Double bromodomain protein BET-1 and MYST HATs establish and maintain stable cell fates in *C. elegans*

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SUMMARY
The maintenance of cell fate is important for normal development and tissue homeostasis. Epigenetic mechanisms, including histone modifications, are likely to play crucial roles in cell-fate maintenance. However, in contrast to the established functions of histone methylation, which are mediated by the polycomb proteins, the roles of histone acetylation in cell-fate maintenance are poorly understood. Here, we show that the *C. elegans* acetylated-histone-binding protein BET-1 is required for the establishment and maintenance of stable fate in various lineages. In most bet-1 mutants, cells adopted the correct fate initially, but at later stages they often transformed into a different cell type. By expressing BET-1 at various times in development and examining the rescue of the Bet-1 phenotype, we showed that BET-1 functions both at the time of fate acquisition, to establish a stable fate, and at later stages, to maintain the established fate. Furthermore, the disruption of the MYST HATs perturbed the subnuclear localization of BET-1 and caused bet-1-like phenotypes, suggesting that BET-1 is recruited to its targets through acetylated histones. Our results therefore indicate that histone acetylation plays a crucial role in cell-fate maintenance.

KEY WORDS: Fate maintenance, BET, MYST HAT, Acetylated histone, *C. elegans*

INTRODUCTION
During animal development, stem cells progressively lose their multipotency and their progeny’s fates are increasingly restricted. Moreover, once cell fate begins to be restricted, including in non-totipotent progenitors, cells maintain their differentiated characteristics and cannot revert to stem cells in vivo, although they can dedifferentiate into stem cells when forced to express certain defined factors in vitro (Takahashi and Yamanaka, 2006). For cell fates to be stably maintained in vivo, the gene expression program must also be maintained. Epigenetic mechanisms, including DNA methylation and histone modifications, are believed to play important roles in maintaining transcriptional programs. Among histone modifications, the roles of histone methylation in the maintenance of cell fate are relatively well understood. For example, in *Drosophila*, the polycomb genes, which include the genes for the histone methyltransferase, E(z), and for methylated histone binding protein (Polycomb), are required for the maintenance of cell fates through the transcriptional silencing of Hox genes (Ringrose and Paro, 2004). In mammals, an H3K4 histone methyltransferase, MLL, is crucial for the maintenance of memory Th2 cell function (Yamashita et al., 2006). However, unlike histone methylation, the well-described roles of histone acetylation are in transcriptional activation per se and in cell differentiation, but its roles in the maintenance of cell fate are poorly understood.

BET-family proteins are evolutionarily conserved and have two bromodomains, which recognize acetylated histones (Florence and Fuller, 2001; Loyola and Almouzni, 2004). In *Saccharomyces cerevisiae*, the BET-family protein Bdf1 has an anti-silencing function that prevents the spreading of the SIR silencing complex from the telomere or mating loci (Ladurner et al., 2003). In *Drosophila*, a BET-family gene, fs(1)h is required for the transcriptional activation of a Hox gene, Ubx (Chang et al., 2007; Florence and Fuller, 2001). BET-family proteins, but not other bromodomain proteins, co-localize with the chromosomes during mitosis (Loyola and Almouzni, 2004), suggesting that they might protect the cell transcriptional state from being disrupted by mitosis. However, it is unknown if BET-family proteins are required for the maintenance of cell fates in multicellular organisms.

In *C. elegans*, histone modification enzymes are shown to be required for normal development. For example, MYS-1, a member of the MYST family of acetyltransferases (MYST HATs), represses the induction of vulval fates (Ceol and Horvitz, 2004). Histone methyltransferase MES-2 represses the expression of Hox genes in inappropriate regions (Ross and Zarkower, 2003) and promotes the loss of developmental plasticity in early embryonic cells (Yuzuyuk et al., 2009). However, the roles of chromatin factors in the maintenance of cell fate have not been established in *C. elegans*.

Here, we found that a *C. elegans* BET-family protein, BET-1, is required for the maintenance of various cell fates. In bet-1 mutants, although cell-fate specification can be abnormal, when cells acquired normal characteristics initially, their fates were not maintained, and they eventually took on the fates of their relatives (e.g. cousin or sister cell) at later stages. Rescue experiments suggested that BET-1 must be expressed both at the time of cell-fate acquisition and later, when aberrant cell transformation occurred in the bet-1 mutants. Disruption of the MYST HATs caused phenotypes similar to those of bet-1 mutants and disturbed the subnuclear localization of BET-1, suggesting that BET-1 is recruited to its target sites through acetylated histones. Our results indicate that histone acetylation plays crucial roles in the establishment and maintenance of stable cell fates.

