2003-08-22

Conserved Nucleosome Remodeling/Histone Deacetylase Complex and Germ/Soma Distinction in *C. elegans*: A Dissertation

Yingdee Unhavaithaya
University of Massachusetts Medical School

CONSERVED NUCLEOSOME REMODELING/HISTONE DEACETYLASE COMPLEX AND GERM/SOMA DISTINCTION IN *C. ELEGANS*

A Dissertation Presented

By

YINGDEE UNHAVAITHAYA

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester, in partial fulfillment of the requirements for the degeree of

DOCTOR OF PHILOSOPHY

August 22, 2003

CELL BIOLOGY

COPYRIGHT INFORMATION

Approval Page

CONSERVED NUCLEOSOME REMODELING/HISTONE DEACETYLASE COMPLEX AND GERM/SOMA DISTINCTION IN *C. ELEGANS*

A Dissertation Presented

By

Yingdee Unhavaithaya

Approved as to style and content by:

_____

Y. Tony Ip, Chair of Committee

_____

Anthony Imbalzano, Member of Committee

_____

Charles Sagerstrom, Member of Committee

_____

Stephen Doxsey, Member of Committee

_____

Sander van den Heuvel, Member of Committee

_____

Craig C. Mello, Dissertation Mentor

_____

Anthony Carruthers, Dean of the Graduate
School of Biomedical Sciences

Department of Cell Biology

ACKNOWLEDGEMENTS

Dr. Craig Mello has been a great advisor and I thank him for his patience, his teaching, and for holding me to a high standard. Similarly, I want to thank the past and present member of the Mello lab for their support and advice. I also would like to acknowledge the people whose contributions are vital to the work featured in this thesis. Many antibodies were received from various laboratories. Susan Strome and Karen Bennett provide P-granules antibody used through out the thesis. The Shin lab provided LET-418 antibody. Yuji Kohara provided cDNA clones which we used for making dsRNA for RNAi. Geraldine Seydoux provided *hsp::pie-1* and the *pes-10::gfp* strain for the analysis of PIE-1 over expression (chapter three) and characterization of *mep-1* (chapter two), respectively. The identification of LET-418 was done in collaboration with John Leszyk of the University of Massachusetts Proteomic Mass Spectrometer Lab. Many worm strains were obtained from the C. elegans Genetics Center, which is funded by a grant from the National Institutes of Health National Center for Research Support. mep-1(q660) strain was given as a gift from A. Puoti, J. Kimble, and B. Barstead. The Shin Lab performed the histone deacetylase assay in chapter three as well as the MEP-1/PIE-1 *in vitro* binding data. In addition, they made the *pie-1;lin-15A* double used in the *pie-1* overexpression assay (chapter III). I also want to thank Tae Ho Shin for teaching and providing supervision during his stay in the Mello lab

Lastly, I would like to thank my girlfriend, Gisela, her sisters, and her parents, for their kindness and help. Most importantly, I want to thank my father, my mother, and my two brothers for their understanding of my career choice, as well as their support.

ABSTRACT

A rapid cascade of regulatory events defines the differentiated fates of embryonic cells, however, once established, these differentiated fates and the underlying transcriptional programs can be remarkably stable. Here, we describe two proteins, MEP-1, a novel protein, and LET-418/Mi-2, both of which are required for the maintenance of somatic differentiation in *C. elegans*. MEP-1 was identified as an interactor of PIE-1, a germ-specific protein required for germ cell specification, while LET-418 is a protein homologous to Mi-2, a core component of the nucleosome remodeling/histone deacetylase (NuRD) complex. 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. We demonstrate that PIE-1 forms a complex with MEP-1, LET-418, and HDA-1. Furthermore, We show that the overexpression of PIE-1 can mimic the *mep-1/let-418* phenotype, and that PIE-1 can inhibit the Histone deacetylase activity of the HDA-1 complex in COS cells. Our findings support a model in which PIE-1 transiently 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.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLE

ABBREVIATIONS

PGCS	Primordial germ cells
pgl	P-granule less
glh	germline helicase
pie	pharynx and intestinal excess
mex	muscle excess
pos	posterior localization/posterior lineage defective
gld	germline differentiation abnormal
skn	**skin** (hypodermis) in excess
gcl	germ cell less
tra	sexual transformer
apx	anterior pharynx in excess
alp	abnormal localization of PIE-1
ubc	ubiquitin conjugating enzyme
Su(HW)	Suppressor of Hairy Wing
mep	mog and ectopic P-granule
YAC	yeast artificial chromosome
RbAp	Rb associated protein
Rb	Retinoblastoma
Mi	Dermatomyositis
let	lethal
mes	maternal effect sterile
lin	lineage
hox	homeobox
EED	embryonic ectoderm development
NURF	nucleosome remodeling factor
RSC	remodeling the structure of chromatin
SWi/SNF	SWitch/Sucrose Non Fermentation mutations

BRM	Bhrama
CHRAC	Chromatin accessibility complex
SAP	Sin3 associated peptide

CHAPTER I

INTRODUCTION

In mammals, the fusion of oocytes and sperm, both of which are haploid gametes, will form the diploid zygote and start the next generation of the organism. The mammalian germ cells undergo cell division to form gametes and are distinguished from all the other types of cells, collectively called the somatic cells, by these criteria: 1.) The germ cell can undergo a specialized cell division called meiosis in which the chromosome number is reduced by half in the daughter cells; 2.) The germ cell has the potential to generate all cell types, termed totipotency (Wylie, 1999); 3.) The germ cell is the only cell which can transmit the genetic material of the parents to the progeny. Thus, the germ cell is the vehicle that facilitates the transfer of inheritable diseases. The study of the germ cell may therefore provide us with insights into the mechanism through which organisms propagate, and may also lay the foundation for the future treatment of sterility and genetic disorders.

The distinction of the germ cells from the somatic cells

A long standing question in biology is how, in various animals including mammals, the pluripotent germ cells are distinguished from the somatic cells. To ask this question, it is important to know the origin of the germ cell during the embryogenesis of respective organisms. In *C. elegans*, the origin of the germ-lineage cell can be traced as far back as one-cell stage of the *C. elegans* zygote (Riddle, 1997). The founder of germ

cells is the P4 cell which divides to generate Z2 and Z3 cell, together called the primordial germ cells. The *C. elegans* germ-cell lineage is illustrated in Figure 1, p.3. In *X. laevis*, primordial germ cells (PGCs), a group of cells considered founders of germ cells, are first identified near the vegetal pole of the 32-cell embryo (Ginsburg et al., 1990). After the fertilization of the oocyte, the four vegetal-pole blastomeres at the 32-cell stage divide asymmetrically, each giving rise to one germ cell and one somatic cell (Whitington and Dixon, 1975). In *D. melanogaster*, early embryos undergo synchronized nuclear division to form a syncitial blastula in which the nuclei share a common cytoplasm. PGCs in *D. melanogaster* are formed in the posterior region of the embryo, and are the first cells to cellularize at the end of the syncitial blastula stage. (Sonnenblick, 1950).

What are the factors which confer germ cells their pluripotency? In many organisms, the specification and function of the germ cells depend on specialized, germ-specific cytoplasm called the germ plasm. The germ plasm is assembled from germ cell-specific factors which the mother deposits into the oocytes. This specialized cytoplasm is localized to the area in the embryos where germ cells will eventually form (Beams and Kessel, 1974; Eddy, 1975). The exact composition of the germ plasm is still a mystery but the function of the germ plasm may possibly be linked to distinctive granules, called the germ granules, which localize specifically to the germline. Germ granules are defined by electron-dense, fibrous particles when examined under the electron microscope. In *C. elegans*, studies show that the germ granules contain RNAs, proteins,

