not shown). Although these embryos often appear to have fewer than the normal embryonic complement of 558 cells, they always exhibit evidence of tissue differentiation and proper organization of the tissue layers. The intestinal cells become polarized and express the birefringent gut granules (Fig. 6b), muscle cells form quadrants along the anterior posterior body axis and undergo muscle contractions in many embryos, and the hypodermal cells spread over and surround the body of the animal (Fig. 6a; data not shown).

We also examined embryos from mothers having received simultaneous injections of RNA prepared from both *rba* genes. These mutant embryos arrest development with ~50–100 large and often multinucleated cells. Despite this early arrest, 70% of these embryos exhibit evidence of intestinal differentiation (data not shown). These observations suggest that the two *rba* genes encode partially redundant functions. The underlying biological cause of the embryonic arrest seen in these single and double injection experiments remains unknown at present.

We were able to obtain cross-reactive antiserum raised against the *Drosophila* RbAp48 homolog p55 [kind gift of J. Tyler and J. Kadonaga (Tyler et al. 1996)]. This α -p55 antiserum detected a prominent nuclear signal present in all embryonic cells beginning at the two-cell stage (Fig. 6; data not shown). Injection of interfering RNA from either *rba* gene diminished but did not eliminate the α -p55 nuclear staining. However, this nuclear staining was completely absent in the embryos obtained from