MATERIALS AND METHODS

Strains and culture
N2 Bristol was used as the wild-type strain. *n4075* (Ceol and Horvitz, 2004), *e1338* (Chalfie and Sulston, 1981) and *os22* (Sawa et al., 2000) were used as the *mys-1*, *mec-3* and *psa-1* mutations, respectively. Animals were
cultured at 22.5°C. To obtain synchronized animals, newly hatched larvae were collected for 1 hour, so larvae ‘1 hr after hatching’ were actually 0 to 1 hour post-hatching.

The following GFP or RFP markers were used (TC and TL indicate transcriptional and translational fusions, respectively): wls51 [scm::gfp] (TC) (Zhang et al., 2003), osEx113 [psa-3::gfp] (TL) (Arata et al., 2006), zldls5[mec-4::gfp] (TC) (Clark and Chiu, 2003; Lai et al., 1996), qsl56[lag-2::gfp] (TC) (Kostic et al., 2003), mnl17[osm-6::gfp] (TL) (Collet et al., 1998), wls33[dop-3::rfp] (TC) (Chase et al., 2004), mnl32[mec-7::gfp] (TC) (Pujol et al., 2000), uIs22[mec-3::gfp] (TC) (Toker et al., 2003), vls1[dat-1::gfp] (TC) (Nass et al., 2002), osdx5[scm::vrm-1::gfp] (TL) (Mizumoto and Sawa, 2007) and qsl74[pys-1::gfp::pop-1] (TL) (Siefried et al., 2004).

Cloning of bet-1

The bet-1(ok364) mutation was located between unc-11 and dpy-5 on LGI by three-factor mapping and between cosmids T01A4 and D1007 by single nucleotide polymorphism (SNP) mapping. Feeding RNAi against Y119C1B.8 in this region caused the Psa phenotype (see Table S1 in the supplementary material), the production of extra distal tip cells (DTCs) and PDE neurons and embryonic lethality. scm::bet-1::gfp and bet-1::bet-1::gfp rescued the Psa and extra-PDE phenotypes of os46 mutants. Therefore, bet-1 is Y119C1B.8.

Plasmid construction

p bet-1::bet-1::gfp comprised a 3 kb genomic fragment between the region 5′ upstream of the first exon to the NcoI site in the first exon, amplified by PCR, and a cDNA fragment from the NcoI site to the codon corresponding to residue 630, ligated to pPD95.77 (a gift from A. Fire, Stanford University, CA, USA). For scm::bet-1::gfp, a fragment from yk1000709 corresponding to almost the entire BET-1 protein (residues 1-630) was inserted between an scm promoter fragment (Koh and Rothman, 2001) and the gfp gene from pPD95.77. For pbs::bet-1::gfp, the yk100709 fragment was inserted between the heat-shock promoter from pPD94.78 (a gift from A. Fire) and the gfp gene from pPD95.77. For pss::dd::bet-1::gfp, the destabilization domain (DD) amplified from pPTuner (Clontech) was inserted between the yk100709 fragment and the heat-shock promoter in pbs::bet-1::gfp. For dat-1::mCherry, a 1.4 kb genomic fragment corresponding to the 5′ upstream region of dat-1 and the mCherry gene were inserted into pGEM-T-easy (Promega). pPD118.17 was used for mec-3::gfp (a gift from M. Chalfie, Columbia University, NY, USA). dat-1::mCherry (25 μg/ml) or pPD118.17 (25 μg/ml) were injected with herring sperm DNA (100 μg/ml) to make complex arrays (Kelly et al., 1997). Ectopic expression of the markers and their increase in adults compared with young larvae was observed using complex arrays (see Table S4 in the supplementary material). Thus, bet-1::GFP is functional. When we expressed BET-1::GFP in the posterior lateral ganglion (PLG) and in the V5.p cell lineage, we observed the right PLG, as the left PLG contains progeny of the Q cell. For heat-shock, animals were transferred to plates pre-heated to 33°C, maintained at 33°C for 30 or 10 minutes, then transferred to 22.5°C.

