

# Two *C. elegans* histone methyltransferases repress *lin-3* EGF transcription to inhibit vulval development

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Studies of *Schizosaccharomyces pombe* and mammalian cells identified a series of histone modifications that result in transcriptional repression. Lysine 9 of histone H3 (H3K9) is deacetylated by the NuRD complex, methylated by a histone methyltransferase (HMT) and then bound by a chromodomain-containing protein, such as heterochromatin protein 1 (HP1), leading to transcriptional repression. A *Caenorhabditis elegans* NuRD-like complex and HP1 homologs regulate vulval development, but no HMT is known to act in this process. We surveyed all 38 putative HMT genes in *C. elegans* and identified *met-1* and *met-2* as negative regulators of vulval cell-fate specification. *met-1* is homologous to *Saccharomyces cerevisiae* Set2, an H3K36 HMT that prevents the ectopic initiation of transcription. *met-2* is homologous to human SETDB1, an H3K9 HMT that represses transcription. *met-1* and *met-2* (1) are each required for the normal trimethylation of both H3K9 and H3K36; (2) act redundantly with each other as well as with the *C. elegans* HP1 homologs; and (3) repress transcription of the EGF gene *lin-3*, which encodes the signal that induces vulval development. We propose that as is the case for Set2 in yeast, MET-1 prevents the reinitiation of transcription. Our results suggest that in the inhibition of vulval development, homologs of SETDB1, HP1 and the NuRD complex act with this H3K36 HMT to prevent ectopic transcriptional initiation.

**KEY WORDS:** SETDB1, Set2, Histone methyltransferase, *C. elegans*

## INTRODUCTION

The diversity of cell types in an organism is generated by cell-fate decisions made throughout development. Cell signaling cascades, such as the receptor tyrosine kinase (RTK)/Ras, Notch and Wnt pathways, direct many of these cell-fate decisions. Each of these pathways regulates the activity of one or more transcription factors, which in turn regulate the transcription of genes that determine cell fates (Korswagen, 2002; Sundaram, 2005). Transcription can be controlled through changes in chromatin structure, thereby altering accessibility of the DNA template to the transcriptional machinery (Jenuwein and Allis, 2001). Thus, chromatin remodeling factors can control cell-fate determination through the transcriptional regulation of cell-fate specification genes (Fisher, 2002).

The development of the *Caenorhabditis elegans* vulva is an excellent system for the study of cell-fate determination. The vulva is dispensable for viability, and cell-fate defects are easily observed using a dissecting microscope (Sternberg and Horvitz, 1991). An epidermal growth factor (EGF)-like signal from a neighboring gonadal cell induces three of a set of six multipotent cells (the vulval equivalence group) located on the ventral surface of the animal to form the vulva (Sulston and Horvitz, 1977; Sulston and White, 1980; Kimble, 1981; Hill and Sternberg, 1992). This EGF signal is transduced by a conserved RTK/Ras pathway that causes those three cells to divide and generate the 22 descendants of the vulva (Kornfeld, 1997). Mutations that reduce or eliminate the activity of the RTK/Ras pathway can result in a vulvaless (Vul) animal in which no cells of the vulval equivalence group express vulval fates; by contrast, mutations that increase the

activity of this pathway can cause ectopic expression of vulval cell fates by the other cells of the vulval equivalence group and result in a multivulva (Muv) animal (Beitel et al., 1990; Han and Sternberg, 1990). The RTK/Ras pathway terminates in the control of at least two transcription factors, LIN-1 and LIN-31 (Beitel et al., 1995; Tan et al., 1998), which regulate the transcription of an unknown set of genes to control the expression of the vulval cell fate.

The vulval cell-fate decision is antagonized by the actions of the synthetic multivulva (synMuv) genes (Fay and Yochem, 2007). These genes have been grouped into three classes: A, B and C (Ferguson and Horvitz, 1989; Ceol and Horvitz, 2004). Animals defective in genes in any two classes have a Muv phenotype, whereas animals defective in genes from a single class are not Muv. Class A genes when mutated cause a Muv phenotype with class B and class C mutations. Class B genes when mutated cause a Muv phenotype with class A and class C mutations. Class C genes when mutated cause a Muv phenotype with class A and class B mutations but can cause a weaker Muv phenotype as single mutants. Many synMuv genes encode homologs of chromatin-remodeling proteins and transcriptional repressors. A subset of the class B synMuv proteins are homologs of a conserved transcriptional repression cascade, including LIN-35/Rb (Lu and Horvitz, 1998), the NuRD-like complex HDA-1/HDAC1, LET-418/Mi2 and LIN-53/RbAp48 (von Zelewsky et al., 2000; Unhavaithaya et al., 2002) and HPL-2/heterochromatin protein 1 (HP1) (Couteau et al., 2002). Some synMuv genes have been shown to act as transcriptional repressors (Cui et al., 2006a). In mammalian cells, the activity of this transcriptional repression cascade is initiated by the recruitment of the NuRD complex by Rb to target genes (Brehm et al., 1998; Brehm et al., 1999). Subsequent deacetylation of histone H3 lysine 9 (H3K9) by a histone deacetylase, methylation of H3K9 by a histone methyltransferase (HMT) and binding of the chromodomain-containing protein HP1 creates a region of repressive chromatin that inhibits transcription (Nakayama et al., 2001; Ayyanathan et

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al., 2003). Of this transcriptional repression cascade, only a gene predicted to encode an HMT is not represented within the cloned synMuv genes.

All lysine-specific histone-tail HMTs contain a SET domain, which is the enzymatic core of these proteins (Kouzarides, 2002; Pirrotta, 2006; Shilatifard, 2006). On the histone H3 N-terminal tail, four lysine residues can be methylated: K4, K9, K27 and K36. The methylation of histone H3 lysines K4 and K36 is generally associated with actively transcribed genes, although H3K36 methylation functions in repression and prevents transcriptional initiation downstream of the promoter (Krogan et al., 2003; Carrozza et al., 2005). The methylation of histone H3 at K9 and K27 is generally associated with repressed transcription. The cysteine-rich domains flanking the SET domain determine the specificity of the HMT. HMTs with a SET domain flanked by PreSET and PostSET domains methylate H3K9. HMTs with a SET domain flanked by AWS and PostSET domains methylate H3K36. HMTs with only a PostSET domain flanking the SET domain methylate H3K4. Enzymes that methylate H3K27 do not have cysteine-rich domains flanking the SET domain. The methylated histone-tail lysines and other modifications have been proposed to regulate the transcription of nearby genes (Jenuwein and Allis, 2001).