HDAC1	1	-----MAQTGGTR	31
YRPD3	1	-----MVYEATPDP	41
Ce-HDA1	1	-----MNSNGLMEHGK	35
Ce-HDA2	1	MSDDKFKLDTLFDND	51
Ce-HDA3	1	-----MSLQHSKSR	30
	32	PHRI RMTHNL LNGLYRKMEIYRPHKANAEEMTKYHSDDYIKFLRSIRPD	82
	42	PHRI RMAHSLIMNYGLYKMEIYRAKPAATKQEMCQHTDEYIDIFLSRVTPD	92
	36	PHRI RMTHHLV LNGLYRNLEI RFPFAS FEDMTRFHSDEYMTFLKSANPD	86
	52	PHRI LVVCHL VSVYEMPKYMTVVE SPK LDAADISVFHTEYVNFQLQVTPK	102
	31	PHRI RMTHSL LNGLYRKLNVMRPARAS FSEI TRYHSDDYINFLRNKVSQ	81
	83	NMSEYSKQMR FNVGDCPV FDGLFEFCQLSTGGSVASAVKLNKQQTDA	132
	93	NLEMFKRRESK FNVGDCPV FDGLFEYCSISGGSGMEGAARLNKRGKDMA	142
	87	NLKS FNKQMLK FNVGDCPL FDGLFEFCQLSTGGSLAAATKLNKQKVDA	136
	103	LGLTMPDDVLRQFNIGDCPI FAGLWDYCTLYAGGSVEGARLNKHMNDIV	153
	82	NMSTFTDQMAR FSVGDCPV EDGMYEFCQLSTGGSLAAARLNKQSELA	131
	133	VNWAGGLHHAHKSEASGFCYNDIVLAILELLKYHQRVLYIDIDIHHGDGV	183
	143	VNWAGGLHHAHKSEASGFCYNDIVLGIILELLKYHQRVLYIDIDVHHGDGV	193
	137	INWVGGLHHAHKSEASGFCYNDIVLGIILELLKYHQRVLYIDIDVHHGDGV	187
	154	INWVGGLHHAHKSEASGFCYNDIVLGIILELLKYHQRVLYIDIDIHHGDGV	204
	132	INWVGGLHHAHKSEASGFCYNDIVLAILELLKYHQRVLYIDIDVHHGDGV	182
	184	EEAFYTTDRYMTVSFHKYGEYFPGTGDLRDIGAGGKGYAVNYP LRDGIDD	234
	194	EEAFYTTDRYMTVSFHKYGEYFPGTGDLRDIGAGGKGYAVNYP LRDGIDD	244
	188	EEAFYTTDRYMTVSFHKYGEYFPGTGDLKDIGAGGKGYAVNYP LRDGITT	238
	205	EEAFYTTDRYMTVSFHKYGEYFPGTGDLKDIGAGGKGYAVNYP LRDGITT	255
	183	EEAFYTTDRYMTVSFHKYGEYFPGTGDLKDIGAGGKGYAVNYP LRDGITT	233
	235	ESYEAIFKPYMSKVMEMEFSAVVLQCGSDSLSGDRLGCFNLITIKGHAKCV	285
	245	ATYRSVIFEPVIAKKIMWYQPSAVVLQCGSDSLSGDRLGCFNLISMEGHAKCV	295
	239	VSYQSIIFKPIIMTKVMERFSAVVLQCGSDSLSGDRLGCFNLITIKGHAKCV	289
	256	EPYLLKLFESVIGFENFPEALIVLQCGSDSLSGDRLGCFNLISMEGHAKCV	306
	234	VTYERILFRTIMGEVMAIRFEPFSAVVLQCGSDSLSGDRLGCFNLITIKGHAKCV	284
	286	EFVKS FNL FLMMLGGGGYTI RNVARCWYETAVALDTEIPNE LPYNDYFEY	336
	296	NYVKS FGI PMMVVGGGGYTI RNVARCWYETAVALDTEIPNE LPYNDYFEY	346
	290	RFFKS YNV PLMMVGGGGYTI RNVARCWYETAVALDTEIPNE LPYNDYFEY	340
	307	KYVKS LGK PLMLVGGGGYTI RNVARCWYETAVALDTEIPNE LPYNDYFEY	357
	285	EYVKS FNV PLMLVGGGGYTI RNVARCWYETAVALDTEIPNE LPYNDYFEY	335
	337	FQDFK LHSIP-SNMTNONTNEYLEKIKQRLFENLRMLPHAFGVQMOAIP	386
	347	YQPDYKLSVRP-SNMFNNTPEYLDKVMNTIFANLENTKYAPSVQNLNHTPR	396
	341	FGENYRLHIES-SNAANENSSDMLAKLQTDVIANLEQLTFVPSVQMRPTPE	390
	358	YETPRLLRPNLVPAKMNDANSAAYLASIEKFTIACLRMIRGAPSVQMQNIVG	408
	336	FIPDYK LHIKPLAKSNTPEFIDQITIVALLENLKQLPHVPSVQMQSIST	386
	387	DAIP-EESGDEDEDPKRISICSSDKRIACEEFSDEEEEGEQQR	431
	397	DA-EDLGDVEEDSAEAKDKGGSQYARDLHVEHND-EFY	433
	391	DALSALNDDSLIADQANPDKRLPPQITDGMIQDDGDFYDGEREGD-DR	437
	409	IRLD-EIEQIEENERLQKSSSIEYEVGKYSKMEIEGCFVEEDSKPP	455
	387	S-CDISIVKTFDEKLIRDHQNDVVRVTFQFEEDVQVEDSAEFVD-QQEPETK	434
	432	KNSSNFKKAKRV--KTE-DEKEKDPEEKKEVTEEEKTKEEKPEAKGVKEE	478
	0	-----	433
	438	RNESDAKRAAQF--ESEGGEKQKTE	461
	456	SFPPGQDPRRIQQYWGYSGLAPPRSHSDVIEEAKYEDRRDKDLNIPGI	506
	435	NIQSMKRHASPICAAQEE-MKKNRIDGGENVA-----	465
	479	VKLA	483
	0	-----	433
	482	-----	463
	507	P-----	507
	0	-----	465