RESULTS

bet-1 encodes a BET-family protein that colocalizes with chromosomes

In a screen for the absence of phasmid socket cells, which are derived from the T cell (Psa phenotype) (Sawa et al., 2000), we isolated three mutant alleles of bet-1: os46, os88 and os118 (see Table S1 in the supplementary material). We cloned bet-1 and found that it corresponds to Y119C1B.8, which encodes a C. elegans BET-family protein with two bromodomains (see Fig. S1A in the supplementary material), a motif that binds acetylated histones (Loyola and Almouzni, 2004).

The os46 bet-1 allele had a nonsense mutation in the region encoding the first bromodomain (see Fig. S1A in the supplementary material). The Psa phenotype and the extra-DTC phenotype (described below) of gk425 (a bet-1 allele from the International C. elegans Gene Knockout Consortium), which had an N-terminal deletion, was weaker than that of os46 (see Table S1 and Fig. S2B in the supplementary material). bet-1 RNAi given at the first larval stage (L1) enhanced the extra-DTC phenotype of gk425 but not that of os46 (see Fig. S2B in the supplementary material). The bet-1 C DNA that we isolated from gk425 encoded a truncated BET-1 protein lacking half of the first bromodomain, when the third methionine (86M) was used as the initiation codon (see Fig. S2A in the supplementary material). Thus, gk425 is a relatively weak allele, whereas os46 shows a strong loss-of-function and is likely to be null. Therefore, we used os46 in all the subsequent experiments.

We analyzed bet-1 expression using gfp fusion genes that encode the BET-1 protein fused to GFP near its C-terminus (Bet-1::GFP). Bet-1::GFP expression using the native promoter rescued the Psa phenotype (see Table S1 in the supplementary material), indicating that Bet-1::GFP is functional. When we expressed BET-1::GFP under the endogenous bet-1 promoter, GFP fluorescence was observed in most, if not all, cells, including the T, V5.pa, Z1/24 and Q cells, and all of their descendants (see Fig. S1Ba-e in the supplementary material). The Bet-1::GFP signal exhibited a granular distribution in the nuclei at interphase. During division, Bet-1::GFP signals were observed along the metaphase plate, indicating that BET-1 colocalized with the chromosomes at mitosis (see Fig. S1Bf,C in the supplementary material), like other BET-family proteins (Loyola and Almouzni, 2004).
To examine whether BET-1 functions cell-autonomously, we performed a mosaic analysis. Genetic mosaics were generated by the spontaneous loss of an extrachromosomal array containing *scm::bet-1::gfp* during embryogenesis in *bet-1* mutants. When BET-1::GFP was expressed in the T, but not the neighboring V6, lineage, the Psa phenotype was usually rescued, but expression in the V6, but not the T, lineage did not rescue it (see Fig. S3 in the supplementary material). Therefore, BET-1 functions cell-autonomously.

**Asymmetry of T-cell division is initially normal, but later disrupted, in bet-1 mutants**

A tail hypodermal cell, the T cell, divides asymmetrically to produce hypodermal cells from the anterior daughter cell (T.a), and neural cells, including the phasmid socket cells, from the posterior one (T.p) (Fig. 1A). Analysis of the cell lineages in *bet-1* mutants revealed that the daughters of the T.p cell (T.pa and T.pp) often adopted a hypodermal fate, as judged by their nuclear morphology and the expression of *scm::gfp* (see Fig. S4J in the supplementary material), consistent with the Psa phenotype of the animals (Fig. 1A-C). These results might mean that the asymmetry of the T-cell division was defective. However, the asymmetric localization of POP-1 and WRM-1 in the daughters of the T cell was normal (n=10 each for POP-1 and WRM-1; see Fig. S4A-F in the supplementary material). *psa-3*, which is a direct target of Wnt signaling and is required for the neural fate of T.p (Arata et al., 2006), was expressed asymmetrically (stronger in T.p and its daughter cells than in the anterior ones) in *bet-1* mutants, as in wild type (Fig. 1D,E; see Fig. S4K in the supplementary material). In addition, the daughters of the T.p cell, but not those of the T.a cell, migrated to interchange their positions in most *bet-1* mutants (Fig. 1A, bottom), as observed in wild type (Sulston and Horvitz, 1977). Furthermore, at the time of the migration, the daughters of the T.p cell in *bet-1* mutants, as in wild type, had a neuroblast-like appearance with smaller nucleoli than the T.a daughters, which had a hypodermal appearance (21 of 21; see Fig. S4H in the supplementary material). These results indicate that the T.p cell and its daughters initially acquired the correct neural fate in most *bet-1* mutants but that the fate was subsequently disturbed in the T.p daughters after their positional interchange.