To identify HMTs that act in vulval development, we used deletion alleles and RNAi to examine the loss-of-function phenotypes of all 38 *C. elegans* genes predicted to encode lysine-specific histone-tail HMTs. We discovered that two HMT genes, which we named *met-1* and *met-2*, caused a synMuv phenotype when inactivated in a class A synMuv mutant background. MET-1 is homologous to *S. cerevisiae* Set2, a histone H3 lysine 36 (H3K36) HMT, and MET-2 is homologous to mammalian SETDB1, an H3K9 HMT. We determined that these two putative HMTs act redundantly with each other and with the presumptive downstream HP1 homologs during vulval development. Additionally, we found that transcription of the synMuv target gene *lin-3* EGF is increased in *met-1*, *met-2* and *hpl-2* mutants. Our results suggest that in *C. elegans*, the trimethylation of histone H3 lysine 36 by MET-1/Set2 promotes a transcriptional repression cascade mediated by a NuRD-like complex and by the trimethylation of histone H3K9 by a SETDB1-like HMT. This cascade leads to the recruitment of HP1 and the inhibition of ectopic *lin-3* transcription during vulval development. We suggest that this mechanism is conserved and that in many organisms the ectopic initiation of transcription downstream of the promoter is inhibited not only by H3K36 trimethylation, but also by the functions of a NuRD-like complex, a SETDB1-like H3K9 HMT and HP1-like proteins.

## MATERIALS AND METHODS

### Strains and genetics

*C. elegans* was grown as described (Brenner, 1974) and maintained at 20°C unless otherwise noted. N2 was the wild-type strain. The mutations and integrants used were: LGI: *met-1(n4337)* (this study), *lin-35(n745)*; LGII: *lin-8(n2731)* (Davison et al., 2005), *dpl-1(n2994)* (Ceol and Horvitz, 2001), *lin-38(n751)*, *trr-1(n3712)* (Ceol and Horvitz, 2004), *lin-56(n2728)* (Thomas et al., 2003); LGIII: *lin-37(n4903)* (Andersen et al., 2006), *met-2(n4256)* (this study); LGV: *mys-1(n3681)* (Ceol and Horvitz, 2004); LGX: *hpl-1(n4317)* (this study), *lin-15A(n433, n767)*, *lin-15B(n744)*, *lin-15AB(n765)*.

Information about *tm* (kindly provided by S. Mitani, Tokyo Women's Medical University, Japan), *gk* and *ok* alleles can be found at [www.wormbase.org](http://www.wormbase.org). For information about all *n* deletion alleles generated in this study (Table 1), see Table S1 in the supplementary material. The following balancer chromosomes were used: *hT2 [qls48]* LGI; LGIII, *nT1 [qls51]* LGIV; LGV, *mIn1 [mIs14]* and *qC1 [nIs189]*. Mutant alleles for which no citation is given have been described previously (Riddle, 1997).

### Scoring of the vulval phenotype

We scored the vulval phenotypes of the progeny of five hermaphrodites raised at 20°C or 25°C. Animals with more than one vulval-like structure (i.e. with one or more ventral protrusions in addition to either a normal vulva or an abnormal vulva) were scored as Muv.

### Determination of gene structures and generation of cDNA constructs

For *met-1*, the sequences of the cDNA clones yk27f9, yk152a5, yk154f7, yk1128b1 and yk1327b12 were determined. 5' rapid amplification of cDNA ends (5' RACE, Invitrogen) was used to determine the 5' end of *met-1*, and an SL1 splice-leader sequence was identified. Clones yk1128b1 and yk1327b12 were generated from a PCR product that inappropriately terminated in a 3' A-rich sequence. Both clones contained a transcriptional start different from that identified in the 5' RACE experiments (data not shown). The 5' RACE products did not contain exon six, indicating that there are two alternatively spliced *met-1* transcripts. Using yeast-mediated ligation (Oldenburg et al., 1997), yk27f9 and the 5' RACE product were combined to make a presumptive full-length *met-1* clone (pEA130), which was transferred to the Gateway System (Invitrogen). For *met-2*, the sequences of three independent cDNA clones, yk6f10, yk29g5 and yk249d10, were determined. 5' RACE identified the same 5' sequence as found in the cDNA clones. Using yeast-mediated ligation, yk249d10 and the 5' RACE product were combined to make a presumptive full-length *met-2* clone (pEA109), which was transferred to the Gateway System. Quickchange (Stratagene) was used to create the clones pEA181 (*met-1*) and pEA110 (*met-2*), which contain two SET-domain mutations that abolish the HMT activities of homologous enzymes: RFVNHSC to GFVNHSA.

### RNAi analyses

RNAi by injection was performed as described (Andersen et al., 2006). For clones used to generate dsRNA for RNAi studies of putative histone methyltransferase genes, see Table S1 in the supplementary material. Yuji Kohara (National Institute of Genetics, Mishima, Japan) kindly provided all yk clones.

### Isolation of deletion alleles

Genomic DNA pools from the progeny of EMS or UV-TMP mutagenized animals were screened for deletions using PCR as described (Ceol and Horvitz, 2001). *hpl-1(n4317)* removes nucleotides 20092 to 21648 of cosmid K08H2. For a complete list of the positions of all HMT deletion alleles, see Table S1 in the supplementary material.

### Germline transformation experiments

Germline transformation experiments were performed as described (Mello et al., 1991). For rescue of the *met-1(n4337)*; *lin-15A(n767)* synMuv phenotype, we injected pEA182 (50 ng/μl). For rescue of the *met-2(n4256)*; *lin-15A(n433)* synMuv phenotype, we injected pEA115 (50 ng/μl). pEA182 and pEA115 have the *met-1* and *met-2* cDNAs, respectively, cloned downstream of the *dpy-7* promoter, which drives expression in the hyp7 syncytium (Gilleard et al., 1997). Each injection included a 1 kb ladder (Invitrogen) at 100 μg/μl and *sur-5::gfp* (Yochem et al., 1998) at 20 ng/μl.

### Quantitative western blot analysis

Protein samples were prepared from embryonic extracts as described (Harrison et al., 2006). The linear range of reactivity for the antisera used in these studies was determined using wild-type extracts with total protein concentrations from 6.25 to 50 μg/μl. Total protein (12.5 μg) was loaded in quadruplicate for each strain tested using quantitative western blots. Levels of histone H3 antibody reactivity (1:1000, Abcam) were normalized to levels of both tubulin (1:1000, DM1A, Sigma) and histone H2A (1:500, Abcam) using fluorescent secondary antibodies (1:500, Cy3 and Cy5, Jackson ImmunoResearch) and a Typhoon Imaging System (GE Healthcare Life Sciences). For each assay, the levels of histone H3 trimethylation were normalized to the levels of total histone H3. The levels of histone H3 lysine 4 trimethylation (H3K4tri, 1:5000, Abcam), H3K9tri (1:1000, Upstate), H3K27tri (1:3000, Upstate) and H3K36tri (1:2000, Abcam) were determined. The data shown are representative of data from at least two independent embryonic protein preparations.