Figure 4. Identification of three *C. elegans* sequences related to mammalian HDAC1 and yeast RPD3. Using the BLAST program, we identified three *C. elegans* sequences from the Sanger *C. elegans* database that are closely related to human and yeast histone deacetylases represented by HDAC1 (Taunton et al. 1996) and RPD3 (Vidal and Gaber 1991), respectively. These three *C. elegans* genes are referred to here as *hda-1*, *hda-2*, and *hda-3*. *hda-1* and *hda-2* correspond to open reading frames C53A5.3 and C08B11.2, respectively. *hda-3* is an open reading frame taken from the cosmid R06C1. The human, yeast, and *C. elegans* sequences were aligned using the PIMA program (Human Genome Center, Baylor College of Medicine, Houston, TX). The regions of amino acid identity are boxed using the SeqVu program, (Garvan Institute of Medical Research, Sydney, Australia).

simultaneous injection of interfering RNA prepared from *rba-1* and *rba-2* (Fig. 6d). An unrelated early embryonic epitope recognized by the P-granule-specific antibody K76 (Strome and Wood 1982), was unaffected (Fig. 6c, d, FITC staining). Similarly, the expression of CBP-1 appeared unaffected (data not shown), suggesting that the interfering RNA specifically blocks expression of the targeted genes. These observations suggest that both *rba-1* and *rba-2* contribute to the expression of the prominent nuclear signal detected by the α -p55 antibodies.

Interfering with expression of HDAC1- and RbAp48-related genes can partially suppress the differentiation defects of *cbp-1(RNAi)* and *skn-1(zu67)* mutant embryos

We first asked whether inhibiting expression of *C. elegans* histone deacetylase-like proteins can reverse the differentiation defects associated with the lack of CBP-1 expression. Significantly, we found that RNA interfer-

ence directed against *hda-1*, *rba-1*, or *rba-2* restored endoderm differentiation in >80% of the *cbp-1(RNAi)* embryos (Fig. 7a,b; data not shown), suggesting that CBP-1 may promote endoderm differentiation by directly or indirectly antagonizing the activities of histone deacetylases.

As described earlier, the endoderm differentiation defects observed in *cbp-1(RNAi)* embryos are similar to those caused by mutations in the transcription factor SKN-1 (Bowerman et al. 1992a; Blackwell et al. 1994). This suggests that CBP-1 may function with SKN-1 or downstream transcription factors to specify endoderm differentiation. Such a hypothesis would predict that inhibiting HDA-1 expression in the *skn-1* mutant background might suppress partially the *skn-1* mutant phenotype. To test this possibility, we performed *hda-1(RNAi)* injection experiments in homozygous *skn-1(zu67)* mutant mothers. We find that with inhibition of *hda-1* expression, endoderm differentiation is restored completely to the resulting *skn-1(zu67)* mutant embryos