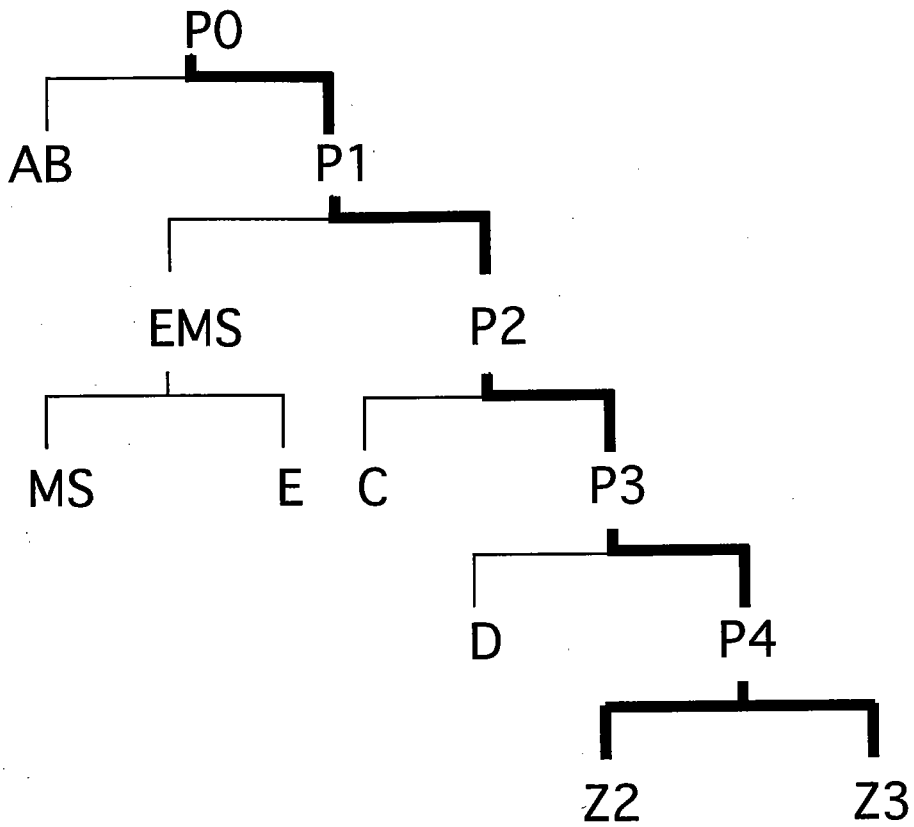


Figure 1

Figure 1. Germ Cell lineage in *C. elegans*.

Early embryonic lineage in *C. elegans*. This tree diagram shows the series of divisions (horizontal lines) of the zygote (P0) into somatic (AB, EMS, C, D) and germline (P1, P2, P3, P4) blastomeres, and the division of P4 into Z2 and Z3 primodial germ cells. Thick lines depict germ-lineage cells.

and are often associated with the germ nuclei (Pitt et al., 2000; Schisa et al., 2001; Seydoux and Fire, 1994; Strome and Wood, 1983). The germ granules are called germinal granules in *X. laevis*, polar granules in *D. melanogaster*, and P-granules in *C. elegans*.

The examination of *Drosophila* mutants with defective germline reveal that the germ cell specification is initiated by genes expressed during oogenesis. Such genes are exemplified by a number of maternal genes (*oskar*, *vasa*, *tudor* , *valois*, *cappuccino*, *spire*, *staufen*, *mago nashi*, *orb*, *homeless*, and *pipsqueak*) (Lehmann and Ephrussi, 1994) which are required for polar granules assembly. Interestingly, the products encoded by these genes have themselves been shown to be polar granule components (Rongo and Lehmann, 1996). It is therefore conceivable that polar granules, and by extension the germplasm, are required for the specification of the germ cell fate. An evidence which suggests that the polar granule can specify the germ cell fate comes from the study of OSKAR, a protein which localizes to the posterior of the embryos and is required for the formation of PGCs (Lehmann and Nusslein-Volhard, 1986). In *Drosophila*, the expression of *oskar* at the anterior region of the embryo can ectopically assemble pole cells which are capable of generating functional germline. (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992). Moreover, the number of primordial germ cells generated by the ectopic expression of *oskar* is correlated to the number of *oskar* copies in the mother (Ephrussi and Lehmann, 1992; Smith et al., 1992). Thus, *oskar* is essential for the formation of the germ cell and is sufficient to initiate all steps required for ectopic germ cell formation. How does the expression of *oskar* instruct the germ cell to be