**Table 1. Deletion or RNAi of some genes encoding proteins with SET domains causes gross abnormalities, including synthetic multivulva and suppression of synthetic multivulva phenotypes**

Gene	Allele or RNAi*	Phenotype as a single mutant	% multivulva <sup>†</sup> in combination with		
			<i>lin-15A(n767)</i>	<i>lin-15B(n744)</i>	<i>lin-15AB(n765)</i>
<i>blmp-1</i>	<i>tm548</i>	WT	0 (196)	0 (188)	100 (99)
<i>lin-59</i>	<i>sa489</i>	WT	0 (188)	0 (102)	100 (143)
<i>mes-2</i>	<i>bn11</i>	Mes	0 (233)	0 (258)	1 (194)
<i>mes-4</i>	<i>bn73</i>	Mes	0 (140)	0 (130)	7 (224)
<i>met-1</i>	<i>n4337</i>	WT	81 (469)	0 (216)	100 (206)
<i>met-2</i>	<i>n4256</i>	WT	100 (350)	0 (349)	100 (273)
<i>set-1</i>	<i>n4617</i>	Emb, Lvl	NA <sup>‡</sup>	NA	NA
<i>set-2</i>	<i>n4589</i>	WT	0 (291)	1 (134)	100 (281)
<i>set-3</i>	<i>n4948</i>	WT	0 (194)	0 (142)	100 (116)
<i>set-4</i>	<i>n4600</i>	WT	0 (361)	0 (236)	99 (202)
<i>set-5</i>	<i>ok1568</i>	WT	0 (170)	0 (157)	100 (104)
<i>set-6</i>	<i>tm1611</i>	WT	0 (151)	0 (139)	100 (111)
<i>ttl-12</i>	RNAi	WT	0 (186)	0 (215)	100 (116)
<i>set-8</i>	<i>tm2113</i>	WT	0 (122)	0 (130)	100 (131)
<i>set-9</i>	<i>n4949</i>	WT	0 (171)	0 (151)	100 (91)
<i>set-10</i>	RNAi	WT	0 (211)	0 (149)	100 (36)
<i>set-11</i>	<i>n4488</i>	WT	0 (473)	0 (275)	100 (241)
<i>set-12</i>	<i>n4442</i>	WT	0 (380)	0 (204)	100 (262)
<i>set-13</i>	<i>n5012</i>	WT	0 (119)	0 (136)	100 (104)
<i>set-14</i>	RNAi	WT	0 (222)	0 (219)	100 (140)
<i>set-15</i>	RNAi	WT	0 (75)	0 (102)	100 (97)
<i>set-16</i>	<i>n4526</i>	Lvl	NA	NA	NA
<i>set-17</i>	<i>n5017</i>	WT	0 (147)	0 (104)	100 (146)
<i>set-18</i>	<i>gk334</i>	WT	1 (171)	0 (161)	100 (143)
<i>set-19</i>	<i>ok1813</i>	WT	0 (163)	0 (101)	100 (111)
<i>set-20</i>	RNAi	WT	0 (211)	0 (195)	100 (147)
<i>set-21</i>	RNAi	WT	0 (235)	0 (176)	100 (113)
<i>set-22</i>	<i>n5015</i>	WT	0 (125)	0 (130)	100 (120)
<i>set-23</i>	<i>n4496</i>	Emb	NA	NA	NA
<i>set-24</i>	<i>n4909</i>	WT	0 (151)	0 (96)	100 (103)
<i>set-25</i>	<i>n5021</i>	WT	0 (183)	0 (117)	100 (170)
<i>set-26</i>	RNAi	WT	0 (227)	0 (159)	100 (90)
<i>set-27</i>	RNAi	WT	0 (123)	0 (139)	100 (123)
<i>set-28</i>	<i>n4953</i>	WT	0 (144)	0 (99)	100 (128)
<i>set-29</i>	RNAi	WT	0 (163)	0 (132)	100 (107)
<i>set-30</i>	<i>gk315</i>	WT	0 (150)	0 (132)	100 (180)
<i>set-31</i>	<i>ok1482</i>	WT	0 (190)	0 (206)	100 (158)
<i>set-32</i>	<i>ok1457</i>	WT	0 (174)	0 (241)	100 (166)

Shading denotes phenotypes that differ from that of the wild type.

WT, wild-type; Lvl, larval lethal; Mes, maternal-effect sterile; Emb, embryonic lethal.

\*For each RNAi experiment, at least two independent cDNA clones were used to make dsRNA for injection.

<sup>†</sup>*lin-15A(n767)* and *lin-15B(n744)* single mutants are non-Muv, and *lin-15AB(n765)* mutants are 100% Muv at 20°C.

<sup>‡</sup>Not applicable because the animals died prior to vulval development.

### Quantitative PCR assays

Synchronized wild-type and mutant animals were grown, and larvae were harvested at or near the L2-to-L3 larval transition, when vulval induction occurs. Total RNA was extracted using Trizol (Invitrogen). First-strand cDNA was prepared from 1 µg total RNA using the SuperScript III First-Strand Synthesis Supermix for qRT-PCR (Invitrogen). Each real-time reverse transcriptase (RT) PCR mix contained 10 ng of RT products, 25 µl of 2× SyBR Green PCR Master Mix (Applied Biosystems) and 0.4 µM of each primer. The real-time PCR was performed in triplicate on a DNA Engine Opticon System (BioRad). Three independent samples of each genotype were prepared, and levels of *lin-3* and *rpl-26* were quantified from each biological replicate. The  $\Delta C_T$  values for *lin-3* were determined using *rpl-26* as the internal reference, and the  $\Delta\Delta C_T$  values were calculated for each genotype by comparison with the wild type (as described in the Applied Biosystems real-time PCR manual). All changes were normalized to the wild type. The error shown is the range of relative *lin-3/rpl-26* ratios for three trials determined from the standard deviations of the  $\Delta\Delta C_T$  values.