hRbAp48	1	- - - MADKEA - AFDD	AVEERVINEEYK	IWKKNTPFLYDL	VMTHALE	41
Drop55	1	MVDRSDNAAESFDD	AVEERVINEEYK	IWKKNTPFLYDL	VMTHALE	45
Ce-RBA1	1	- - - - - MSE	AESCEETSKEHR	IWKKNTPFLYDL	VMTHALE	34
Ce-RBA2	1	- - - - -	- - - - -	- - - - -	MTALE	6
	42	WPSLTAQWLDPDVT	RPEGKDFS	IHLVGLGHT	SDEQNHLV	IASVQ
	46	WPSLTAQWLDPDVT	KQDGKDYSVHR	ILGHT	SDEQNHLV	IASVQ
	35	WPSLSVQWMPDVT	KTENS	DSMHRM	IHGHT	CGVQNHLMI
	7	WPSLSVQWLPDVA	KDNS	DHTIHL	ILGHT	SDEQNHLV
						SKIC
	86	IPNDDAQFDASHYDS	EKG	EFGGFG	SVSGKIE	EIKINHEGEVNR
	90	LPSEDAQFDGSHYDNE	KGE	EFGGFG	SMCGKIE	EIKINHEGEVNR
	90	ITDTPEFDDAKWDS	ERE	EFGGFG	YGE	GSAAKWDTE
	50	MPITDDAQFDASRYDTE	ERS	EYGGFG	AVNGKVEP	DLRINHEGEVNR
						93
	130	ARYMPONPC	LIATKT	PSSDVLV	FDYTKHPS	KDPSPGECNPD
	134	ARYMPONAC	VIATKT	PSSDVLV	FDYTKHPS	KDPSPGECNPD
	125	ARYMPHNP	FIASRG	PSSDVLV	FDYTKHPS	KDPSPGECNPD
	94	ARYMPQKSN	LIATKT	PSSDVLV	FDYTKHPS	KDPSPGECNPD
						174
	175	GHOKEGYGLSWNP	NLSGH	LLSASDDHT	ICLWD	ISAVPKEGKVVD
	179	GHOKEGYGLSWNP	NLSGH	LLSASDDHT	ICLWD	ISAVPKEGKVVD
	169	GHEGEGYGLSWNP	NLSGH	LLSASDDHT	ICLWD	ISAVPKEGKVVD
	138	GHTKEGYGLSWNP	NLSGH	LLSASDDHT	ICLWD	ISAVPKEGKVVD
						219
	220	KTIFTGHTAVVEDVS	WHLLHES	LFGSVA	DDQKLM	IWDTRSNNTSK
	224	KNIFTGHTAVVEDVS	WHLLHES	LFGSVA	DDQKLM	IWDTRSNNTSK
	214	QSKFKGHSSNAEDVS	FHALHNFV	FSGVGD	DRKLN	IWDTRSNNTSK
	183	KDVFKGHSSNAEDVS	FHALHNFV	FSGVGD	DRKLN	IWDTRSNNTSK
						255
	265	PSHSVDAHTAEVNCL	SFN	PNYSEFILATGS	ADKTVALWD	LRNLK
	269	PSHSVDAHTAEVNCL	SFN	PNYSEFILATGS	ADKTVALWD	LRNLK
	256	PQLTAVGHTAEVNCL	SFN	PNYSEFILATGS	ADKTVALWD	LRNLK
	225	PGHCIDAHTAEVNCL	SFN	PNYSEFILATGS	ADKTVALWD	LRNLK
						309
	310	LHSFESHKDE	IFQVQWSPHNET	ILASSGTD	RRLN	VWDLISKI
	314	LHSFESHKDE	IFQVQWSPHNET	ILASSGTD	RRLN	VWDLISKI
	301	MYTLKHND	DEIFQVQWSPHNET	ILASSGTD	RRLN	VWDLISKI
	270	LHSFESHKDE	IFQVQWSPHNET	ILASSGTD	RRLN	VWDLISKI
						352
	353	EQSPEDAEDGPP	ELLFIHGHTAK	ISDFSWNP	NEPWW	ICSVSEDN
	357	EQSPEDAEDGPP	ELLFIHGHTAK	ISDFSWNP	NEPWW	ICSVSEDN
	348	SSSAASSDSV	PEVIF	IHA	GHG	TKVAD
	313	DQSAEDAEDGPP	ELLFIHGHTAK	ISDFSWNP	NEPWW	ICSVSEDN
						397
	398	IMQVWQMAEN	IYND	EDPE	-GSVDPEGQGS	
	402	IMQVWQMAEN	IYND	EDPE	-IPASELENTAV	
	391	ALQVWQMAEN	IYND	EDPE	-TEM	
	358	ILQVWQMAEN	IYND	EDPE	-TPADVVERQQ	

Figure 5. Identification of RbAp48-related proteins in *C. elegans*. Using the BLAST program, we identified two *C. elegans* sequences from the Sanger *C. elegans* database that are closely related to human RbAp48 (Qian et al. 1993), RbAp46 (Qian and Lee 1995), and Drosophila p55 (Tyler et al. 1996). The two *C. elegans* genes are referred to here as *rba-1* and *rba-2*, respectively. *rba-1* and *rba-2* correspond to open reading frames K07A1.11 and K07A1.12. The sequences of human RbAp48, the Drosophila p55, *rba-1*, and *rba-2* were aligned using the PIMA program (Human Genome Center, Baylor College of Medicine, Houston, TX). The regions of amino acid identity are boxed using the SeqVu program, (Garvan Institute of Medical Research, Sydney, Australia).

