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MEP-1 and a Homolog of the NURD Complex Component Mi-2 Act Together to Maintain Germline-Soma Distinctions in *C. elegans*

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Summary

A rapid cascade of regulatory events defines the developmental fates of embryonic cells. However, once established, these developmental fates and the underlying transcriptional programs can be remarkably stable. Here, we describe two proteins, MEP-1 and LET-418/Mi-2, required for maintenance of somatic differentiation in *C. elegans*. In animals lacking MEP-1 and LET-418, germline-specific genes become derepressed in somatic cells, and Polycomb group (PcG) and SET domain-related proteins promote this ectopic expression. MEP-1 and LET-418 interact in vivo with the germline-protein PIE-1. Our findings support a model in which PIE-1 inhibits MEP-1 and associated factors to maintain the pluripotency of germ cells, while at later times MEP-1 and LET-418 remodel chromatin to establish new stage- or cell-type-specific differentiation potential.

Introduction

The segregation of the germline cells away from the more specialized somatic cells is among the earliest and most fundamental of developmental events in many animal species. In some cases, embryonic germline cells appear to be insulated from differentiation-inducing signals by transcriptional repression. For example, germline specification in both *C. elegans* and *Drosophila* embryos has been correlated with the global repression of RNA polymerase II (pol II)-dependent transcription (van Doren et al., 1998). In *C. elegans*, this transcriptional silencing requires a maternally expressed zinc-finger protein called PIE-1 (Mello et al., 1996; Seydoux et al., 1996). PIE-1 protein is present in germline nuclei and also in the cytoplasm from the 1-cell stage until approximately the 200-cell stage and functions to prevent somatic differentiation in response to maternally encoded transcriptional activators that are also present in the germline during this time (Bowerman et al., 1993; Mello et al., 1992, 1996). The mechanism by which PIE-1 induces transcription repression is not known but may involve direct interactions between PIE-1 and the general transcriptional machinery (Batchelder et al., 1999).

The disappearance of PIE-1 in later stage embryos correlates with the activation of transcription in the germline cells, which then undergo proliferation, meiosis, spermatogenesis, and oogenesis in hermaphrodite animals. These processes depend in part on the activities of the maternal effect sterile (MES) proteins (reviewed in Seydoux and Strome, 1999), which include proteins related to the *Drosophila* Polycomb group (PcG) and Trithorax group (TrxG) proteins. PcG- and TrxG-related proteins have been shown to maintain active and/or inactive transcriptional states of homeotic genes and other genes in *Drosophila* and vertebrates, and this function is mediated in part by core histone modifications and nucleosome remodeling (reviewed in Brock and van Lohuizen, 2001; Francis and Kingston, 2001; Pirrotta, 1998). Similarly, the MES proteins are thought to influence transcription through the regulation of chromatin organization in germline cells (reviewed in Pirrotta, 2002).

In the present study, we describe a putative chromatin-remodeling complex that interacts with the MES proteins and also with PIE-1 to maintain germline/soma distinctions during *C. elegans* embryogenesis. This complex contains a conserved Krüppel-type zinc-finger protein named MEP-1 and two *C. elegans* homologs of mammalian proteins that function in the nucleosome remodeling and histone deacetylase (NURD) complex, LET-418/Mi-2 and HDA-1/HDAC-1 (von Zelewsky et al., 2000; Shi and Mello, 1998). Interestingly, whereas PIE-1 is required to prevent somatic development in the germline, we show that MEP-1 and LET-418 activities are required to prevent germline development in the soma. Our findings support a model in which PIE-1 inhibits the activities of MEP-1, LET-418, and HDA-1 to prevent chromatin remodeling and to preserve MES-protein-dependent germline pluripotency during the early embryonic phase of somatic cell-fate specification.

Results

MEP-1 Is Required for Fertility and the Suppression of Germline Differentiation in Somatic Cells

To investigate the role of PIE-1 in germline specification in *C. elegans*, we searched for proteins that interact with PIE-1 using a yeast two-hybrid screen. Clones encoding positive interactors were then analyzed by RNA interference (RNAi) for phenotypes relevant to germline specification or PIE-1 localization. Several clones identified in this assay define the *mep-1* locus. RNAi targeting *mep-1* induces an L1 larval arrest phenotype that appears to reflect the combined maternal and zygotic loss-of-function phenotype (see Experimental Procedures). A null allele, *mep-1*(*q660*), causes a sterile phenotype with defects in gonad development and oocyte production (Belfiore et al., 2002).

The *mep-1* L1 arrest phenotype was correlated with

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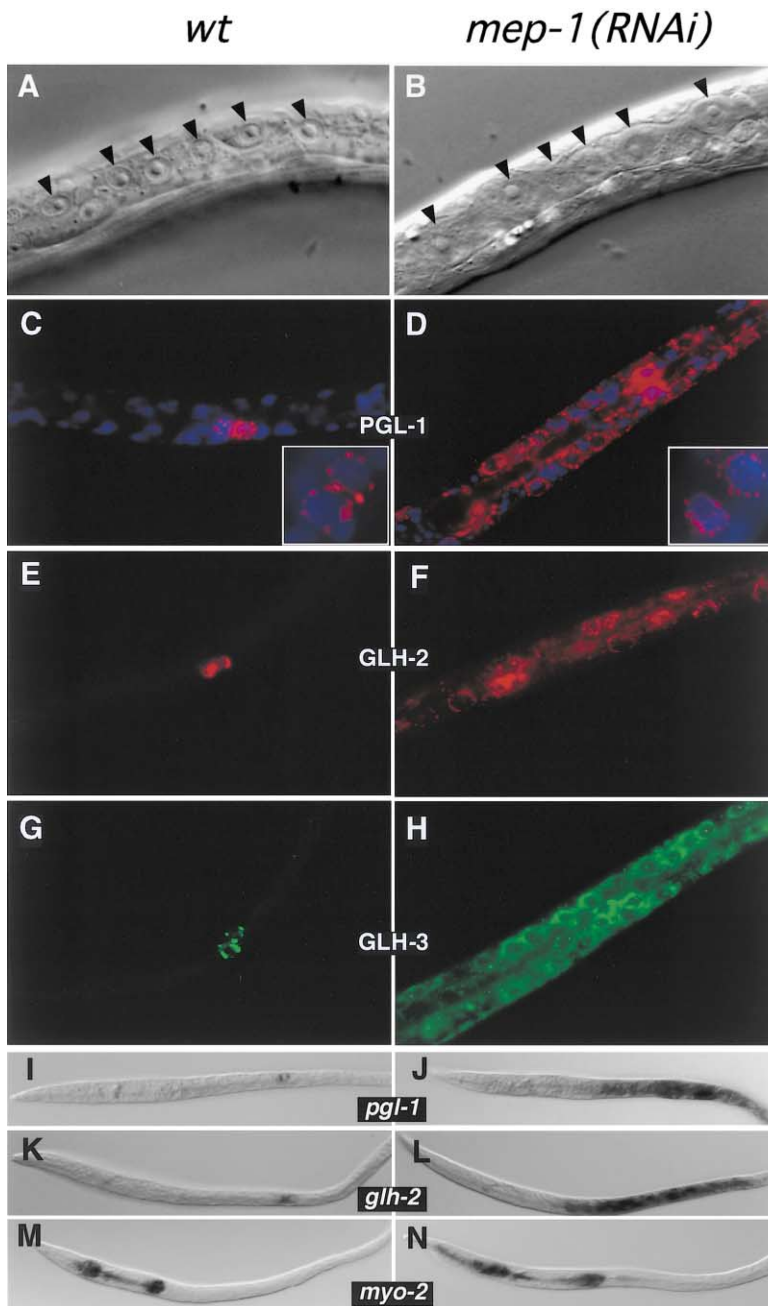