**Aberrant increase in the numbers of specific cells during the development of bet-1 mutants**

In addition to the Psa phenotype, we found that the *bet-1* mutation caused the ectopic expression of markers for particular cell types (PDE, DTC and AVM/PVM). First, in the adult posterior lateral ganglion (PLG) of *bet-1* mutants, multiple cells expressing *dat-1::gfp* or *osm-6::gfp* (markers for the PDE neuron) were observed (Fig. 2B,D,M) compared with the one positive cell (PDE neuron) in wild type (Collet et al., 1998; Nass et al., 2002). Second, in the anterior or posterior half of the gonad, bet-1 adults often had more than one distal tip cell (DTC) that expressed *lag-2::gfp*, whereas there was only one DTC in wild type (Fig. 2E,F,M). All the DTCs in *bet-1* mutants, as in wild-type animals, were positioned at the tip of the gonad arms and had a cup-like shape (see Fig. S5A in the supplementary material), indicating that extra DTC cells had been produced rather than the marker simply being expressed ectopically. Third, *mec-4* and *mec-7* are normally expressed in the touch neurons, including AVM and PVM (Fig. 2G,I,M) (Challie et al., 1994; Lai et al., 1996). In *bet-1* adults, ectopic *mec-4::gfp* or *mec-7::gfp*-positive neurons were observed. Their positions and morphology indicated that they were ectopic AVM and PVM neurons (see below for more evidence; Fig. 2H,J,M). As the extra AVM phenotype was more frequently observed than the extra PVM one, we mostly analyzed the AVM phenotype in the following experiments. Because the *bet-1* mutation caused the ectopic induction of multiple characteristics for each cell type (i.e. the expression of multiple markers or, for DTCs, appropriate morphology and marker expression), BET-1 might regulate the expression of cell-fate determinants that are required for multiple cell-specific characteristics. Although the determinant(s) for the PDE or DTC is unknown, the LIM homeodomain transcription factor MEC-3 is expressed in the AVM (Fig. 2K,M) and functions as its fate determinant (Way and Challie, 1988). Consistent with our hypothesis, we observed ectopic *mec-3::gfp*-positive neurons in the anterior right side of *bet-1* adults, where the AVM is positioned in wild type (Fig. 2L,M), and found that the production of all of the AVM-like cells in *bet-1* mutants was completely dependent on *mec-3* (Fig. 2M). These results suggest that BET-1 regulates the expression of cell-fate determinants, such as MEC-3, to prevent the excessive production of certain types of cells.
Interestingly, we also found that the number of these ectopic cells increased during development. In wild-type animals, these cells are produced at early larval stages (PDE at L2, A VM at mid-L1 and DTC at L1). We compared the expression patterns of markers (dat-1::gfp for PDE, lag-2::gfp for DTC, and mec-4::gfp and mec-3::gfp for AVM) between the adult stage and middle-larval ones (PDE at L3, DTC at L2, A VM at late L1), which are the stages occurring shortly after the production of these cells in wild type (Fig. 2N). At the larval stages, some bet-1 mutants already expressed the marker genes in multiple cells but the number of cells expressing the marker genes was smaller than in adults (Fig. 2N; data for DTCs represent the number of DTCs in the anterior or posterior half of the gonad). To confirm the increases in these cell types, the number of ectopic cells was first counted in individual animals at the larval stages and then, after their recovery and growth, recounted at the adult stage. This experiment showed that the number of PDEs (dat-1::gfp), DTCs (lag-2::gfp) and AVMs (mec-4::gfp) increased in 38% (n=64), 20% (n=40) and 35% (n=20) of individual bet-1 mutants, respectively. We did not observe any cells that lost marker expression in these experiments. In addition, in contrast to the larval stages, the number of GFP-positive cells was the same between 1- and 2-day-old adults (data not shown). These results indicated that these cells are ectopically produced in the larval, but not the adult, stages.

**Transformation of cell fates to those of relatives in bet-1 mutants**

Because the transformation from neuroblast to hypodermal cells was observed in the T cell lineage, we suspected that a similar fate transformation might cause the increase in GFP-positive cells in other lineages in bet-1 mutants. To test this hypothesis, all of the
GFP-positive cells near the normal position of these cell types were ablated by laser during the middle-larval stages. After the ablation, new GFP-positive cells appeared until the adult stage in bet-1 mutants but not in wild type (see Table S2 in the supplementary material). These results indicated that GFP-negative cells started to express GFP during the larval stages in bet-1 mutants.