formed? One likely hypothesis is one in which *oskar* is the nucleus around which the other polar granules assemble, and in the process induce germ cell formation. It is yet unclear through which mechanism OSKAR functions.

In *C. elegans*, the germ plasm segregated along with the germ cell precursor as the zygote undergoes asymmetric cell division to generate somatic cell and another germ cell precursor. Asymmetric division will eventually stop when the germ cell precursor divides symmetrically, distributing their germ plasm to both Z2 and Z3 cells. The proteinaceous components of P-granules can be roughly categorized as constitutive or transient. Constitutive components localize to the P-granules throughout the life cycle of the worm while transient components localize briefly to the P-granules in the early embryos.

Known constitutive components of the P-granules are encoded by the gene *pgl-1*, *glh-1*, *glh-2*, *glh-3*, *glh-4*, and are required for postembryonic gonadal development. (Gruidl et al., 1996; Kawasaki et al., 1998; Kuznicki et al., 2000; Roussell and Bennett, 1993) . *glh* genes encode homologs of *vasa* while *pgl-1* encodes an RGG-box RNA binding protein. These genes are required for proper P-granule assembly and mutations in *glh-1* or *pgl-1* results in temperature-sensitive sterility in which the germline is underproliferated and gametogenesis is perturbed, as well as defect in P-granule assembly (Kawasaki et al., 1998). Interestingly, work by Schissa et.al. suggests that PGL-1 is functioning in mRNA transport from the nucleus into the P-granule (Schisa et al., 2001). This observation implies that the mRNA export factors might interact with the PGL and GLH proteins. Consistent with this hypothesis, mRNA transport in *D. melanogaster*

appears to involve a subcellular structure called the sponge bodies which is implicated in the possible assembly and transport of RNA protein complex . Sponge bodies surround the nuage, a possible polar granule precursor (Wilsch-Brauninger et al., 1997).

Numerous studies have been conducted on the transient protein components of P-granules. These components are encoded by the gene *pie-1*, *mex-1*, *mex-3*, *pos-1*, *gld-1*, and are required for cell polarity and cell fate determination (discussed below). The significance of the association of these proteins to P-granules is not clear.

Data from previously mentioned organisms suggest that the specification of the germline relies on the localization of the germplasm. In contrast, germ cell specification in mammalian embryo appears to depend on positional cues (Ginsburg et al., 1990; Saffman and Lasko, 1999; Tam and Zhou, 1996; Wylie, 1999). In mammalian embryos, all the cells during the first few cleavages retain their totipotency (Gardner and Rossant, 1979; Lawson and Hage, 1994; Snow, 1981). As the mammalian embryos proceed through gastrulation, the capacity to form germ cell are gradually lost from the somatic cells and the totipotency is restricted to only germ cells (Lawson and Hage, 1994; Snow, 1981). Despite this difference, similarity exists between mammals and other organisms in how the germ cells are specified. Although germ granules or germ plasm are not yet identified in mammals, the successful cloning of various mammals such as mice and sheep indicates that the cytoplasm of mammalian germ cells contain the necessary instruction required for the germ cell development (Campbell et al., 1996; Wakayama et al., 1998). Similarly, the transplantation (Ikenishi, 1987; Illmensee and Mahowald, 1974) of the somatic nuclei of *X. laevis* into eunucleated eggs can produce a new organism with

functional germline (Gurdon, 1974). This likely existence of the cytoplasmic germ cell determinants in the germ cell progenitor, and the inheritance of these determinants by the daughter cells, indicate that the mammalian germline specification may be similar to that in *Drosophila, C. elegans*, and *Xenopus.*