Figure 1. Soma to Germline Transformations in *mep-1(RNAi)* Larvae

Comparison of wild-type L1 (left) and *mep-1(RNAi)* L1 larvae (right). (A and B) Light micrographs. The arrowheads indicate several hypodermal nuclei in each animal. In (B), these nuclei appear larger and flatter and are surrounded by a more granular cytoplasm. (C–H) Immunofluouresence micrographs of larvae stained for expression of P granule components, PGL-1, GLH-2, and GLH-3 (as indicated). In (C) and (D), the nuclei are revealed by Dapi staining (blue), and the higher magnification (insets) reveal the punctate perinuclear distribution of the PGL-1 protein. (I–M) In situ staining to detect mRNA levels of *pgl-1*, *glh-2*, and the pharyngeal-specific myosin *myo-2* (as indicated).

a change in the appearance of somatic cells. The hypodermal and intestinal cells in the arrested larvae exhibited abnormal nuclear and cellular morphologies. For example, hypodermal cells often exhibited a larger than normal nucleus and rounded cellular outlines (compare Figures 1A and 1B) and had an overall appearance that resembled early mitotic germline cells (Figure 1B, and data not shown). To ask if these cells exhibit other features in common with germline cells, we stained the arrested larvae to detect proteins normally expressed strictly in germline cells. The one such protein named PGL-1 is a component of P granules, which are part of the *C. elegans* germ plasm (Kawasaki et al., 1998). We found that the *mep-1(RNAi)*-arrested larvae express PGL-1 protein ectopically in all intestinal cells and in many hypodermal cells (Figures 1C and 1D, and data not shown). The ectopic PGL-1 accumulates in perinuclear structures as does PGL-1 in the germline cells of wild-type animals (compare insets in Figures 1C and 1D), raising the possibility that the P granules themselves may be ectopically assembled. Consistent with this idea, immunofluorescence staining also detected ectopic expression of two additional components of P granules, GLH-2 and GLH-3, both homologous to the conserved germline helicase Vasa (Gruidl et al., 1996). Like PGL-1, these proteins accumulated in the hypodermal and intestinal cells of the *mep-1(RNAi)*-arrested larvae, where they assembled into P-granule-like structures (Figures

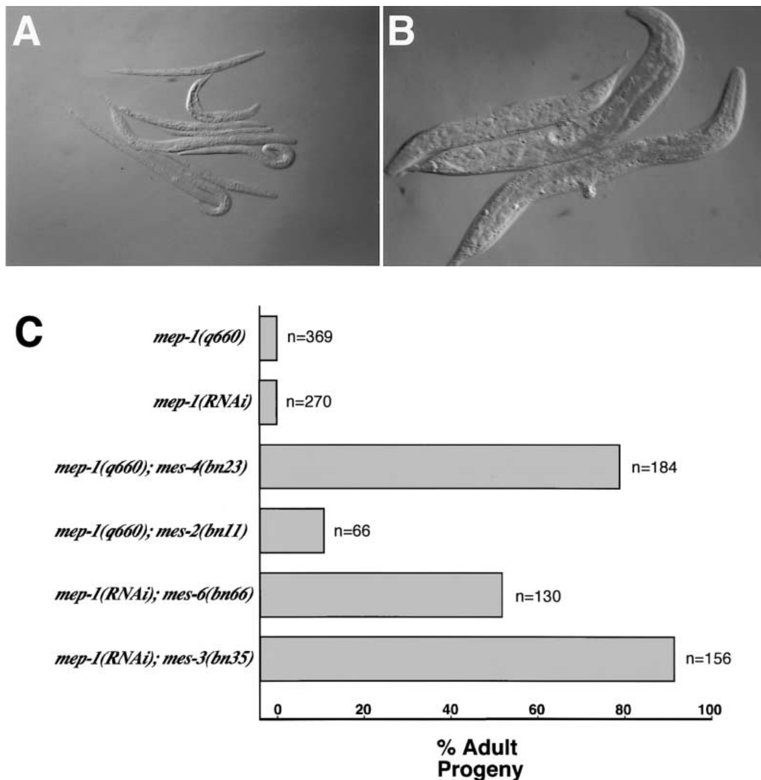


Figure 2. *mes* Genes Interact Genetically with *mep-1*

(A and B) Light micrographs showing animals lacking MEP-1 (A) or lacking both MEP-1 and MES-4 (B). The animals in (A) are arrested at the L1 stage, while the animals in (B) have matured to form sterile adults that resemble *mep-1(q660)* homozygotes. (C) Graphic representation of genetic interactions between *mep-1* and four *mes genes*. For each single and double mutant combination listed at the left of the graph, the progeny of *mep-1(RNAi)* animals or the GFP-negative progeny of *mep-1(q660); mep-1::gfp-Ex* transgenic animals were identified as larvae and monitored for development to the adult stage (% Adult Progeny).

1E–1H). GLH-3 was also visible diffusely in the cytoplasm (Figure 1H). Taken together, these findings suggest that the loss of *mep-1(+)* activity causes normally germline-specific gene products to accumulate in somatic cells.

Despite these abnormalities, the hypodermal and intestinal cells in *mep-1(RNAi)* animals do not completely convert to germline fates. For example, somatic genes such as *jam-1* and *pes-10* that are normally expressed in differentiated hypodermal and intestinal cells (Francis and Waterston, 1991; Mohler et al., 1998; Seydoux and Fire, 1994) are expressed at apparently normal levels in *mep-1(RNAi)* embryos. These and several other somatic genes assayed continued to exhibit normal levels of protein and mRNA expression after hatching and even in the arrested larvae (data not shown). Thus, *mep-1* is required for the repression of germline differentiation in the somatic tissues but does not appear to be necessary for the expression of soma-specific genes.

We found that the maternally expressed P granules exhibited a wild-type distribution in early *mep-1(RNAi)* embryos (data not shown). Ectopic PGL-1 expression was first observed at or shortly after the two-fold stage of embryogenesis (data not shown), at approximately the same time that zygotic expression of PGL-1 normally begins in germline cells of wild-type embryos (Kawasaki et al., 1998). We therefore used in situ hybridization to follow the expression of *pgl-1* and *glh-2* mRNAs in *mep-1(RNAi)*-arrested larvae. We found that the arrested larvae accumulated high levels of *pgl-1* and *glh-2* mRNAs in the somatic tissues, while in contrast, these mRNAs were detectable only in germ cells in wild-type larvae (Figures 1I–1L). In contrast, a muscle myosin specific to the pharynx exhibited a wild-type pattern of mRNA expression in *mep-1(RNAi)* embryos (Figures 1M and 1N). These observations suggest that ectopic P granules in *mep-1(RNAi)* animals result from transcriptional derepression during late embryonic and early larval stages. To further test this possibility, we used a genetic cross to bring a *pgl-1::gfp* transgene into *mep-1(RNAi)* embryos via the sperm. We found that PGL-1::GFP was abundantly expressed in somatic cells of the arrested larvae derived from this cross (data not shown), demonstrating that de novo transcription of the paternally provided *pgl-1::gfp* contributed to PGL ectopic expression.

In summary, *mep-1(RNAi)* appears to cause the ectopic expression of germline genes in somatic cells, with timing that coincides with the normal onset of zygotic gene expression in germline cells. Somatic genes, meanwhile, appear to continue to be expressed with proper spatial and temporal distributions.