### RESULTS

#### A survey of all 38 *C. elegans* putative HMT genes identified two genes required for viability

To characterize the in vivo roles of the HMT genes, we generated or collected loss-of-function mutations for 29 of the 38 predicted *C. elegans* genes encoding proteins with SET domains and inactivated each of the remaining nine genes by RNAi. We found that loss-of-function of five of the 38 HMT genes caused obvious abnormalities (Table 1). It was known previously that mutations in *mes-2* and *mes-4* cause maternal-effect sterility (Holdeman et al., 1998; Fong et al., 2002), and that RNAi of *set-1* causes embryonic lethality (Terranova et al., 2002). We found that null mutations of *set-16* and *set-23* caused lethality. SET-16 is homologous to human MLL3, which is mutated in mixed-lineage leukemias (Ruault et al., 2002) and is associated with H3K4 methylation (Lee et al., 2006). *set-23* is homologous to a primate



gene that encodes a SET-domain protein (Cordaux et al., 2006) and is named SETMAR for the SET-mariner fusion, because it has a mariner transposon insertion (Robertson and Zumpano, 1997).

#### Four HMT genes regulate vulval cell-fate specification

We also investigated possible more subtle roles for the HMT genes during vulval development. We constructed multiple mutants carrying HMT deletions and loss-of-function mutations in the class A synMuv gene *lin-15A*, the class B synMuv gene *lin-15B* or both. For the nine genes without deletion alleles, we used RNAi to inactivate them, as above (Table 1). Because the synMuv phenotype is temperature-sensitive (Ferguson and Horvitz, 1989), we also scored the vulval phenotypes of our mutant strains at 25°C (see Table S1 in the supplementary material).

This survey identified two synMuv genes, which we named *met-1* and *met-2* (*met*=histone methyltransferase-like, Table 1). Previously, *met-2* but not *met-1* was identified as a class B synMuv gene in a whole-genome RNAi-feeding screen (Poulin et al., 2005). We found that a role for *met-1* in vulval development can be observed using RNAi by injection (data not shown) or in a deletion mutant but not using RNAi by feeding. Additionally, we identified two genes, *mes-2* and *mes-4*, that when inactivated suppressed the synMuv phenotype (Table 1). Subsequently, *mes-2*, *mes-3*, *mes-4* and *mes-6* were reported to be suppressors of the synMuv phenotype (see Table S2 in the supplementary material) (Cui et al., 2006b).

#### *met-1* and *met-2* are synMuv genes that encode homologs of Set2 and SETDB1, respectively

Deletion mutations of *met-1* or *met-2* caused no vulval abnormalities (Table 2). A synMuv phenotype resulted when each deletion was combined with a loss-of-function mutation of each class A gene. Loss of *met-2* function caused a more severe synMuv phenotype than did loss of *met-1* in combination with null mutations in each of the class A genes, indicating that *met-2* might more strongly inhibit the vulval cell-fate decision. Double mutants of a *met-1* or *met-2* mutation and one of several class B mutations did not have a synMuv phenotype. A *met-2* deletion, but not a *met-1* deletion, enhanced the incompletely penetrant Muv phenotype of the class C synMuv mutant *trr-1(n3712)* and caused a synMuv phenotype with the class C synMuv mutation *mys-1(n3681)*. Because it is synMuv in combination with mutations in both class A and C genes, *met-2* is a class B synMuv gene. Unlike the class C genes, the *met-1* deletion did not cause a synMuv phenotype in combination with class B synMuv mutations. Because a *met-1* mutation did not cause a synMuv phenotype with class B or class C mutations but did cause a synMuv phenotype with class A mutations, *met-1* might define a novel class of synMuv gene.

Using database searches, we determined that MET-1 is similar to yeast Set2 and human HYPB, both of which are H3K36 HMTs (Strahl et al., 2002; Sun et al., 2005). MET-1 has AWS, SET, PostSET and WW domains (Fig. 1). Set2 inhibits transcription from genes that are actively being transcribed (Carrozza et al., 2005; Keogh et al., 2005). Specifically, Set2 prevents transcription from initiating downstream of the promoter region by recruiting the Rpd3S histone deacetylase (HDAC) complex through interaction of trimethylated H3K36 with the chromodomain-containing protein Eaf3 (Joshi and Struhl, 2005). The Rpd3S complex removes acetyl groups from histone H3K9, thereby preventing transcription. Set2 is recruited to actively transcribed genes by interaction with the carboxyl-terminal domain (CTD) of RNA polymerase II

**Table 2. *met-1* and *met-2* are synthetic multivulva genes**

Genotype	% multivulva (n)
<b><i>met-1</i> and <i>met-2</i> single mutants</b>	
<i>met-1(n4337)</i>	0 (247)
<i>met-2(n4256)</i>	0 (170)
<b><i>met-1</i> and <i>met-2</i> interactions with class A mutations</b>	
<i>met-1(n4337); lin-8(n2731)</i>	89 (185)
<i>met-1(n4337); lin-15A(n433)</i>	20 (291)
<i>met-1(n4337); lin-15A(n767)</i>	81 (469)
<i>met-1(RNAi); lin-15A(n767)</i>	50 (263)
<i>met-1(n4337); lin-38(n751)</i>	52 (313)
<i>met-1(n4337); lin-56(n2728)</i>	78 (330)
<i>lin-8(n2731); met-2(n4256)</i>	89 (341)
<i>met-2(n4256); lin-15A(n433)</i>	94 (234)
<i>met-2(n4256); lin-15A(n767)</i>	100 (350)
<i>met-2(RNAi); lin-15A(n767)</i>	99 (347)
<i>lin-38(n751); met-2(n4256)</i>	100 (349)
<i>lin-56(n2728); met-2(n4256)</i>	100 (263)
<b><i>met-1</i> and <i>met-2</i> interactions with class B mutations</b>	
<i>met-1(n4337); lin-15B(n744)</i>	0 (216)
<i>met-1(n4337); lin-35(n745)</i>	0 (146)
<i>met-1(n4337); lin-37(n4903)</i>	0 (131)
<i>met-1(n4337); dpl-1(n2994)</i>	0 (435)
<i>met-2(n4256); lin-15B(n744)</i>	0 (349)
<i>lin-35(n745); met-2(n4256)</i>	0 (391)
<i>lin-37(n4903); met-2(n4256)</i>	0 (114)
<i>dpl-1(n2994); met-2(n4256)</i>	0 (105)
<b><i>met-1</i> and <i>met-2</i> interactions with class C mutations</b>	
<i>met-1(n4337); mys-1(n3681)*</i>	0 (241)
<i>met-1(n4337); trr-1(n3712)<sup>†</sup></i>	12 (22)
<i>met-2(n4256); mys-1(n3681)*</i>	24 (93)
<i>trr-1(n3712); met-2(n4256)<sup>†</sup></i>	29 (36)

\*The vulval phenotypes of these animals were scored at 25°C, at which temperature the single mutants *met-1(n4337)*, *met-2(n4256)* and *mys-1(n3681)* are 0% Muv.