To determine which cells became ectopic PDEs, AVMs and DTCs in bet-1 mutants, we ablated their normal precursors (V5.pa, QR or Z1/Z4, respectively). These cell types were not produced in the ablated animals (see Table S2 in the supplementary material), indicating that all of the ectopic cells of each cell type in bet-1 mutants were produced from the same precursor that normally produces these cell types. This experiment also confirmed that the ectopic mec-4- and mec-7-expressing cells were AVM-like neurons derived from QR rather than other touch neurons like ALM.

To analyze the fates of the V5.pa progeny, we followed the lineages of the V5.pa cell in bet-1 mutants carrying osm-6::gfp, which is expressed earlier than dat-1::gfp. We began our observation during the L2 stage and followed the cell fates until late L2, when osm-6 expression begins in wild-type animals. The lineage analyses showed that the cell-division patterns during the L2 stage were abnormal in 4 of 11 bet-1 mutants, suggesting that cell fates might not be correctly specified in these animals (see Fig. S6Bh-k in the supplementary material). However, in the remaining 7 animals, in terms of cell-division patterns, the daughters of the V5.paa cell acquired distinct fates, as in wild type. In 5 of these 7 animals, osm-6::gfp expression was observed only in V5.paaa, which is the PDE neuron in wild-type animals (see Fig. S6A,Ba-e in the supplementary material). When these five animals were recovered from the slide glasses and allowed to grow to the adult stage, ectopic osm-6::gfp expression was observed in cells with the positions of the nieces of the original PDE (V5.papa or V5.paap; see Fig. S6Ba-e in the supplementary material). Therefore, in these animals, V5.papa (the sister of PDE and the mother of PVD in wild type) and its progeny appeared to behave normally in terms of their cell divisions and their initial non-expression of osm-6::gfp, but they subsequently adopted the PDE fate. Consistent with the initially normal behavior of these cells, most of bet-1 mutants (93%, n=30) had one mec-3::gfp (marker for the PDE neuron)-positive cell, similar to wild-type animals (97%, n=30) (Way and Chalfie, 1988), in the right PLG at the early L3 stage (see Fig. S7A,B in the supplementary material), suggesting that the PVD fate was specified normally. Our results indicate that cells that initially acquired non-PDE fates (e.g. the PVD fate) subsequently adopted the PDE fate, similar to the fate transformation observed for the T.p progeny. Consistent with the possible transformation from the PVD to the PDE fate, the PVD marker mec-3::gfp was not expressed in 38% (n=29) of the bet-1 adult animals.

The above results for the V5.pa lineage indicate that cells that were initially osm-6 (a PDE marker)-negative at the L2 stage became osm-6-positive by the adult stage. However, unexpectedly, we found that the number of osm-6-positive cells differed slightly between L3 and the adult stage, despite the increase in dat-1 (another PDE marker)-positive cells (these markers are expressed in equivalent numbers of cells at the adult stage; see Table S3 in the supplementary material). Similarly, although the number of mec-3 (a PVD marker)-positive cells decreased after the L3 stage, bet-1 adults had more cells that were positive for dop-3::rfp (another PVD marker) than did the L3 larvae. This phenotype can be explained by considering that OSM-6 (intraflagellar transporter required for cilia formation) (Collet et al., 1998) and MEC-3 (transcription factor) are early differentiation markers, whereas DAT-1 (dopamine transporter) (Nass et al., 2002) and DOP-3 (dopamine transporter) (Chase et al., 2004) are terminal differentiation markers required for neuronal function. In fact, mec-3::gfp and osm-6::gfp expression started earlier than the expression of dop-3::rfp and dat-1::gfp in wild type. Our lineage analyses indicated that, in bet-1 mutants, cells that initially differentiated as non-PDE cells, which might have included PVD-like neurons, tended to be transformed to the PDE fate; the beginning of the PDE differentiation program was marked by the expression of osm-6. However, the fates of these cells might still have been unstable until they fully differentiated to express dat-
l; that is, between the L3 and adult stages they could be transformed to other cell types, including PVD-like neurons, causing a loss of osm-6-positive cells and a gain of dop-3-positive cells.