The MES Proteins Are Required for Ectopic Germline Fates

In *C. elegans*, the *mes-2*, *mes-3*, *mes-4*, and *mes-6* genes were originally identified in a screen for maternal-effect sterile mutants and are thought to mediate transcriptional control essential for proper development of the germline (Capowski et al., 1991; Garvin et al., 1998; Seydoux and Strome, 1999). Because the *mes* genes are required for germline development in wild-type embryos, we hypothesized that the *mes* genes might also be required to promote ectopic germline-like development in the somatic cells of *mep-1*-arrested larvae. Consistent with this hypothesis, we found that the removal of each *mes(+)* activity resulted in partial suppression

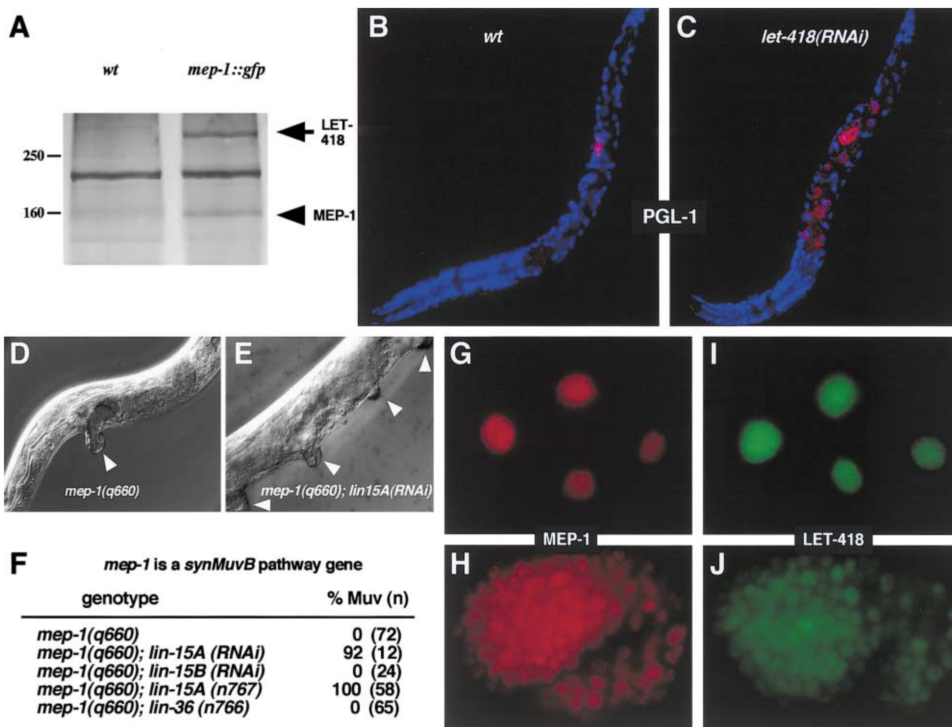


Figure 3. The Nuclear Proteins MEP-1 and LET-418 Interact In Vivo and Have Similar Developmental Activities

(A) MEP-1 and LET-418 interact in vivo. Extracts prepared from wild-type *(wt)* and from *mep-1::gfp* transgenic animals were immunoprecipitated with a GFP-specific monoclonal antibody. Eluted proteins were subjected to SDS-PAGE followed by silver staining. MEP-1::GFP protein migrating at 165 kDa and a second protein (LET-418) migrating at 280 kDa were detected in the transgenic strain, but not in wild-type *(wt)* (as indicated). (B and C) *let-418(RNAi)* induces soma to germline transformations. Immunofluoresence micrographs of a wild-type L1 larva (B) and a *let-418(RNAi)* L1 larva (C) stained for PGL-1 protein (red staining) and Dapi (blue staining). (D–F) *mep-1(q660)* is a synMuvB pathway mutant. (D and E) Light micrographs of an adult *mep-1(q660)* homozygote (D) and of an adult *mep-1(q660);lin-15A(RNAi)* animal (E). (D and E) The arrowheads indicate regions of vulval development in both animals. (G–J) MEP-1 and LET-418 are broadly expressed nuclear proteins. Immunofluorescence micrographs of four cell-stage embryos (G and I) and of comma-stage embryos (H and J) stained with antibodies raised against MEP-1 and LET-418 (as indicated).

of ectopic PGL-1 expression and a partial rescue of *mep-1* larval lethality (Figure 2, and data not shown). This effect was strongest for *mes-3*, *mes-4*, and *mes-6*, which suppressed ~50% or more of the L1 lethality caused by *mep-1* loss of function, resulting in the production of viable but sterile adults (compare Figures 2A and 2B). The *mes-2(bn11)* mutant induced only a slight (but significant) suppression of the L1 arrest, resulting in four viable *mep-1* homozygous adults among 66 animals assayed (as opposed to zero out of 369 for the *mep-1(q660)* single mutant strain; Figure 2C).

The MES proteins are present in somatic cells at the stage immediately preceding the first ectopic expression of PGL-1 protein in *mep-1*-depleted embryos (Holdeman et al., 1998; Korf et al., 1998; Paulsen et al., 1995). The MES-2 and MES-6 proteins are also expressed at low levels in intestinal nuclei at later stages (Korf et al., 1998). Perhaps the particularly intense activation of germline-specific genes in the intestine (see Figure 1) reflects this relatively higher level of MES-2 and MES-6 expression. We found that the MES-2, MES-3, MES-4, and MES-6 proteins each exhibited a wild-type abundance and localization in *mep-1(RNAi)* animals (data not shown). Thus, MEP-1 appears to antagonize the activity, rather than the expression, of the MES proteins.

MEP-1 and LET-418 Interact and Function Together in Development

The predicted MEP-1 protein contains seven zinc-finger motifs (Belfiore et al., 2002). Each finger is comprised of a $C(X)_2C(X)_{10\text{-}12}H(X)_4H$ motif except for the third finger, which contains a cysteine residue in place of the terminal histidine. These features and a glutamine-rich sequence between the third and the fourth zinc-fingers are all well conserved in the predicted MEP-1 ortholog of *Caenorhabditis brigsae*, a sister nematode species, and in the protein product of the CG1244 gene in *Drosophila* (Lukacsovich et al., 2001). No other proteins in the current database show significant overall similarity to MEP-1.

To gain further insight into MEP-1 function, we immunoprecipitated a rescuing GFP-tagged MEP-1 protein from *C. elegans* extracts and analyzed associated proteins using MALDI-TOF mass spectrometry. This analysis identified a 280 kDa protein (Figure 3A), identified as the product of the gene *let-418*. LET-418 is a *C. elegans* homolog of Mi-2/CHD3 (von Zelewsky et al., 2000), a core component of the conserved nucleosome remodeling and histone deacetylase (NURD) complex (Taunton et al., 1996).