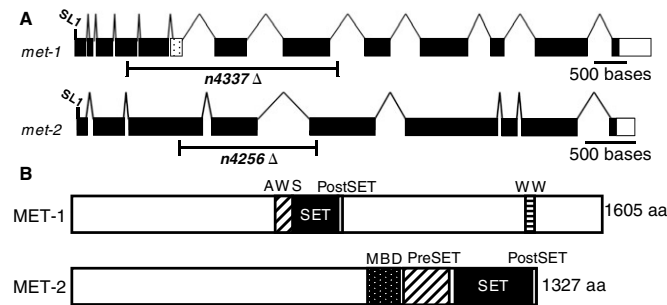
<sup>†</sup>*trr-1(n3712)* is 11% Muv as a single mutant.

phosphorylated on serine five (Li et al., 2002; Krogan et al., 2003; Li et al., 2003; Xiao et al., 2003). HYPB and MET-1 are similar throughout their lengths (27% identity), especially in the enzymatic HMT domains (46% identity).

MET-2 is similar to human SETDB1, which is an H3K9 methyltransferase that plays a role in euchromatic transcriptional repression and the formation of heterochromatin (Schultz et al., 2002). MET-2 has PreSET, SET, PostSET and methylated DNA-binding domains (Fig. 1). MET-2 and SETDB1 share sequence similarity throughout their length (19% identity) but are most similar in the enzymatic HMT domains (50% identity).

#### *met-1* and *met-2* might act redundantly to inhibit vulval cell fates through the trimethylation of the N-terminal tail of histone H3

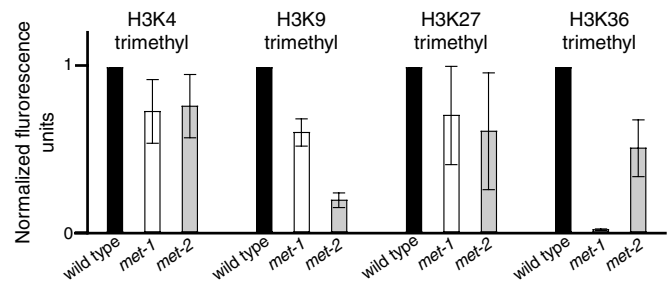
We observed that a *met-1; met-2* double mutant had an incompletely penetrant synMuv phenotype (Table 3). The *met-1; met-2* synMuv phenotype is recapitulated by RNAi of either *met* gene combined with a deletion of the other, indicating that this synMuv phenotype was caused specifically by loss of *met-1* and *met-2* gene function and not by a linked mutation. Additionally, we found that the HMT genes most similar to *met-1* and *met-2* (*set-12* and *set-11*, respectively) did not act redundantly with either *met* gene during vulval development (see Table S3 in the supplementary material). Thus, not all predicted H3K9 and H3K36 HMT genes act redundantly with *met-1* or *met-2*. Additionally, the *met-1; met-2*



**Fig. 1. *met-1* and *met-2* gene structures, mutations and predicted protein structures. (A)** The genomic structures of *C. elegans met-1* and *met-2*. Exons are represented by black boxes, 3' untranslated regions by white boxes. The alternatively spliced exon six of *met-1* is depicted as a white, stippled box. The locations of the deletion alleles are shown. **(B)** The domain structures of the MET-1 and MET-2 proteins.

double mutant displayed a mortal germline (Mrt) phenotype (Ahmed and Hodgkin, 2000) in which the strain was almost completely sterile when derived from heterozygotes and became 100% sterile after 3-11 generations (see Fig. S3 in the supplementary material). By contrast, *met-2* mutants were Mrt after 18-28 generations and *met-1* mutants were not Mrt (data not shown). We conclude that *met-1* and *met-2* act partially redundantly in the inhibition of vulval cell fates and in promoting the immortality of the germline.

The class B synMuv defects of *met-1* or *met-2* mutants could be rescued by expressing *met-1* or *met-2*, respectively, under the control of the *dpy-7* promoter, which is expressed in the hypodermal tissue that neighbors the vulval cells. Specifically, in eight independent lines, expression of *met-1* reduced the penetrance of the *met-1*(n4337); *lin-15A*(n767) synMuv phenotype from 81% to 2, 6, 14, 14, 15, 15, 32 and 34%, and in seven independent lines expression of *met-2* reduced the penetrance of the *met-2*(n4256); *lin-15A*(n433) synMuv phenotype from 94% to 0, 0, 0, 3, 3, 6 and 9%. The addition of two missense mutations known to abolish enzymatic function of homologous HMTs (Rea et al., 2000; Landry et al., 2003) reduced this phenotypic rescue of either *met-1* or *met-2*. Specifically, in seven independent lines, expression of such a *met-1* SET mutant gene caused a synMuv phenotype that was 44, 50, 51, 52, 54, 60 and 70% penetrant, and in eight independent lines expression of such a *met-2* SET mutant gene caused a synMuv phenotype that was 91, 91, 93, 94, 95, 97, 98 and 100% penetrant. Therefore, the methylation activity of the SET domain is necessary for most of the functions of *met-1* and *met-2* during vulval development. The rescue of the *met-1*; *lin-15A* synMuv phenotype was not disrupted completely by the SET-domain mutations, suggesting that *met-1* might have other functions in addition to



**Fig. 2. *met-1* and *met-2* are required in vivo for normal levels of histone H3K36 and H3K9 trimethylation, respectively.** The levels of histone H3 trimethylation at K4, K9, K27 and K36 were assayed using quantitative western blots and normalized to levels of histone H3 (see Materials and methods). Relative histone H3 trimethylation levels of *met-1*(n4337) mutants (white) and *met-2*(n4256) mutants (gray) were normalized to the trimethylation levels of wild-type *C. elegans* (black) for each experiment. The levels of histone H3 were measured (see Fig. S1 in the supplementary material), and the specificity of the H3K9 and H3K36 trimethylation antisera were confirmed using dot blots of methylated histone tail peptides (see Fig. S2 in the supplementary material). Normalized units of fluorescence and standard deviations are shown.

histone methylation. Our results indicate that histone methylation mediated by the SET domains of MET-1 and MET-2 regulates vulval development.