To observe cell-fate transformation in more animals and the possible transformation between the PDE and PVD fates, we analyzed strains carrying both osm-6::gfp and dop-3::rfp and compared their expression between the L3 and adult stages. To detect changes in marker expression in individual cells, we only scored animals in which it was easy to identify the same cells at both stages by their spatial patterns (30 of 49 bet-1 mutant animals). In wild-type animals, cells expressing one of these markers at the L3 stage lost the marker expression in about half of the adults (osm-6::gfp-positive cells were found at the same position at the adult stage and still expressed the marker (Fig. 3B; arrowhead). osm-6::gfp-positive by the adult stage (Fig. 3B, arrowhead).

Consistent with the slight decrease in osm-6-positive cells during the L3 to adult stages described above, we observed animals (50%) in which cells that expressed osm-6::gfp at the L3 stage lost the expression by the adult stage (arrowhead in Fig. 3C). In about half of these adults, dop-3::rfp-expressing cells were found at the same position as the osm-6::gfp-expressing cells at the L3 stage (Fig. 3D), suggesting a transformation from PDE to PVD (23% of the total animals scored). In addition, in 10% of these animals, we observed cells that expressed both markers at the L3 stage (see Fig. S7C in the supplementary material). These might represent cells in transition to other cell types, including PVD-like neurons, causing a loss of osm-6-positive cells and a gain of dop-3-positive cells.

To confirm the PDE-to-PVD transformation, we ablated the osm-6-negative cells and osm-6 dop-3 double-positive cells in the PLG at the early L3 stage, then observed the marker expression at the adult stage. Although all the cells in the PLG had expressed osm-6, but not dop-3, after the ablation at the L3 stage, dop-3-positive osm-6-negative cells were observed at the adult stage (4 of 29 animals; see Fig. S7D in the supplementary material). Taken together, our data show that in bet-1 mutants, the cell fates were unstable and the cells could be transformed into other cell types even after the initiation of their differentiation program.

**bet-1 functions to establish and maintain stable cell fates**

To determine when bet-1 is required for the neural differentiation of T.p progeny, BET-1::GFP expression was induced using a heat-shock promoter at various times during development. Heat-shock treatment at 3 hours after hatching caused the initial GFP fluorescence to be detected 4-5 hours after hatching, which is the time of T.p cell division. We found that the heat shock treatment before or at 3 hours after hatching rescued the defect in the neural differentiation of T.p progeny in bet-1 mutants (Fig. 4A). By contrast, heat-shock treatment given at 4 hours or later did not rescue the defect. This result indicated that BET-1 must be expressed at the time T.p is produced, when the cells acquire neural fates through the expression of psa-3. This is much earlier than the stage of the fate transformation that we observed in bet-1 mutants.

Similarly, the multiple-AVM phenotype of bet-1 mutants was rescued by heat-shock treatment 4 hours after hatching, which is before the division of the AVM mother (QR.pa), but not by treatment given at 6 hours or later (Fig. 4B), again indicating that BET-1 must be expressed much earlier than the time when the bet-1 phenotype arose (14 hours after hatching at the earliest). Taken together, our results suggest that BET-1 functions when cells acquire their fates to stabilize them. Without BET-1 function at this time, the fates of the cells are transformed at later stages.

In the experiments described above, we could not determine the requirement for BET-1 at the later stages, when cell fates are maintained, because some of the BET-1 protein that was transiently induced by heat shock was likely to persist in the cells for a long time. In fact, fluorescence from the scm::BET-1::GFP transgene...
transcribed in the V5.\textit{p} seam cell (but not in the neural V5.\textit{pa} descendants) at early L2 stage could be detected even in adult animals and the transgene could efficiently rescue the multiple-PDE phenotype of \textit{bet-1(os46)} mutants (6% defective, \textit{n}=50). To circumvent this problem, we used a BET-1 protein tagged with a destabilization domain (DD), which induces the proteasome-dependent degradation of tagged proteins (Banaszynski et al., 2006). When DD::BET-1::GFP was induced by a 10-minute heat-shock treatment given 16 hours after hatching, the percentage of animals with GFP fluorescence in the V5.\textit{pa} descendants had greatly decreased by 8 hours after the heat shock, compared with the percentage at 3 hours post-heat shock (see Fig. S8 in the supplementary material). A similar decrease was not observed in animals expressing BET-1::GFP without the DD. Therefore, the DD destabilized the tagged protein in \textit{C. elegans}.