To determine if LET-418 functions along with MEP-1 in development, we analyzed the phenotype induced by

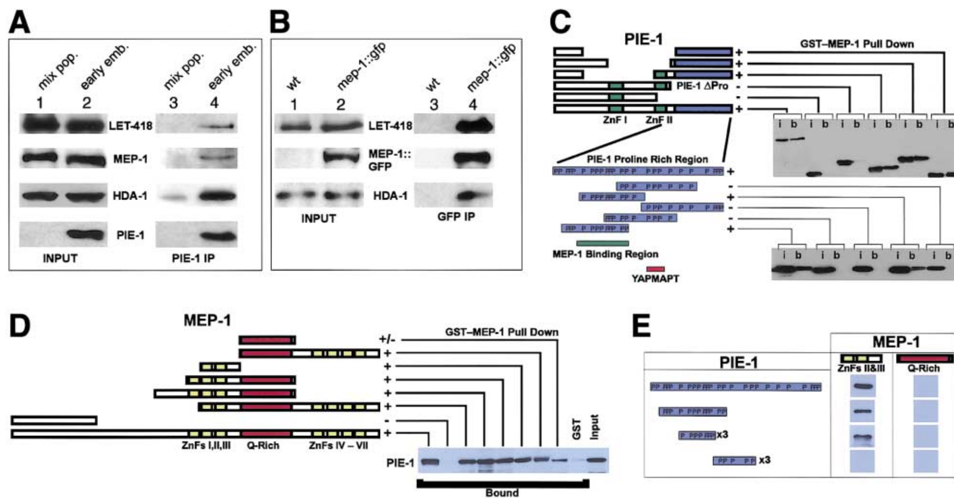


Figure 4. PIE-1 Interacts with LET-418, MEP-1, and HDA-1

(A) Endogenous PIE-1 interacts with LET-418, MEP-1, and HDA-1 in vivo. Extracts from control, mixed-stage populations (mix pop.) that contain undetectably low levels of PIE-1 protein and from early embryo populations (early emb.) that contain abundant PIE-1 were immunoprecipitated using PIE-1-specific monoclonal antibodies. Total lysates (INPUT) and the precipitates (PIE-1 IP) were then subjected to Western analysis and blotted for LET-418, MEP-1, HDA-1, and PIE-1 (as indicated). (B) MEP-1::GFP interacts with LET-418 and HDA-1 in vivo. Extracts from nontransgenic (wt) animals and from animals carrying a rescuing *mep-1::gfp* transgene were immunoprecipitated using GFP-specific monoclonal antibodies. Total lysates (INPUT) and the precipitates (GFP IP) were then subjected to Western analysis and blotted for LET-418 and HDA-1 as indicated. (C–E) In vitro binding and domain analysis of the PIE-1, MEP-1 interaction. (C) PIE-1 binds directly to GST-MEP-1 in vitro. GST pull-down experiments were performed using in vitro translated HA-tagged PIE-1 constructs illustrated in the schematic diagrams. The input (i) and bound (b) fractions were subjected to Western analysis and probed for HA-PIE-1 (as indicated). (D) MEP-1 Zinc-Fingers II and III and a glutamine-rich (Q-rich) region interact with PIE-1 in vitro. The GST-fused truncations and deletions of MEP-1 illustrated in the schematic diagram were tested for their ability to precipitate PIE-1 as described in (C). (E) The proline-rich domain of PIE-1 interacts with Zinc-Fingers II and III of MEP-1. Fragments of MEP-1 comprising fingers II and III and the Q-rich region of MEP-1 were tested for interactions with proline-rich fragments of PIE-1 (as illustrated), including constructs that contain three tandem copies of small segments of the PIE-1 protein.

let-418(RNAi). A previous study reported that the loss of both maternal and zygotic *let-418(+)* activity causes developmental arrest at the L1 stage and that zygotic loss of function results in sterile animals with vulval defects (von Zelewsky et al., 2000). We found that the larval arrest phenotype induced by *let-418(RNAi)* was similar to the phenotype caused by *mep-1(RNAi)*. Consistent with this idea, we found that these larvae exhibited ectopic PGL-1 expression identical to that observed in *mep-1(RNAi)*-arrested larvae (Figures 3B and 3C). Thus, LET-418 appears to function along with MEP-1 to repress germline-specific genes in the soma.

Homozygous *mep-1* and *let-418* mutants also exhibit similar sterile and vulval defects. For example, the homozygous *mep-1* and *let-418* mutants produce malformed vulvae and occasional ectopic pseudo-vulvae (data not shown). The *let-418* gene has been shown to participate in the synMuvB pathway that negatively regulates the induction of primary and secondary vulval cell fates (von Zelewsky et al., 2000). To determine whether *mep-1* acts similarly to *let-418* in this respect, we made double mutants between *mep-1(q660)* and a synMuvA mutant, *lin-15A(n767),* and also between *mep-1(q660)* and *lin-36(n766)*, a synMuvB mutant. One hundred percent of the *mep-1(q660); lin-15A(n767)* double mutant animals exhibited a synMuv phenotype while in contrast, *mep-1(q660); lin-36(n766)* double mutants failed to show extra vulval induction (Figures 3D–3F). These genetic interactions suggest that MEP-1 functions with LET-418 in the synMuvB pathway. Consistent with the finding that MEP-1 and LET-418 function together in both the embryo and larva, antibodies raised against each protein detected similar nuclear proteins expressed in all interphase nuclei throughout development (Figures 3G–3J).

The MEP-1, LET-418, HDA-1, and PIE-1 Proteins Interact

The *C. elegans* HDA-1 protein functions along with MEP-1 and LET-418 in the synMuvB pathway (the present study and Solari and Ahringer, 2000). Furthermore, HDA-1 interacts with the LET-418 homolog, Mi2, in vertebrate cells (Zhang et al., 1998). We therefore decided to ask if PIE-1 interacts with MEP-1, LET-418, and HDA-1 in vivo. PIE-1 monoclonal antibody was used in immunoprecipitation assays on extracts prepared from populations of mixed-stage animals and on populations of early *C. elegans* embryos (Figure 4A). Antibodies raised against MEP-1, LET-418, and HDA-1 were then used to probe Western blots for coprecipitation with PIE-1. We found that all three proteins coimmunoprecipitate with PIE-1 from early-embryo extracts (Figure 4, lane 4). Identical immunoprecipitation assays on extracts prepared from mixed-stage animals where PIE-1 protein itself is undetectable (Figure 4A, lane 1) failed to recover detectable quantities of MEP-1 and LET-418 (Figure 4A, lane 3), indicating that the observed interaction depends on the presence of PIE-1. A small quantity of HDA-1 was recovered from mixed-stage extracts (Figure 4A, lane 3) and could reflect a background of nonspecific immu-

noprecipitation under these conditions or might reflect coprecipitation with the low levels of PIE-1 present in this extract. Based on the efficiency of the IP and the relative amounts of each protein in the input and pellet, we estimate that between 0.5% and 5% of the total MEP-1, LET-418, and HDA-1 coimmunoprecipitates with PIE-1. This is a very significant interaction considering that PIE-1 is present in an average of only one cell (the germline cell) in every 28 cells within the early embryo population used in these assays, while the other proteins are present in all cells.

The MEP-1, LET-418, and HDA-1 sera were not suitable for immunoprecipitation assays. However, Western blots on proteins recovered using anti-GFP antisera to immunoprecipitate MEP-1::GFP confirmed that both HDA-1 and LET-418 interact with MEP-1::GFP in vivo (Figure 4B, see lane 4). It was not possible to analyze the GFP precipitates for PIE-1 protein due to incompatability of the anti-PIE-1 and anti-GFP monoclonal sera.

In order to look for direct interactions between PIE-1 and MEP-1, we carried out an in vitro protein binding assay. We found that in vitro translated PIE-1 can interact with a GST-MEP-1 protein purified from *E. coli* (Figure 4C). This interaction requires a C-terminal proline-rich region of PIE-1 that was both necessary and sufficient for the interaction (Figure 4C). The minimal MEP-1 binding region identified in these assays includes residues 240–268 (Figure 4C). This MEP-1 binding region lies adjacent to, but does not include, the YAPMAPT motif, which has previously been implicated in the transcriptional repression of reporter genes in HeLa cells (Batchelder et al., 1999).

In reciprocal experiments, we identified at least two separate regions in MEP-1 as major sites of PIE-1 interaction (Figure 4D). The first region consists of Zn-fingers II and III, and the second includes the adjacent glutamine-rich region. Further experiments suggest that the proline-rich domain of PIE-1 interacts primarily with the second and third Zn-fingers of MEP-1 (Figure 4E).