Using quantitative western blots, we tested which residues of the histone H3 tail are methylated in the wild type and in *met-1* and *met-2* mutants. MET-1 is predicted to methylate histone H3K36 based on its homology to yeast Set2, and MET-2 is predicted to methylate histone H3K9 based on its homology to mammalian SETDB1. We probed embryonic protein extracts for levels of histone H3 lysine trimethylation (Fig. 2, and see Figs S1, S2 and S5 in the supplementary material), because HP1 has been shown to bind more strongly to trimethylated than to dimethylated or monomethylated histone tails (Bannister et al., 2001; Nielsen et al., 2002). Our results suggest that levels of H3K4 and H3K27 trimethylation were not significantly different from those in the wild type. However, *met-1* embryos showed a striking defect in histone H3K36 trimethylation and a ~50% decrease in histone H3K9 trimethylation. *met-2* mutants showed a defect in H3K9 trimethylation and a ~40% decrease in H3K36 trimethylation. We conclude that, consistent with their homologies, MET-1 is likely to trimethylate H3K36, and MET-2 is likely to trimethylate H3K9. The *met-1*; *met-2* double mutant had an incompletely penetrant sterile phenotype that increased to 100% penetrance after 3-11 generations (see Table S3 in the supplementary material). We were unable to collect sufficient quantities of histones to measure the levels of lysine trimethylation in the double mutant.

### ***met-1* and *met-2* act redundantly with the *C. elegans* HP1 homologs in vulval cell-fate determination**

Because the trimethylation of lysines on histone H3 tails creates binding sites for HP1, we investigated the role of the two *C. elegans* HP1 homologs HPL-1 and HPL-2 in vulval cell-fate specification. The *C. elegans* HP1 homolog *hpl-2* has been reported to be a class B synMuv gene (Couteau et al., 2002). We found that although an *hpl-2* single mutant did not have a Muv phenotype at 20°C (Table 4), at 25°C it had a 99% penetrant Muv phenotype (see Table S4 in the supplementary material). At 20°C, a presumptive null allele of

**Table 3. *met-1* and *met-2* act redundantly to control the vulval cell-fate decision**

Genotype	% multivulva (n)
<i>met-1</i> (n4337)	0 (247)
<i>met-2</i> (n4256)	0 (170)
<i>met-1</i> (n4337); <i>met-2</i> (n4256)*	29 (126)
<i>met-1</i> (n4337); <i>met-2</i> (RNAi)	22 (34)
<i>met-1</i> (RNAi); <i>met-2</i> (n4256)	11 (48)

\*The *met-1*; *met-2* double mutant is synthetically sterile with an Mrt germline phenotype, so these animals were descended from *met-1*+/+; *met-2*+/+ heterozygotes.

*hpl-2* caused a class B synMuv phenotype with null or strong mutations of each of the class A synMuv genes (see Table S4 in the supplementary material). By contrast, an *hpl-1* deletion did not cause a synMuv phenotype when combined with class A or B mutations, nor did it enhance a synMuv double mutant phenotype. At 20°C, *hpl-2*; *hpl-1* double mutants had a 24% penetrant synMuv phenotype (Table 4), showing that *hpl-1* and *hpl-2* act redundantly during vulval development (Schott et al., 2006).

If *met-1* or *met-2* act in the same pathway as the *C. elegans* HP1 homologs, then one would not expect mutations in the *hpl* and *met* genes to enhance the synMuv phenotypes of other *hpl* or *met* mutants when combined in multiple mutants. However, null mutations in *met-1* or *met-2* enhanced the Muv phenotype of *hpl-2* (Table 4). Triple mutants in which the *met-1*; *met-2* double mutant was combined with either *hpl-1* or *hpl-2* had a more severe phenotype than each of the *met hpl*, *met-1*; *met-2* or *hpl-2*; *hpl-1* double mutant combinations. Triple mutants in which the *hpl-2*; *hpl-1* double mutant was combined with either *met-1* or *met-2* had a more severe phenotype than each *met hpl*, *met* or *hpl* double mutant combination. The quadruple mutant *met-1*; *met-2* *hpl-2*; *hpl-1* had a more severe synMuv phenotype than any double or triple mutant combination. The synMuv phenotype of the quadruple mutant was completely penetrant when derived from the *met-2 hpl-2* heterozygote, unlike the triple mutant synMuv phenotypes, which were maternally rescued (Table 4 and data not shown).

We conclude that the *C. elegans* MET-1 and MET-2 HMTs and the proteins thought to be recruited to the methylated residues created by these HMTs can act independently to inhibit the expression of ectopic vulval cell fates.

### ***met-1*, *met-2* and *hpl-2* mutants display pleiotropic defects distinct from canonical class B synMuv mutants**

Many of the class B synMuv genes control aspects of a germline-versus-soma cell-fate decision process (Unhavaithaya et al., 2002; Wang et al., 2005). Defects in this process can be observed as the ectopic expression of germline markers in the soma, enhanced sensitivity to RNAi, silencing of repetitive transgenes (Tam phenotype) and the germline-like appearance of somatic cells in *mep-1* and *let-418* arrested larvae. We found that *met-1*, *met-2*, *hpl-1* and *hpl-2* single mutants were not hypersensitive to RNAi (see Fig. S3 in the supplementary material) and did not have a Tam phenotype (data not shown). However, *met-1*; *met-2* and *hpl-2*; *hpl-1* double mutants were sensitive to RNAi (see Fig. S4 in the supplementary material) but were not Tam (data not shown).

We also tested the ectopic activation of a *lag-2::gfp* reporter construct; some class B mutants show such activation (Dufourcq et al., 2002; Poulin et al., 2005; Coustham et al., 2006; Schott et al., 2006). *met-1*, *met-2* and *hpl-2* but not *hpl-1* mutations caused ectopic activation of the *lag-2* reporter in the intestine and the posterior of the animal (data not shown), suggesting that these genes might normally repress transcription from the *lag-2* promoter. Additionally, some class B synMuv mutations suppress the vulval defects of *mat-3(ku233)* mutants, presumably through the ectopic activation of *mat-3* transcription from the promoter mutant *ku233* (Garbe et al., 2004). We found that the *met* and *hpl* null mutations suppressed the cell-cycle-like vulval defects of *mat-3(ku233)* mutants (see Table S5 in the supplementary material). Many class B synMuv mutants are hypersensitive to RNAi, have a Tam phenotype, ectopically express GFP from a *lag-2* reporter and suppress *mat-3(ku233)* vulval defects. Given that *met-1*, *met-2* and *hpl-2* mutations

**Table 4. The *C. elegans* HP1 homologs act redundantly with each other and with the *Met* genes to control the vulval cell-fate decision**