Using this construct, we first confirmed that BET-1 was required at the time of fate acquisition, by demonstrating that a 30-minute heat-shock induction of DD::BET-1::GFP significantly rescued the multiple-PDE phenotype of \textit{bet-1} mutants before (16 hours after hatching), but not after (24 hours after hatching), the production of V5.\textit{pa}-derived neural cells, which are born 19-22 hours after hatching (Fig. 4C). Weak induction by a 10-minute heat-shock treatment given 16 hours after hatching was not sufficient to provide significant rescue. However, an additional 10-minute heat-shock given 24 hours after hatching provided significant rescue of the phenotype (Fig. 4C; 10 minutes \times 2). These results indicated that BET-1 is required for cell-fate maintenance after the acquisition of cell fate.

**Disruption of MYST HATs causes the bet-1-like phenotype**

Because the bromodomain is known to bind acetylated histone, reducing the histone acetylation by disrupting certain histone acetyltransferases (HATs) might cause a Bet-1-like phenotype. Therefore, we performed inactivation of the \textit{C. elegans} HAT genes, the Gcn5 family gene, \textit{pcaf-1}, or the MYST family genes \textit{mys-1} (Ceol and Horvitz, 2004), \textit{mys-2(K03D10.3)}, \textit{mys-3(R07B5.8)} and \textit{mys-4(C34B7.4)}, by mutation (\textit{mys-1}) or RNAi. We could not examine the p300/CBP family gene, \textit{cbp-1}, because its inactivation by RNAi caused embryonic lethality. The \textit{mys-1} mutants showed the extra-DTCs phenotype, which was enhanced by \textit{mys-2(RNAi)} (Fig. 5A; see Fig. S5B in the supplementary material). As in \textit{bet-1} mutants, the extra-DTCs phenotype was weaker at the larval stage. Furthermore, when the DTCs were laser-ablated during the L2 stage, DTCs were still produced, indicating that cells initially fated to be non-DTCs transformed into DTCs (see Table S2 in the supplementary material). In addition, \textit{mys-2(RNAi)} treatment in the \textit{mys-1} mutants caused the Psa phenotype (3.6%) and induced multiple \textit{dat-1::gfp}-positive cells in the PLG (7%), as observed in \textit{bet-1} mutants. Finally, \textit{mys-1(RNAi)} enhanced the extra DTC phenotype of the weaker \textit{bet-1} allele, \textit{gk425}, but not that of the putative null allele \textit{os46} (see Fig. S9 in the supplementary material), indicating that the function of \textit{mys-1} is dependent on the \textit{bet-1} activity. These results indicate that \textit{MYS-1} and \textit{MYS-2} function in the BET-1-mediated maintenance of cell fates.

**bet-1 subnuclear localization is dependent on MYST HATs**

MYST HATs primarily acetylate histone H4 (Clarke et al., 1999; Kimura and Horikoshi, 1998). We found that BET-1 bound strongly to a histone H4 peptide acetylated at four Lys residues (K5, K8, K12 and K16) but only weakly to the unacetylated H4 peptide or to the acetylated or unacetylated H3 peptide in vitro (see Fig. S10 in the supplementary material). Interestingly, BET-1 bound much more weakly to H4 peptide that was singly acetylated at any residue or diacetylated (K5 and K12 or K8 and K16) than to the tetra-acetylated form, suggesting that acetylation had a synergistic effect on BET-1 binding. These results indicate that BET-1 recognizes acetylated histone H4.

To determine whether the BET-1 localization depends on \textit{MYS-1} and \textit{MYS-2}, the BET-1::GFP distribution was observed in \textit{mys-1}- and \textit{mys-2}-deficient animals at late larval stages in seam cells, which have large nuclei and are suitable for observing subnuclear localization. In wild-type animals, many puncta indicating the BET-1::GFP localization in hypodermal cells, including seam cells (arrows), at the L3/L4 stage in wild type (B) and \textit{mys-1(n4075)} \textit{mys-2(RNAi)} (C) and \textit{psa-1(os22)} (D) mutants. Anterior is to the left, ventral is to the bottom. Scale bars: 5 \textmu m.

**DISCUSSION**

**bet-1 establishes and maintains stable cell fates**

In a small percentage of \textit{bet-1} animals, cell fates were initially abnormal, suggesting that \textit{bet-1} is required for correct fate specification. In the majority of \textit{bet-1} mutants, however, cells acquire the correct cell fates initially but cannot maintain them,
indicating that bet-1 is required for the maintenance of acquired fates. Counterintuitively, our heat-shock experiments showed that bet-1 expression is required at the time of cell-fate acquisition for cell fates to be stably maintained at later stages. Therefore, bet-1 functions are most likely involved in the stabilization of the acquired fates, which must occur soon after their acquisition. Theoretically, such a fate-stabilization step can be thought of as ‘fate-determination’, i.e. the step in which specified cell fates become irreversible (Gilbert, 2003; Livesey and Cepko, 2001). The invariant cell lineage of C. elegans might suggest that fate determination occurs soon after fate specification so that the fates of most cells are not altered in normal development. With this assumption, our results suggest that BET-1 functions in the fate-determination processes.