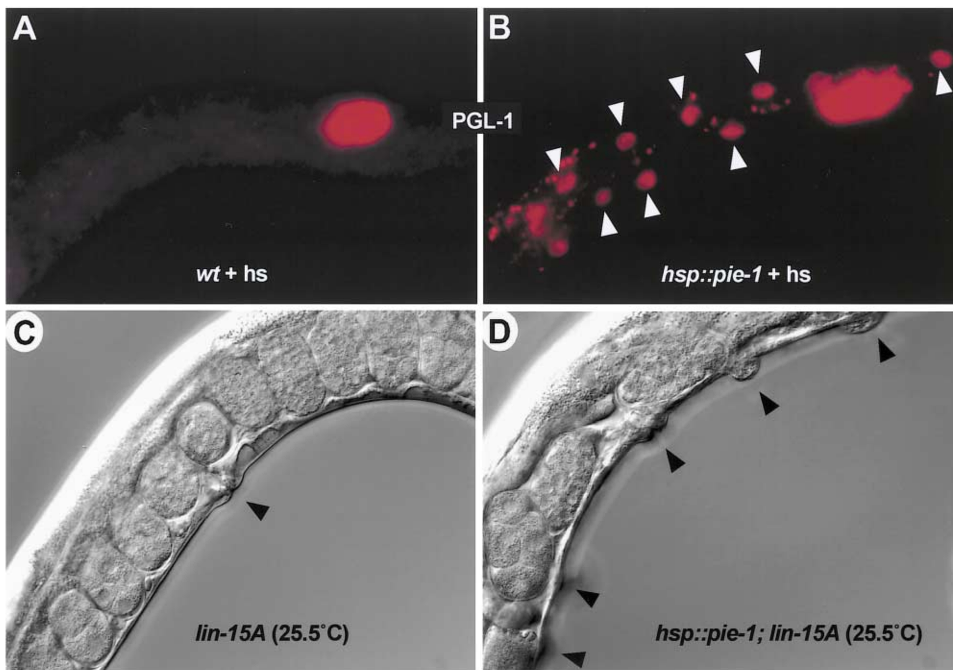
Ectopic Expression of PIE-1 in Somatic Cells Mimics *mep-1* Loss of Function

Loss-of-function mutations in *pie-1* and *mep-1* induce striking and nearly opposite effects on germline-soma distinctions, suggesting that the genes are likely to have an antagonistic relationship. However, the studies described so far leave unresolved the question of what functional significance might underlie the physical interaction between the PIE-1 and MEP-1 proteins. MEP-1 protein localization was not altered in *pie-1* mutant embryos and, vice versa, PIE-1 protein was not mislocalized or expressed ectopically in *mep-1* mutant embryos or arrested larvae (data not shown). Furthermore, *pie-1;mep-1* double mutants exhibit characteristics of both mutants. For example, *mep-1(RNAi);pie-1(zu154)* mothers produce arrested embryos that appear identical to *pie-1* single mutants, producing excess somatic tissues at the expense of germline (Mello et al., 1992, and data not shown). And while the overall terminal phenotype resembles that of *pie-1,* the *mep-1* phenotype of somatic expression of PGL-1 is also observed in the double mutant embryos (data not shown). Thus, our analysis of loss-of-function phenotypes associated with *pie-1* and *mep-1* was not informative about potential interactions between these genes.

We therefore decided to ask if the forced expression of PIE-1 in somatic cells could induce functional consequences consistent with an interaction with MEP-1. We found that PIE-1 protein expressed in somatic cells via the heat-shock promoter (*hsp16-1,* Seydoux et al., 1996) interacts in vivo in coimmunoprecipitation assays with MEP-1 and LET-418 (data not shown). Previous work has shown that the ectopic expression of PIE-1 protein in the somatic cells of early embryos can inhibit transcription, causing embryonic arrest. However, we found that induction of PIE-1 for 2 hr beginning at the bean stage of embryogenesis did not prevent terminal differentiation and morphogenesis in a portion of the embryos. Interestingly, we found that 75% (n = 346) of the resulting hatched larvae had arrested development and appeared phenotypically similar to the *mep-1(RNAi)* and *let-418(RNAi)*-arrested larvae (data not shown). Furthermore, we found that the PGL-1 protein was expressed ectopically in the cytoplasm and nuclei of intestinal cells in 98% (n = 100) of the *hsp::pie-1*-arrested larvae, but not in heat-shock-treated wild-type embryos or larvae (compare Figures 5A and 5B), indicating that forced expression of PIE-1 in somatic cells causes ectopic expression of PGL-1.

However, the ectopically expressed PGL-1 did not accumulate in P granule-like structures at the nuclear periphery, suggesting that the expression of PIE-1 in somatic cells failed to induce other essential components of P granules. Consistent with this view, we found that GLH-2 was not detectable in the *hsp::pie-1* arrested larvae, and a previous report has shown that the localization of PGL-1 to P granules requires *glh-1* and *glh-4* activity (Kuznicki et al., 2000). This difference in the extents to which germline-specific genes were derepressed in the absence of MEP-1/LET-418 and in the ectopic presence of PIE-1 may be due in part to the fact that *hsp::pie-1* can induce transient repression of transcription under these conditions (Seydoux et al., 1996). Nonetheless, these findings are consistent with a model in which the ectopic expression of PIE-1 can at least partially inhibit the function of MEP-1 (and LET-418) in the repression of germline-specific genes.

We next asked if ectopic expression of PIE-1 can induce the synMuv phenotype characteristic of MEP-1, LET-418, and HDA-1 inhibition. To do this, we constructed *lin-15A(n767)* mutant strains that carry either a full-length *hsp::pie-1* transgene or a *pie-1* transgene bearing a deletion of the domain required for MEP-1 binding (*hsp::pie-1*Δ*pro*; see Figure 4C). Strikingly, we found that even with mild heat shock (continuous culture at 23–26°C), a large fraction of the *lin-15A* animals bearing the full-length *pie-1* transgene exhibited a multivulva phenotype (Figures 5C–5E). In contrast, although PIE-1ΔPro was expressed at a level similar to that of full-length PIE-1 in the somatic nuclei of transgenic animals (data not shown), PIE-1ΔPro failed to induce the multivulva phenotype (Figure 5E), even under conditions of strong heat shock (data not shown). We did not observe any change in the abundance or localization of the MEP-1 or LET-418 proteins in *hsp::pie-1* embryos and larvae (data not shown), suggesting that ectopic PIE-1 expres-



E

Ectopic expression of PIE-1 induces a *synMuv* phenotype.

phenotype	temp. (˚C)	% Muv (n)
hsp::pie-1	15	2 (n=54)
hsp::pie-1	25.5	0 (n=64)
lin-15 A (n767)	15	0 (n=120)
lin-15 A (n767)	25.5	0 (n=120)
hsp::pie-1;lin-15A(n767)	21	0 (n=200)
hsp::pie-1;lin-15A(n767)	23.5	46 (n=200)
hsp::pie-1;lin-15A(n767)	25.5	99 (n=200)
hsp::pie-1ΔPro;lin-15A(n767)	21	0 (n=50)
hsp::pie-1ΔPro;lin-15A(n767)	23.5	0 (n=50)
hsp::pie-1ΔPro;lin-15A(n767)	25.5	0 (n=50)

Figure 5. Somatic Expression of PIE-1 Induces the Expression of PGL-1 Protein and a synMuv B Phenotype

(A and B) Immunofluorescence micrographs of wild-type L1 (A) and *hsp::pie-1* L1 larvae (B) stained with the anti-PGL-1 antibody (K76) to detect PGL-1 protein expression (arrowheads). (C–E) Ectopic expression of PIE-1 induces a synMuv phenotype. (C and D) Light micrographs of a *lin-15A(n767)* adult (C) and of a *hsp::pie-1;lin-15A(n767)* adult animals (D). Regions of vulva development are indicated with arrowheads. (E) Tabular representation of the data depicted in (D).

sion inhibits the activity of, rather than the expression of, MEP-1 and LET-418.