Genotype	% multivulva (n)*
<b><i>hpl</i> and <i>met</i> single mutants are not Muv at 20°C</b>	
<i>hpl-1(n4317)</i>	0 (312)
<i>hpl-2(tm1489)</i>	0 (161)
<i>met-1(n4337)</i>	0 (247)
<i>met-2(n4256)</i>	0 (170)
<b><i>hpl-2</i> acts redundantly with <i>hpl-1</i>, <i>met-1</i> and <i>met-2</i></b>	
<i>hpl-2(tm1489); hpl-1(n4317)</i>	24 (203)
<i>met-1(n4337); hpl-1(n4317)</i>	0 (165)
<i>met-2(n4256); hpl-1(n4317)</i>	0 (276)
<i>met-1(n4337); hpl-2(tm1489)</i>	17 (81)
<i>met-2(n4256) hpl-2(tm1489)</i>	87 (286)
<b><i>met-1</i> and <i>met-2</i> act redundantly with the <i>C. elegans</i> HP1 homologs</b>	
<i>met-1(n4337); met-2(n4256)</i>	29 (126)
<i>met-1(n4337); hpl-2(tm1489); hpl-1(n4317)</i>	84 (205)
<i>met-2(n4256) hpl-2(tm1489); hpl-1(n4317)</i>	100 (52)
<i>met-1(n4337); met-2(n4256); hpl-1(n4317)</i>	57 (7)
<i>met-1(n4337); met-2(n4256) hpl-2(tm1489)</i>	100 (126)
<i>met-1(n4337); met-2(n4256) hpl-2(tm1489); hpl-1(n4317)</i>	100 (68) <sup>†</sup>

\*These vulval phenotypes were scored at 20°C.

<sup>†</sup>These homozygotes were derived from *met-2/+ hpl-2/+* heterozygotes because the quadruple mutant is synthetically sterile. This mutant did not show any maternal rescue of the synMuv phenotype, unlike the *met-2(n4256) hpl-2(tm1489); hpl-1(n4317)* and *met-1(n4337); met-2(n4256) hpl-2(tm1489)* triple mutants.

all cause strong mutant phenotypes and share only the last two attributes, these genes are likely to represent a distinct subset of class B synMuv genes.

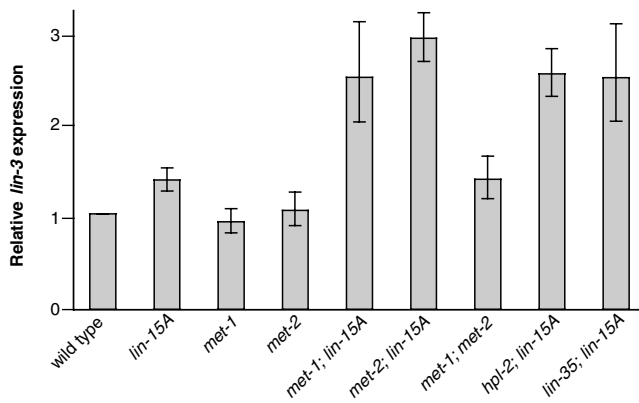
### ***met-1*, *met-2* and *hpl-2* regulate the transcriptional repression of the synMuv target gene *lin-3***

Recently, it was reported that some synMuv proteins repress the transcription of the EGF gene *lin-3* (Cui et al., 2006a). *lin-3* is normally expressed in the gonadal anchor cell, and LIN-3 activates the RTK/Ras pathway in the cells of the vulval equivalence group closest to the anchor cell to cause these cells to adopt vulval cell fates (Hill and Sternberg, 1992; Kornfeld, 1997). Single class A or class B mutants do not have significantly increased levels of *lin-3* expression, whereas class AB double mutants have greater levels than the wild type or either single synMuv mutant (Cui et al., 2006a).

We quantified *lin-3* expression from *met-1*, *met-2* and *hpl-2* synMuv mutants (*hpl-1* mutants are not synMuv) during the time of vulval induction (Fig. 3). *met-1* and *met-2* single mutants did not have increased levels of *lin-3*. However, *met-1*, *met-2* or *hpl-2* mutations combined with the synMuv class A mutation *lin-15A(n767)* showed increased levels of *lin-3* as compared with that in the wild type. The *met-1*; *met-2* double mutant had slightly higher levels of *lin-3* than the wild type. *lin-3* expression was lower in the *met-1*; *met-2* double mutant than in the *met* double mutants with *lin-15A*. This result might reflect the less penetrant Muv phenotype of *met-1*; *met-2* animals (Table 2). Alternatively, the *met-1*; *met-2* double mutant Muv phenotype might not be caused by an increase in *lin-3* expression.

In short, the vulval HMT genes *met-1* and *met-2* as well as the HP1 gene *hpl-2* control the transcriptional repression of the synMuv target gene *lin-3* redundantly with the class A synMuv genes, and





**Fig. 3. *met-1* and *met-2* are each required to prevent ectopic *lin-3* expression in a *lin-15A*(n767) mutant background.** Real-time RT-PCR experiments were performed using RNA samples from *C. elegans* of the genotypes shown. Mean  $\Delta\Delta C_T$  values were used to calculate relative changes in *lin-3* expression normalized to levels of *rpl-26* (see Materials and methods). Mean values and ranges of relative *lin-3*/*rpl-26* ratios for three trials are shown.

mutations in each of these genes can cause increased levels of *lin-3* transcription. That MET-1, MET-2 and HPL-2 are homologous to transcriptional repressors suggests that these proteins control *lin-3* levels by acting directly as transcriptional repressors of *lin-3*.

## DISCUSSION

### The class B synMuv genes *met-1/Set2* and *met-2/SETDB1* regulate both H3K9 and H3K36 trimethylation

Whereas *met-1* and *met-2* single mutants are normal in vulval development, *met-1; met-2* double mutants have an incompletely penetrant Muv phenotype, indicating that these two genes act redundantly to inhibit the expression of vulval cell fates. Because both *met-1* and *met-2* encode presumptive HMTs and because an active HMT enzymatic domain is required for the function of each, *met-1* and *met-2* might function similarly in vivo. Furthermore, *met-1* and *met-2* mutants are both defective in both H3K9 and H3K36 trimethylation. These observations are all consistent with the hypothesis that *met-1* and *met-2* control functionally redundant activities. We discuss below several hypotheses that could explain the redundancy observed between *met-1* and *met-2*.