The maintenance of the germ cell

In addition to pluripotency, the germ cell also exhibits an intriguing ability to remain undifferentiated during embryogenesis. Although the *C. elegans* germ cell precursor has the potential to develop into various tissues (Laufer et al., 1980), the germ cell precursor and the primordial germ cell are kept undifferentiated while all the other cells in the embryos differentiate to form somatic tissues. Upon the proper signal to initiate gonadogenesis, the primordial germ cell will proliferate to form the gonad syncitium (Saffman and Lasko, 1999). The molecular mechanism which functions to keep the germ cell undifferentiated during embryogenesis is presently not clear, though evidence in *D. melanogaster* and *C. elegans* suggests that this mechanism is accomplished at the transcriptional level. First, transcription in the germ cell appears to be inhibited during early embryogenesis. The somatic cells of *D. melanogaster* start to incorporate [^{3}H]UTP, an indication of active transcription, at around stages 3-4 of the embryo as opposed to the initiation of transcription by the germline around the beginning of stage 28 embryo (Edgar et al., 1994; Lamb and Laird, 1976; Zalokar, 1976). In the *C. elegans* embryo, the incorporation of [^{3}H]UTP starts as early as the 16 cell embryo

(Edgar et al., 1994). However, an examination of a collection of genes shows that the expression of any embryonic gene is repressed in the germline cells, with the exception of the RNApolI-dependent rRNA genes which has been shown to be active in both somatic and germline cells (Seydoux and Dunn, 1997; Seydoux et al., 1996). Additional evidence that the germ cell quiescence is regulated at the transcriptional level is a correlation between the repression of gene expression and the absence of the activated form of the C-terminal domain (CTD) of the RNApolII large subunit (Seydoux and Dunn, 1997). The phosphorylation of the RNApolII CTD has been correlated with transcription elongation (Dahmus, 1996) and the RNApolII phospho-CTD-specific epitope can detected in the somatic blastomeres as early as the 4-8 cell stage of the *C. elegans* embryo, while the germ cell expression of the phospho-CTD epitope starts at the 100-cell stage (Kobayashi et al., 1996). Moreover, the germline repression of RNApolII-dependent gene expression in *C. elegans* can prevent the activity of the transcription activator SKN-1 which is present in the germ cell (Mello et al., 1992). Similarly, the expression of a strong transcriptional activator VP16 in *Drosophila* can not activate transcription when targeted in the pole cell (Van Doren et al., 1998). These data suggest a global repression of RNapolII-dependent transcription in the germ cell as a mechanism through which the germ cell achieves quiescence. A good example for the evidence of this mechanism is demonstrated by mutations in the *C. elegans* gene *pie-1*. Mutations in *pie-1* result in the derepression of embryonic gene expression in the germ line, resulting in the germ line cell adopting a somatic cell fate (Mello et al., 1992).