Under conditions that induced 99% synMuv animals, strains carrying the *hsp::pie-1* gene produced only low levels of PIE-1 protein, barely detectable by immunofluorescence microscopy (data not shown). When grown continuously under these conditions, the *hsp::pie-1* transgenic strain remained viable and fertile, indicating that PIE-1 does not induce significant transcriptional repression under these conditions. These findings suggest that inhibition of MEP-1 and LET-418 is achieved at relatively low levels of PIE-1 protein, levels that are apparently below the threshold for inducing transcriptional silencing.

PIE-1 Inhibits the Histone Deacetylase Activity of HDA-1

The studies described above suggest that PIE-1 inhibits the function of MEP-1 and LET-418. PIE-1 also interacts with the histone deacetylase, HDA-1. Therefore, we next decided to ask if PIE-1 can inhibit the enzymatic activity of HDA-1. For this purpose, an epitope-tagged HDA-1 was expressed in COS-7 cells, either by itself or together with PIE-1. The HDA-1 protein was then immunoprecipi-

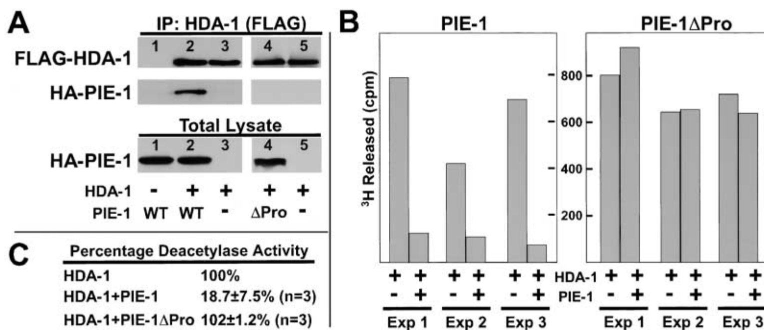


Figure 6. PIE-1 Inhibits HDA-1 Deacetylase Activity in COS-7 Cells

(A) PIE-1 interacts with HDA-1 in COS-7 cells. Extracts prepared from COS-7 cells transfected with combinations of full-length *flag::hda-1*, *ha::pie-1* and a C-terminal truncation of *pie-1, ha::pie-1ΔPro* (ΔP) were immunoprecipitated with anti-FLAG antibody and analyzed by Western analysis for coprecipitation of HA::PIE-1 (as indicated). Note that the total quantity of FLAG-HDA-1 is not reduced in lysates from animals expressing full-length PIE-1 ([A], lane 2). (B–C) PIE-1 inhibits HDA-1 histone deacetylase activity. Equal amounts of HDA-1 protein complex were prepared from three separate transfection experiments, and associated histone deacetylase activity was analyzed using a radio-labeled (^{3}H) acetylated synthetic peptide corresponding to the N terminus of histone H4. (B) The counts per minute (cpm) of radiolabeled acetate released were measured as indicated by the bars. (C) Table indicating the percent deacetylase activity relative to that observed for HDA-1 alone. The values and standard deviations were calculated from the data shown graphically in (B).

tated via the epitope tag. We found that PIE-1 coimmunoprecipitated with HDA-1 in these assays, indicating that PIE-1 can form a complex with HDA-1 in mammalian cells (Figure 6A, lane 2). We found that PIE-1 does not bind HDA-1 in a GST-pull-down assay (data not shown), suggesting that the association of the two proteins may depend on endogenous factors in COS-7 cells that are functionally analogous to MEP-1 and LET-418. Consistent with this view, we found that the PIE-1 protein lacking its C-terminal region, which mediates its interaction with MEP-1, does not coimmunoprecipitate with HDA-1 in these assays (Figure 6A, lane 4).

In order to analyze the histone deacetylase activity of HDA-1, we subjected approximately equal amounts of the immunoprecipitated HDA-1 protein to a histone deacetylase assay, using an acetylated synthetic peptide corresponding to the N terminus of histone H4. As expected, we found that HDA-1 exhibits sodium butyrate-sensitive deacetylase activity when prepared from extracts containing no PIE-1 (Figure 6B). In contrast, samples immunoprecipitated in the presence of full-length PIE-1 protein showed significantly reduced deacetylase activity (Figures 6B and 6C), indicating that PIE-1 inhibits the histone deacetylase activity of HDA-1. The truncated form of PIE-1 (PIE-1ΔPro), which does not coimmunoprecipitate with HDA-1, had no effect on HDA-1 histone deacetylase activity (Figures 6B and 6C), suggesting that the proline-rich region of PIE-1 and/or the ability of PIE-1 to interact with HDA-1 are important for PIE-1's inhibitory function.

The expression of PIE-1 did not alter the levels of HDA-1 protein or of endogenous β-actin in COS-7 cells (Figure 6A, lane 2, and data not shown). Cells expressing PIE-1 appeared morphologically normal, and the overall abundance of proteins was similar between protein extracts prepared from PIE-1-expressing cells and nonexpressing cells. Taken together, these findings suggest that PIE-1 does not induce a general repression of transcription in COS-7 cells under these conditions and further support the model that PIE-1 interacts with and inhibits HDA-1 activity.

Discussion

Here, we have shown that the nuclear C2H2 zinc-finger protein MEP-1 inhibits the expression of germ-plasm components in somatic cells of *C. elegans* embryos and larvae. Embryos lacking MEP-1 protein complete embryogenesis but arrest development shortly after hatching and begin to express gene products normally restricted to the germline. MEP-1 functions along with LET-418, a *C. elegans* homolog of Mi-2/CHD3. Mi-2 and CHD3 belong to a family of highly conserved chromodomain proteins implicated in chromatin remodeling and transcriptional repression in eukaryotes and function as core components of the nucleosome remodeling and histone deacetylase (NURD) complex (Zhang et al., 1998). In addition to LET-418, we have shown that MEP-1 also interacts with HDA-1 the *C. elegans* ortholog of HDAC-1, a conserved histone deacetylase and NURD complex component (Taunton et al., 1996).

Regulation of Stage-Specific Transcription by MEP-1, LET-418, and the MES Proteins

Our genetic studies suggest that the repressive functions of MEP-1 and LET-418 in preventing the expression of germline genes in somatic cells antagonize positive inputs from MES-2, MES-3, MES-4, and MES-6. These MES proteins are *C. elegans* homologs of the PcG and TrxG groups of chromatin regulators (reviewed in Pirrotta, 2002). Taken together, our findings suggest an intriguing model for the developmental interactions between MEP-1, LET-418, and the MES proteins (Figure 7). According to this model, stage-specific patterns of chromatin organization are established sequentially within each cell lineage in the developing animal through the concerted action of transcriptional activators and repressors. Maintenance of these domains in the germline is controlled at least in part through the action of the MES proteins, while other PcG- and TrxG-related proteins may serve this function in other tissues. The MEP-1 and LET-418 proteins are proposed to function along with HDA-1 and, perhaps, with other components of the NURD complex at or after the onset of succeeding differentiation events to modify the distribution of these maintenance factors and thereby to allow the stable specification of new stage-specific chromatin domains (Figure 7).