First, MET-1 and MET-2 might each methylate both H3K9 and H3K36. However, MET-1 and MET-2 are homologous to an H3K36 HMT and an H3K9 HMT, respectively. One simple model is that MET-1 primarily methylates H3K36 but also methylates H3K9, and MET-2 primarily methylates H3K9 but also methylates H3K36. In the absence of either MET-1 or MET-2 function, the activity of the other results in a sufficient level of methylation for some biological function. Thus, neither single mutant has a Muv phenotype. However, in vitro studies of Set2 and SETDB1 have not shown such dual specificity (Schultz et al., 2002; Strahl et al., 2002), suggesting either that the in vitro results do not recapitulate the in vivo functions of these proteins, that MET-1 and MET-2 function differently than their homologs, or that this simple model is incorrect.

Second, the transcriptional repression of vulval target genes could depend on the concerted action of MET-1 and MET-2 to methylate H3K9 and H3K36. For example, in *met-1* mutants H3K36 trimethylation is strongly reduced. The trimethylation of H3K9 also could be impaired because the H3K9-HMT activity of MET-2 is

dependent on the methylation of H3K36 by MET-1. The reciprocal methylation activity of MET-1 might also require the H3K9 activity of MET-2. Although partially deficient in both H3K9 and H3K36 methylation, the single mutants might not show a Muv phenotype because their levels of methylation are sufficient for wild-type vulval development. Methylation of H3K36 has been associated with transcriptional repression in mammalian cells (Strahl et al., 2002), and the possible dependence of this repression on H3K9 methylation has not been investigated.

Third, the redundancy between *met-1* and *met-2* could be caused solely by defects in the level of either H3K9 or H3K36 trimethylation. One possibility is that the level of H3K9 trimethylation is the major methylation event for inhibiting the vulval cell fate, with MET-2 primarily methylating H3K9 and MET-1 indirectly providing some H3K9 trimethylation by promoting the expression of another H3K9-specific HMT. In support of this hypothesis, the severity of the *met-1* or *met-2* class B synMuv defect is more closely correlated with the level of H3K9 trimethylation than with the level of H3K36 trimethylation (Table 2 and Fig. 2). Thus, the redundancy between *met-1* and *met-2* might be caused by a reduction in H3K9 trimethylation below the threshold needed to prevent ectopic vulval development. In *met-1* or *met-2* single mutants, there would still be sufficient H3K9 trimethylation to repress *lin-3* expression in the hypodermis, so wild-type vulval development would occur. In the *met-1; met-2* double mutant, H3K9 trimethylation would drop below the threshold needed to repress *lin-3* transcription, and a synMuv phenotype would result.

### The *C. elegans* HP1 homologs can act independently of histone methylation mediated by MET-1 and MET-2

Because HP1 is an effector of methylation-dependent transcriptional repression (Hediger and Gasser, 2006), we expected the HP1 homologs to act downstream of either or both of the *met* genes. However, we found that the *met* genes could act redundantly with the *hpl* genes. Perhaps the *C. elegans* HP1 proteins act at sites other than the methylated histone tails generated by MET-1 and MET-2. One observation indicates that HP1 proteins might act independently of histone methylation: in *Drosophila*, HP1 can bind naked DNA and nucleosomal DNA with histones without N-terminal tails in vitro (Zhao et al., 2000).

Alternatively, the functional redundancy between the *met* and *hpl* genes during vulval cell-fate determination could be caused by an incomplete loss of HMT or HP1-like gene functions. For example, in the *met-1; met-2* double mutant, a third HMT could provide some histone methylation important for the localization of the HPL proteins. Besides *met-1* and *met-2*, one or more of the other 36 HMT genes could have subtle roles not detected in our assays. For example, we could not assess the roles of the four HMT genes required for viability in vulval development. Furthermore, there are 20 genes in *C. elegans* that encode proteins with at least one chromodomain (E.C.A. and H.R.H., unpublished). Other chromodomain-encoding genes could function redundantly with the *hpl* genes in vulval cell-fate determination.

### The synMuv genes encode conserved chromatin remodeling activities that prevent ectopic initiation of *lin-3* transcription during vulval development

In *S. cerevisiae*, Set2 is localized to actively transcribed genes and methylates H3K36 through interactions with RNA polymerase II (Li et al., 2002; Krogan et al., 2003; Li et al., 2003; Xiao et al., 2003).

Methylated H3K36 is bound by Eaf3 (Joshi and Struhl, 2005) and subsequently recruits an HDAC complex to prevent inappropriate transcriptional initiation downstream of the promoter (Carrozza et al., 2005; Keogh et al., 2005). Eaf3 is a part of the NuA4 complex, which, through a distinct mechanism, also prevents ectopic transcriptional initiation (Morillon et al., 2005).

This mechanism of inhibiting inappropriate transcriptional initiation might be identical to that controlling *lin-3* expression during vulval development. In addition, our data indicate that other processes are also involved, including H3K9 trimethylation, the binding of a NuRD-like complex and HP1-like proteins. Specifically, we propose that MET-1 inhibits transcriptional initiation downstream of the *lin-3* promoter, acting much as Set2 does in *S. cerevisiae*. H3K36 methylated by MET-1 is bound by the *C. elegans* NuRD-like complex, which contains the chromodomain-containing LET-418/Mi2 subunit and the HDA-1 histone deacetylase subunit (von Zelewsky et al., 2000; Unhavaithaya et al., 2002). Subsequently, the NuRD-like complex deacetylates histone H3K9, and MET-2/SETDB1 methylates H3K9, thereby creating a site for HPL-1 and HPL-2 to bind and prevent the inappropriate initiation of *lin-3* transcription. The methylation of H3K36 might also recruit the *C. elegans* NuA4-like complex, which contains class C synMuv proteins (Ceol et al., 2006), to inhibit inappropriate transcriptional initiation of *lin-3*. Consistent with this model, H3K9 trimethylation and HP1 $\gamma$  have recently been found to be enriched in actively transcribed genes in human cells (Vakoc et al., 2005). We propose that the unidentified human HMT mediating H3K9 trimethylation of histones in the promoters of such actively transcribed genes is the MET-2 homolog SETDB1.

Genes that act antagonistically to the synMuv genes have been identified as suppressors of the synMuv phenotype; two such genes encode homologs of the NURF chromatin-remodeling complex (Andersen et al., 2006). In mammalian cells, the NURF complex has been shown to promote the initiation of transcription (Wysocka et al., 2006). We suggest that the synMuv suppressor NURF-like complex and the synMuv proteins antagonize each other by oppositely regulating the initiation of *lin-3* transcription during vulval development. More generally, we propose that pathways involving H3K36 trimethylation, NuRD histone deacetylase activity, SETDB1 H3K9 trimethylation and HP1 opposed by a NURF complex might be conserved in other organisms, including humans, and serve as important and general mechanisms for the regulation of transcriptional initiation.

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#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/16/2991/DC1>

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