(Fig. 7c,d). Similarly, inhibiting the expression of either *rba-1* or *rba-2* also restored completely endoderm differentiation in the *skn-1(zu67)* mutant (data not shown).

Because the *skn-1(zu67)* mutation is an early stop codon (C. Schubert, unpubl.) and because the expression of CBP-1 protein was not restored by inhibiting expression of HDA-1 (data not shown), the above findings suggest that blocking the expression of *C. elegans* HDAC1 or either of the RbAp48-related genes can bypass the need for CBP-1 and SKN-1 in the specification of endoderm. These findings suggest that the histone deacetylase related factors analyzed in this study function to regulate endoderm differentiation negatively and that SKN-1 may recruit CBP-1 to overcome this repressive effect. Although it is not clear whether this genetic relationship reflects a direct competition between these factors for acetylation and deacetylation of histones at specific promoters, nevertheless these results suggest that the ability of CBP-1 to antagonize histone deacetylase activity (directly or indirectly) is likely to be important for its differentiation-promoting functions.

Discussion

In the present study, we have shown that a gene closely

related to the human p300 and CBP transcriptional co-factors functions in the early *C. elegans* embryo as a general activator necessary for the proper specification of essentially all early somatic cell fates. Remarkably, in the absence of CBP-1, many of the somatic lineages of the embryo undergo extra cell divisions and exhibit evidence of ectopic neuronal differentiation in later development. We have used the genetic context provided by these studies to examine the relationship of CBP-1 to factors related to HDAC1 and RbAp48, which are components of a conserved mammalian histone deacetylase. Our findings suggest that CBP-1 promotes endoderm differentiation by directly or indirectly antagonizing the repressive function of histone deacetylases.

A role for CBP-1 in differentiation

Studies in vertebrate systems have shown that embryonic cells appear to adopt neuronal cell fates unless instructed to do otherwise (for review, see Hemmati-Brivanlou and Melton 1997). The present study is consistent with this view and suggests that CBP-1 may represent a component that is common to nearly all non-neuronal developmental pathways in *C. elegans*. Therefore, in the future, it will be interesting to determine

Figure 6. Histone deacetylase is essential for *C. elegans* embryonic development. Nomarski (a) and gut birefringence images (b) for terminal stage *hda-1(RNAi)* embryos showing one-fold stage arrest (A very similar phenotype was observed in *rba-1(RNAi)* and *rba-2(RNAi)* embryos.) The birefringent granules visualized in b are storage products that accumulate during the terminal differentiation of gut cells. (c-d) Expression of RBA proteins in a four-cell embryos from wild-type animals before (c) and after (d) injection of a mixture of interfering RNAs corresponding to *rba-1* and *rba-2*. Embryos were stained with affinity-purified polyclonal antibodies, anti-p55, that were raised against the *Drosophila* RbAp48 homolog, p55, and visualized by a secondary antibody conjugated to rhodamine (red). A second antibody, K76, that recognizes the germ-line-specific P-granules was included as a control, and was visualized using an FITC conjugated secondary antibody (green). Prominent anti-p55 specific (red) nuclear staining is visible in wild type (c), but is absent after blocking both *rba-1* and *rba-2* expression (d). The P-granule-specific staining was not affected.

whether developmental factors that block or delay differentiation (or which promote neurogenesis) do so by blocking CBP-1 activity. It remains possible, however, that CBP-1 may be needed for a subset of neuronal differentiation pathways that we were unable to score. In addition to neurogenesis, we have observed that pathways regulating apoptosis and germ-line specification do not appear to require CBP-1. These observations suggest that either independent factors, or basal activities can promote these alternative cell fates in the absence of CBP-1. Alternatively, it is possible that the RNA-mediated interference assay is for some reason unable to block CBP-1 activity in some cell lineages. Therefore, the role of CBP-1 if any, in germ-line specification, apoptosis, and neurogenesis will have to await the analysis of *cbp-1* null mutations.