This model can also explain the role of MEP-1, LET-418, and HDA-1 in regulating the competency of vulval precursor cells (VPCs). The MES proteins are not expressed in the VPCs and their inhibition does not sup-

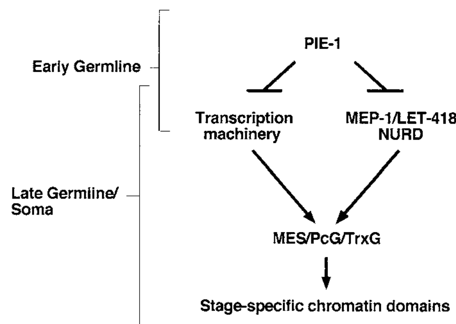


Figure 7. Model

A model to explain the interactions between PIE-1, MEP-1, LET-418, and the MES proteins. During early embryogenesis PIE-1 negatively regulates both transcription and chromatin remodeling in order to counteract the influence somatic transcription factors present in germline cells during this time. In somatic cells and after PIE-1 is gone in the germline, the concerted action of the transcriptional machinery and the MEP-1/LET-418 complex modify chromatin and concomitantly modify the distribution of chromatin-associated proteins including the MES proteins and possibly other PcG and TrxG related proteins, to establish new stage-specific chromatin domains. Arrows are shown converging on MES/PcG/TrxG proteins to indicate that the above mechanisms act through these factors. However, specific interactions are expected to be both positive and negative as some chromatin domains are rendered active and others silent.

press the synMuv phenotype of *mep-1* (data not shown). Thus, we propose that when the VPCs are specified during the L1 stage, other chromatin-maintenance factors, perhaps including additional PcG- and TrxG-related proteins (Chamberlin and Thomas, 2000; Holdeman et al., 1998; Korf et al., 1998; Xu and Strome, 2001), establish a stable chromatin conformation that is competent to respond to vulval induction and to execute vulval differentiation. When the gonad signals the VPCs to initiate vulval differentiation, synMuvB components, including MEP-1, LET-418, and HDA-1, are either down-regulated in the three VPCs nearest the gonadal signal or are activated in more distal VPCs. SynMuvB activity in the three VPCs not selected to undergo vulval development inactivates the previously set chromatin-based potential to undergo vulval differentiation and ensures that these cells instead undergo the alternative pathway of fusion with the hypodermal syncytium. Thus, just as embryonic somatic cells require MEP-1 and LET-418 to stably inactivate germline potential, we propose that the activities of MEP-1 and LET-418 and, by extension, other synMuvB proteins including HDA-1 are required during larval development for VPCs to stably inactivate the potential to undergo vulval differentiation.

Thus, chromatin remodeling by MEP-1, LET-418, and HDA-1 could function through PcG- and TrxG-related proteins to sequentially erase and establish new differentiation competent chromatin domains throughout development. The interplay between these activities may be of special importance when founder cells are specified many hours before they initiate terminal cell-fate-specific differentiation, as is true for both the germline precursors and the vulval precursors.

A Role for PIE-1 in Regulating MEP-1, LET-418, and HDA-1

The PIE-1 protein is localized to germline cells where it functions to prevent somatic development during early embryogenesis. Here, we have shown that endogenous PIE-1 protein interacts with MEP-1, LET-418, and HDA-1 in vivo in early *C. elegans* embryos and also interacts directly with MEP-1 in in vitro protein binding assays. The finding that PIE-1, which functions in global repression of transcription in early germline cells, interacts with components of a conserved histone deacetylase complex (the NURD complex), raises the question of whether PIE-1 recruits this complex to mediate transcriptional repression in the germline. However, the genetic evidence does not support such a model. Inactivation of *mep-1, let-418* (the present study), and of *hda-1* (Shi and Mello; 1998) does not inhibit germline specification or lead to ectopic somatic differentiation in the early embryonic germline as would be expected if PIE-1 acts through these factors to repress transcription in the germline. In contrast, as discussed above, inhibition of *mep-1* and *let-418* leads to ectopic germline gene expression in somatic cells.

Our findings instead support a model in which PIE-1 transiently inhibits MEP-1, LET-418, and HDA-1 activities to prevent chromatin remodeling in the germline and to thus insure the proper activation of germline gene expression during later development. The embryonic germ cells, Z2 and Z3, are completely absent in *pie-1* mutants due to PIE-1's earlier function in preventing somatic gene expression in early germline blastomeres (Mello et al., 1992). Therefore, it is not possible to directly examine the genetic relationship between *pie-1* and *mep-1* in the control of gene expression in Z2 and Z3. Instead, we investigated the consequences of the ectopic expression of PIE-1 at later developmental times. Consistent with a negative regulatory relationship, we found that the inhibition of MEP-1 and LET-418 activities and the forced expression of PIE-1 in somatic cells caused similar phenotypes—the derepression of germline-specific genes and the induction of ectopic vulval cell fates. Finally, we have shown that PIE-1 interacts with the histone deacetylase, HDA-1, and significantly reduces its activity when both proteins are coexpressed in vertebrate cells.

Taken together, the above findings are consistent with a model in which PIE-1 inactivates a complex that contains MEP-1, LET-418, and HDA-1 in early germline cells (Figure 7). According to this model, PIE-1 protects the germline not only from soma-specific transcription (Seydoux et al., 1996), but also from soma-specific chromatin remodeling. At later times in the germline, once the factors that promote somatic differentiation are no longer present in germline cells, PIE-1 is no longer needed to inhibit MEP-1 and LET-418, allowing them to function along with the MES proteins and other germline-specific regulators and chromatin maintenance factors to promote germline developmental events such as mitosis, meiosis, and gametagenesis.

MEP-1 and LET-418 May Have Additional Functions in the Germline after PIE-1 Disappears

Both MEP-1 and LET-418 are required for fertility and although PIE-1 is proposed to inactivate them during

early embryogenesis, as discussed above, at later times they may be required for proper germline development. At these later times, MEP-1 and LET-418 may function in chromatin remodeling in the germline, and consistent with this idea, a recent report suggests that the X chromosome undergoes changes in histone modification indicative of chromatin remodeling during early to mid pachytene of meiosis in adult hermaphrodites (Kelly et al., 2002).

Interestingly, a recent report has identified MEP-1 as a binding partner with the putative DEAH-box RNA-helicase proteins MOG-1, MOG-4, and MOG-5 that are critical for the translational regulation of the *fem-3* 3′ untranslated region (Belfiore et al., 2002). A related helicase has been implicated in transgene and transposon silencing in Chlamydomonas (Wu-Scharf et al., 2000). Thus, another intriguing possibility is that MEP-1 and, perhaps, other components of the MEP-1 complex function in both transcriptional and posttranscriptional repression. PIE-1 itself may have roles in both transcriptional and translational control of germline gene expression (Tenenhaus et al., 2001). The study of RNAi and germline transgene silencing has also drawn intriguing connections between posttranscriptional and transcriptional silencing mechanisms in *C. elegans* (Tabara et al., 1999). And posttranscriptional and transcriptional silencing phenomena are clearly connected to one another in both plants and animals (reviewed in Baulcombe, 2002; Dernberg and Karpen 2002). However, the specific nature of the connection remains unknown. Thus, in the future, it will be interesting to learn if proteins like MEP-1 and PIE-1 function at the interface between transcriptional and posttranscriptional gene-silencing pathways.