Our experiments using DD::BET-1 showed that bet-1 expression at fate-acquisition periods is not sufficient for the establishment of stable fates, indicating that BET-1 is also required after fate acquisition, at the time cell transformation was observed in the bet-1 mutants. Because BET-1 is an acetylated histone-binding protein, it might bind to the chromatin in the regulatory regions of genes whose expression is required for the adoption of given fates soon after fate acquisition and continue to bind these regions to protect the epigenetic information and stabilize gene expression. BET-1 might exert such functions even during cell divisions, at least in the case of the T-cell lineage, in which the neural fate of T.p must be maintained in its progeny. Our finding that BET-1 was associated with chromatin even during mitosis is consistent with this hypothesis.

The nearly ubiquitous BET-1 expression suggests that BET-1 might function in many lineages. In fact, we observed a variety of defects in bet-1 mutants: protruding vulvae, abnormal male tail structure, multiple anchor cells and extra VC neurons, as judged by the expression of chd-3::gfp (Y.S. and H.S., unpublished data). In addition, an RNAi of bet-1 caused embryonic lethality at the morphogenesis stage after the completion of most embryonic divisions. The observation that bet-1 mutants did not show drastic developmental defects like embryonic lethality might be owing to the presence of the maternally supplied gene product. Therefore, bet-1 might be required to establish and maintain stable fates in many embryonic and postembryonic lineages.

How does BET-1 function in cell-fate maintenance?

Based on our results and observations in other organisms, we can propose a possible model for how BET-1 and histone acetylation might maintain cell fates. We suggest that when cells acquire new fates, cell-fate regulators recruit HAT to the promoters of fate-determinant genes to create the initial acetylated nucleosome(s). Because a Brd2 that is most similar to BET-1 in mammals associates with a histone H4-specific HAT activity, possibly by MYST HAT (Sinha et al., 2005), the initial acetylation of promoters might recruit BET-1–MYST HAT complexes, which acetylate the histone H4 of adjacent nucleosomes. Repeating this process might create larger and more stable acetylated genomic regions that help to maintain the transcriptional state for a longer period. A similar mechanism was proposed for the function of a complex containing SUVAR39H1 and HP1, which are a histone methyltransferase and a protein that recognizes methylation on lysine 9 of histone H3, respectively, in fission yeast (Bannister et al., 2001). During the period of fate maintenance following fate acquisition, BET-1 might protect acetylated histones from HDACs, given that yeast BdFl1 protects histone H4 from deacetylation by Sir2/HDAC both in vitro and in vivo (Ladurner et al., 2003).

Epigenetic regulation of cell-fate maintenance through histone acetylation

Although chromatin hypoacetylation is observed during mitosis in mammalian cells, the level of acetylation on lysines 12 and 16 of H4 (H4K12Ac and H4K16Ac) is preserved, even during mitosis (Kruhlak et al., 2001), suggesting that these acetylation patterns function to maintain the transcriptional status, similar to histone methylation. In fact, in dosage compensation in Drosophila, the acetylation of H4K16 by a MYST HAT called MOF is required for the global upregulation of X-linked genes in males (Hilfiker et al., 1997; Kelley et al., 1995). Although the developmental roles of the BET-family proteins and MYST HATs remain largely elusive in vertebrates, the Drosophila BET-family protein FSH is implicated as an antagonist of polycomb genes that function in cell-fate maintenance (Chang et al., 2007). Therefore, BET-family proteins and MYST HATs might also play fundamental roles in maintaining cell fates and preventing aberrant cell transformation in other organisms.

Acknowledgements

We thank the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health National Center for Research Resources, the C. elegans Gene Knockout Consortium, Yui Kohara, Martin Chalfie, Randy Blakely and Cynthia Kenyon for strains and reagents, and members of the Sawa laboratory and Junichi Nakayama for helpful discussions and comments. This work was supported by the Special Postdoctoral Researchers Program, Riken (Y.S.) and by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology (Y.S. and H.S.).

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.042812/-/DC1

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