Genes required for the distinction of the *C.elegans* germ cell

In *C. elegans*, the maternally contributed factors are required for a range of functions, from the specification of the body patterning and cell fate specification to the localization of other maternal factors (Bowerman et al., 1992; Gotta and Ahringer, 2001; Kemphues, 2000; Mello et al., 1992; Priess, 1994; Schubert et al., 2000). Mutagenesis screens for embryonic defects in axis polarity and patterning yield mutations in many maternal genes, among which is *pie-1*. *pie-1* mutations cause the germ cell precursor, P2, to adopt the somatic fate of its sister cell, EMS. *pie-1* loss-of-function mutation results in embryos which produce extra pharynx and intestinal tissues at the expense of the germ cells. This pharynx/intestinal tissue differentiation by the germ cell is due to the transcriptional activity of bZIP domain transcription factor SKN-1 which is needed for the induction of the pharyngeal and intestinal tissue (Mello et al., 1992). Thus, *pie-1* mutant phenotype indicates that wild-type *pie-1* activity is required to protect the germ cell from *skn-1*-dependent transcriptional activity that leads to somatic differentiation. Whether PIE-1 is directly inhibiting transcription is not clear, though several lines of evidence suggest that PIE-1 may do so directly. First, PIE-1 protein demonstrates germ-specificity through its exclusive localization in the germ-lineage, disappearing at about the 100-cell stage. Second, *pie-1* encodes a CCCH type Zn finger protein. A number of proteins with this motif (Suppressor of Sable, Cleavage and polyadenylation specificity factor, and U2AF35) have been shown to bind to RNA and are implicated in RNA regulation (Barabino et al., 1997; Murray et al., 1997; Rudner et al., 1998). Third,

Betchelder et.al. showed that *pie-1* can repress the expression of a reporter gene in cell culture when PIE-1 is fused to a DNA-binding domain. This repressive activity of PIE-1 is dependent on a YAPMAPT motif which is similar, minus the phosphoreceptor residues, to the YSPMSPT motif contained in the RNA PolII CTD (C-terminal domain), a repeat which couples transcription initiation to elongation during mRNA transcription (Batchelder et al., 1999). These findings raise the possibility that PIE-1 may mimic CTD and titrate away the effector kinase. Consistent with this notion, Peterlin et. al. suggest that PIE-1 binds to cyclin T, a protein required for the phosphorylation of CTD by P-TEFb (CDK-9/Cyclin T) kinase, and may compete it away from the CTD (Zhang et al., 2003). The localization of PIE-1, its inverse relationship with the phospho-CTD epitope, and its phenotype make *pie-1* a very good candidate for the factor which confer germline its quiescence.

Germ cell transcriptional quiescence appears to be conserved in other organisms such as *Drosophila* and mammal. In *Drosophila*, the failure to establish quiescence in nuclei of cells destined to become pole cells is strongly correlated with the failure of those cells to form pole cells (germ cell precursor in *Drosophila*). *nanos*, *pumilio*, and *gcl*, work together, though apparently at different stages, to achieve transcriptional silencing during pole cell formation (Asaoka et al., 1998; Asaoka-Taguchi et al., 1999; Deshpande et al., 1999; Leatherman et al., 2002). The mouse OCT-4 is expressed only in germ cells and totipotent cells of the mouse and is downregulated upon the differentiation of the totipotent cells. *In vivo* ablation of *oct-4* causes the cells to lose pluripotency and differentiate into soma-destined trophectoderm (Niwa et al., 2000).

However, *nanos*, *pumilio*, *gcl*, and *oct-4* appear to be functioning in a different mechanism than *pie-1*. Whereas *pie-1* has been shown to be required for the inhibition of all PolII-dependent transcripts assayed thus far, *nanos*, *pumilio*, *gcl*, and *oct-4* repress only a subset of genes (Asaoka et al., 1998; Asaoka-Taguchi et al., 1999; Deshpande et al., 1999; Leatherman et al., 2002).