Conservation of Mi-2 Function in Maintaining Germline-Soma Distinctions

Finally, it is interesting to note that the *Arabidopsis* homolog of Mi-2, *pickle (pkl)*, exhibits a mutant phenotype similar to that reported here for its homolog, *let-418* (Eshed et al., 1999; Ogas et al., 1997). *pkl* is required for the suppression of embryonic characteristics in root meristem cells. In fact, the root tissues in *pkl*-mutant plants spontaneously generate new embryos and entire new plants (Ogas et al., 1997). Thus, in *Arabidopsis*, Mi-2-related proteins appear to function not only in the maintenance of somatic differentiation, but also indeed in the suppression of totipotency, the potential to make a whole new organism. Conceivably, the germline-like somatic cells in *mep-1(RNAi)*-arrested and *let-418(RNAi)*-arrested larvae have the potential to act as true germline cells but lack gonadal signals and supportive functions required for gametogenesis. If this role for Mi-2 is conserved in vertebrates, transient inhibition of Mi-2 could prove useful as a means for preventing stem cells from adopting differentiated fates or perhaps for resetting cells to more immature stem-cell fates.

A long-standing question in developmental biology centers on where the specificity lies for differences in the cell-type-specific or stage-specific outcomes of developmental signaling pathways. Our findings suggest that the interplay between chromatin modifiers and chromatin maintenance factors can help explain this specificity. In *C. elegans*, PcG- and TrxG-related proteins appear to maintain latent transcriptional potential. MEP-1, LET-418, and HDA-1 in turn appear to act through these PcG- and TrxG-related proteins to alter this transcriptional potential and, thus, to prepare chromatin to respond appropriately to future differentiation signals. It will be important to learn the molecular details that underlie these regulatory interactions and to learn whether similar relationships exist between homologous factors in other organisms.

Experimental Procedures

Strains

The mutations and balancer chromosomes used in this study are listed by chromosomes as follows: *LGI*: *mes-3(bn35), dpy-5(e61), sDP2(I,f)*; *LGII*: *mes-2(bn11), unc-4(e120), mnCI*; *LGIII*: *lin-36(n766), unc-32(e189)*; *LGIV*: *mep-1(q660), mes-6(bn66), dpy-20, DnTI (IV;V)*; *LGIV*: *mes-4(bn23), dpy-11(e224), unc-76(e911)*; *LGX*: *lin-15A (n767), lin-15AB (e1763)*. The bristol strain N2 was used as the wild-type strain. *mep-1(q660)* and *lin-15AB(e1763)* were kindly provided by A. Puoti (University of Fribourg) and R. Horvitz (Massachusetts Institute of Technology), respectively.

Transgene Construction and DNA Transformation

The *mep-1::gfp* and *pgl-1::gfp* transgenes were constructed in yeast artificial chromosomes (YACs) as described in Rocheleau et al. (1999). Briefly, a GFP cassette containing the yeast selectable marker, sup4°, inside a synthetic *C. elegans* intron was inserted in frame just before the stop codon into a ~1 kb 3′ fragment of each *C. elegans* gene cloned in bacterial vectors. The fusion gene fragments were then excised from the plasmid DNA as linear molecules and were used to tranform yeast bearing the corresponding *mep-1* and *pgl-1* YACs, Y51D1, and Y43B11 respectively. Total yeast genomic DNA was purified (as described previously, Rocheleau et al., 1997) from each recombinant yeast strain and coinjected at a final concentration of 200 μg/ml yeast DNA and 100 μg/ml of the marker plasmid pRF4. Approximately one in five transgenic worm lines exhibited GFP expression. As described previously (Rocheleau et al. 1999), the use of a YAC as a transgene vector partially prevents epigenetic silencing of the GFP transgene in *C. elegans* germ cells.

Two-Hybrid Screen and RNAi

The full-length PIE-1 protein was fused to the GAL4 DNA binding domain in the vector pAS1 and transformed into the yeast host strain AH109 (James et al., 1996), obtained from CLONTECH (Palo Alto, CA). Screen was carried out according to the manufacturer's instructions. The *C. elegans* cDNA libraries made in the vector pACT were a generous gifts from R. Barstead (Oklahoma Medical Research Foundation) and Z. Zhou (Baylor College of Medicine). A total of 20 PIE-1 binding proteins were isolated and examined by RNAi. MEP-1 (GenBank Accession AAL27004) corresponds to a predicted product of M04B2.1 and was represented by ten independent clones obtained in this screen. RNAi was performed by microinjection of dsRNA into young hermaphrodite adults as previously described (Rocheleau et al., 1997).

The L1 arrest phenotype induced by RNAi was compared to the phenotype of *mep-1(q660)*. Althougth *mep-1(q660)* animals were completely sterile, rescued strains that carry an extrachromosomal transgene (described above) were found to segregate mosaic animals that produce germ cells completely lacking the MEP-1 activity. Adults with mosaic germlines segregated approximately 10% arrested larvae with a phenotype identical to that of *mep-1(RNAi)*.

Antibody Production, Immunoblotting, and Immunofluorescence Staining

Antibodies specific for MEP-1 and LET-418 were produced by subcutaneous injection of bacterially produced and purified GST-MEP-1 into rabbits and GST-LET-418 proteins into rats, respectively (Alpha Diagnostics, San Antonio, TX). The resulting antibodies were purified using GST-MEP-1 or GST-LET-418 as an affinity matrix. The affinity-purified anti-MEP-1 and anti-LET-418 antibodies (used at 1:100–1:250 dilutions) recognize endogenous worm proteins of approximately

110 kDa and 280 kDa in size, respectively. Affinity-purified sera were used for immunofluorescence staining at a 1:100 dilution. Embryos and larvae were permeablized for staining by a freeze-crack method and fixed in methanol/acetone according to the standard procedures (Epstein et al., 1988). Mouse monoclonal anti-PGL-1 antibodies (Kawasaki et al., 1998) and chicken anti-GLH-2 and anti-GLH-3 antibodies (Gruidl et al., 1996) were kindly provided by S. Strome (University of Indiana) and K. Bennett (University of Missouri), respectively.

In Situ Hybridization

In situ hybridization was performed essentially according to the method of Tabara et al. (1996). (http://watson.genes.nig.ac.jp/db/method/insitu_larvae.html) with the following modifications: after proteinase K digestion, the slides were incubated first in 0.1 M triethanolamine (pH 8.0) for 2 min, then in 0.15% acetic anhydride, 0.1 M triethanolamine (pH 8.0) for 10 min. The slides were then washed twice with PBT (PBS, 0.1% Tween-20) for 2 min each before the fixation with formaldehyde. To generate large quantities of MEP-1-depleted larvae, homozygous *mep-1(q660)* animals carrying the rescuing *mep-1::gfp* transgene were subjected to RNAi by feeding bacterial strain, which expresses dsRNA for GFP (a kind gift from A. Fire [Carnegie Institute of Washington]).

Immunoprecipitation and Mass Spectrometry

Embryos or mixed populations of worms were homogenized in the buffer containing 25 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 1 mM EDTA-NaOH, 1 mM DTT, 0.5% Triton X-100, 10% glycerol, and protease inhibitor cocktail (Amersham Biosciences, Piscataway, NJ), using the method described in Rocheleau et al. (1999). Approximately 3.5 mg of protein were used for immunoprecipitation, using the mouse monoclonal anti-GFP antibody 3E6 (Qbiogen, Carlsbad, CA) or the mouse monoclonal anti-PIE-1 antibody P4G5 (Mello et al. 1996). For mass spectrometry, the immunoprecipitated proteins were resolved on a 6% SDS/PAGE gel and visualized by silver staining. The bands of interest were excised and analyzed by MALDI-TOF mass spectrometry at the core protein facility at the University of Massachusetts Medical Center.

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