Mechanisms that underlie the differentiation-promoting function of CBP-1

In recent studies, a correlation has been documented between the steady state of histone acetylation and the transcriptional potential of the genome; in most cases hyperacetylation is correlated with increased transcription, whereas hypoacetylation is correlated with transcriptional repression or silencing (for review, see

Brownell and Allis 1996; Roth 1996; Wolffe 1996; Grunstein 1997; Pazin and Kadonaga 1997; Wade and Wolffe 1997). The identification of nuclear HATs and histone deacetylases, and the revelation that some of these proteins are previously identified transcription factors, provide a molecular connection between HAT and transcriptional regulation. The mammalian CBP/p300 transcriptional cofactors are among the newly identified nuclear HATs. Taken together, these observations lead to a simple hypothesis that part or all of p300 activity in transcriptional activation may involve its ability to acetylate histones at the targeted promoters. A prediction of this model is that the repressive effects of histone deacetylase activities may be antagonized by CBP/p300 function in vivo.

Indeed, we have observed that a block in endoderm differentiation caused by depletion of CBP-1 can be overcome by simultaneously inhibiting the expression of the *C. elegans* HDAC1 homolog HDA-1 (or the RbAp48-related proteins RBA-1 and RBA-2). Similarly, blocking the expression of HDAC1 or RbAp48-related genes can bypass the need for SKN-1 in endoderm differentiation. These genetic interactions, along with molecular data from other systems, are consistent with a molecular model in which the SKN-1 transcription factor may recruit CBP-1 to specific promoters to overcome differentiation repressive effects caused by a *C. elegans* HDAC1/RbAp48-like histone deacetylase complex. A future challenge is to determine whether the ability of CBP-1 to antagonize histone deacetylase is dependent on its inherent or associated HAT activities.

In mammalian cells, the list of sequence-specific DNA-binding transcription factors that may use CBP/p300 as cofactors is growing steadily (for review, see Eckner 1996; Janknecht and Hunter 1996; Goldman et al. 1997; Shikama et al. 1997). Our findings suggest that

Figure 7. Rescue of endoderm differentiation by blocking the activity of histone deacetylase. (a-d) Fields of terminally differentiated embryos visualized with polarized light that detects the endoderm-specific birefringent gut granules. One of 15 *cbp-1(RNAi)* embryos is positive (a). Twelve of 14 *cbp-1(RNAi);hda1(RNAi)* embryos are positive (b). Six of 15 embryos are positive in *skn-1(zu67)* mutant embryos (c). All 23 *skn-1(zu67);hda1(RNAi)* embryos are positive in this view (d).

CBP-1 may exhibit similarly broad specificity in vivo during *C. elegans* embryogenesis. However, it is clear that CBP-1 is not required as a cofactor for all transcriptional events. For instance, CBP-1 is not necessary for transcription of the neuronal-specific genes such as *unc-86*, *mec-7*, and *hlh-2* (Fig. 2), and is also not required for transcription of an early zygotic gene *pes-10* (data not shown). These findings indicate that CBP-1 does not function as a promiscuous transcriptional cofactor.

In summary, our findings for the first time place a homolog of the human proteins CBP and p300 at the base of several major differentiation pathways in *C. elegans*. These results further suggest that CBP-1 specifies differentiation by functioning along with the early cell-fate determining transcription factors, and by antagonizing the functions of factors related to a conserved histone deacetylase complex.

Materials and methods

Strains and plasmids

The Bristol strain N2 was used as the standard wild-type strain. HLH1::GFP, HLH2::GFP, and *pes-10*::GFP were kind gifts from Michael Krause (NIH/NIDDK/LMB, Bethesda, MD) and Geraldine Seydoux (Johns Hopkins School of Medicine, Baltimore, MD), respectively. *cbp-1*, *hda-1*, *hda-3*, *rba-1*, and *rba-2* correspond to open reading frames R10E11.1, C53A5.3, R06C1 (reading frame number unassigned), K07A1.11, and K07A1.12, respectively. The EST clones for *cbp-1* (yk6f6), *hda-1* (yk101g6), *rba-1* (yk117c9), and *rba-2* (yk187f5) were provided by Yuji Kohara (NIG, Japan) and *hda-3* (cm18g11) by the St. Louis *C. elegans* Genome Project. *hda-2* (corresponding to open reading frame C08B11.2) was obtained by reverse transcription coupled with PCR. Interfering RNAs were synthesized in vitro using T7 or S6 polymerases.

Microinjection

Microinjection experiments were performed, as described by Mello et al. (1991), by injecting RNA directly into both arms of the adult gonad. Recently, however, we have discovered that it is not necessary to inject into the gonad to induce interference among progeny of the injected mother. For several genes, the body cavity or intestinal cytoplasm appears to be a more effective target (Fire et al. 1998). With the RNAi technique, it is possible, depending on the timing of the injection and the expression properties of the gene, to observe phenotypes that correspond to distinct temporal functions of the targeted gene. For genes that function both zygotically and maternally, the first few embryos made after injection may have already incorporated the maternal gene product and thus “escape” the maternal RNAi defect. If a zygotic activity exists, these escapers often exhibit a phenotype that corresponds to a block in the zygotic activity of the gene (C. Mello, unpubl.). For example, in the case of *glp-1*, which is required zygotically for fertility, the escapers mature to form sterile adults (the zygotic phenotype for the *glp-1* mutant).

In the case of *cbp-1*(RNAi), we observed a small window in the injected animal's brood consisting of 10 or so embryos that arrest development with morphogenesis defects but otherwise fairly normal tissue differentiation. This type of embryo occurs only in the early part of the brood after injection and is eliminated if the injections are performed into younger adults or

fourth stage larvae. After this brief period, the remainder of embryos produced by the injected mother (often as many as 200–300 embryos) exhibit the stronger phenotype that we infer reflects the combination of zygotic and maternal inhibition. In principal, determining which phenotypes represent a zygotic function for *cbp-1*(RNAi) will have to await isolation of null mutations for the gene.

Immunostaining of *C. elegans* embryos

Antibodies against human CBP (sc-1211) were purchased from Santa Cruz Biotechnology, Inc. The α -UNC-86, α -MEC-7, and α -p55 antibodies were kind gifts from the laboratories of G. Ruvkun (MGH, Boston) and J. Kadonaga (UCSD), respectively. The K76 antibody that recognizes the P-granule has been reported previously (Strome and Wood 1982). Embryos were permeabilized by freeze-cracking and fixed in MeOH for 5 min, and rinsed in acetone for 5 min at -20°C . Antibodies were applied directly to the air-dried samples and incubated at room temperature for 1 hr. The samples were washed in the buffer containing 100 mM Tris (pH 7.5) and 0.5% Tween-20.

Microscopy and lineage analysis

Microscopy and lineage studies were performed as described (Mello et al. 1992). Light and immunofluorescence micrographs were taken electronically using a Photometrics Image Point, digital camera. The identity of differentiated cell types in experimental embryos were assigned based on morphological criteria in the light microscope, followed in most cases by analysis with differentiation-specific antibodies: muscle tissue was scored by mAB 5.6 (Miller et al. 1983), intestinal cells with 2CB7 (Bowerman et al. 1992), pharyngeal tissue with 921 or 3NB12 (Miller et al. 1983; Priess and Thomson 1987), and neurons with α -UNC-86 and α -MEC-7. Criteria for assigning muscle, intestinal, pharyngeal, and hypodermal cell fates were as previously described (Bowerman et al. 1992b; Mello et al. 1992).

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