In addition to the regulation of transcription, post-transcriptional control is apparently required to regulate the development of the *C. elegans* germline. Two groups of proteins which contain motifs implicated in RNA binding have been shown to be required for the specification and development of the germ cell precursor: 1.) *mex-1*, *pos-1*, *mex-5* and *mex-6* encode proteins containing *pie-1*-type CCCH Zn fingers. Mutations in *mex-1* result in defective germ cell development and improper segregation of P-granules during early embryogenesis (Guedes and Priess, 1997). *pos-1* mutations result in the transformation of germ cell to somatic cells. The function of *pos-1* in the formation of the germ cell fate is due in part to the translational regulation of the *apx-1* mRNA. (Asaoka-Taguchi et al., 1999; Crittenden et al., 1997; Mello et al., 1996; Ogura et al., 2003; Tabara et al., 1999a). *mex-5* and *mex-6* encode homologous proteins and are required to inhibit anterior expression of germline genes. 2) *gld-1*, *gld-3*, and *mex-3* encode KH-domain proteins. *gld-1* binds to *tra-2* 3'UTR and represses its expression (Jan et al., 1999), and is essential for oogenesis, spermatogenesis and suppression of unchecked germ cell proliferation (Francis et al., 1995). The *gld-3* (*alp-1*) gene product is an interactor of PIE-1 and is required for its localization (Shin and Mello, unpublished data). In addition, *gld-3* has also been found to interact physically and genetically with

FBF, an RNA binding protein which binds to the 3' UTR of various sex determination genes and represses their expression (Eckmann et al., 2002). MEX-3 localizes to the cytoplasm of the somatic blastomere and contributes to axis polarity by specifying the posterior blastomeres (Draper et al., 1996).

CHAPTER II

IDENTIFICATION AND CHARACTERIZATION OF *MEP-1*

Results and Discussion

In order to study 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. Among several positive interactors identified were proteins encoded by the gene *alp-1*, *pie-1*, *ubc-9*, and *mep-1*. Preliminary examination of these candidates suggested that they are relevant in the PIE-1-related biological process. ALP-1 is a homolog of *D. melanogaster* Bicaudal-C, a KH domain protein required for anterior patterning of the *Drosophila* embryo and also for follicle cell migration (Mahone et al., 1995). Abrogation of the activity of bicaudal-C results in the persistence of *oskar* mRNA in the anterior of the embryo, consistent with the concurrent model that KH domain proteins function as RNA binding proteins (Burd and Dreyfuss, 1994; Chen et al., 1997). Mutations in ALP-1 in *C.elegans* result in the mislocalization of PIE-1 in sister cells of the P-lineage cells in early embryos. These *alp-1(RNAi)* animals develop into sterile, but otherwise normal adults (Shin and Mello, unpublished results). Another positive clone examined is *CeUBC-9*, a *C. elegans* homolog of the vertebrate E2 ubiquitin-conjugating enzyme, and ortholog of the *S.cerevisiae* Ubc9p. Ubc9p mediates the covalent attachment of the small ubiquitin-related modifier (SUMO)

to various substrates including Ran-GAP1 and p53 (Gostissa et al., 1999; Rodriguez et al., 1999; Saitoh et al., 1998). *CeUBC-9(RNAi)* results in embryonic defects in which approximately 20% of the embryos produce extra pharyngeal tissue from the germline blastomere P2, a defect also seen in *pie-1* mutants. This observation suggests that CeUBC-9 is required for PIE-1 to function. Consistent with this hypothesis, *CeUBC-9* and *pie-1* interact genetically. Whereas a *pie-1* heterozygote normally makes 100% viable progeny, *CeUBC-9(RNAi)* in a *pie-1* heterozygote results in a nearly fully penetrant *pie-1* (extra pharynx and intestine) phenotype (Tae Ho Shin, unpublished data). Several clones identified in this assay also define the *mep-1* locus. Microinjection of dsRNA 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). The zygotic phenotype of *mep-1* is a defect in gonadogenesis.

MEP-1 protein contains seven zinc-finger motifs (Figures 2 A and B, p.16). 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 (Figure 2B, p.16). These features as well as the presence of a long 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 CG1244, an essential but otherwise uncharacterized gene in *Drosophila* (Saitoh et al., 1998). Zinc-fingers similar to those of MEP-1 occur in several previously characterized proteins in divergent species, including a *Drosophila* protein, Suppressor of Hairy Wing (Su(HW)). Su(HW) is a DNA-binding protein, and its binding to the *gypsy* element is required for

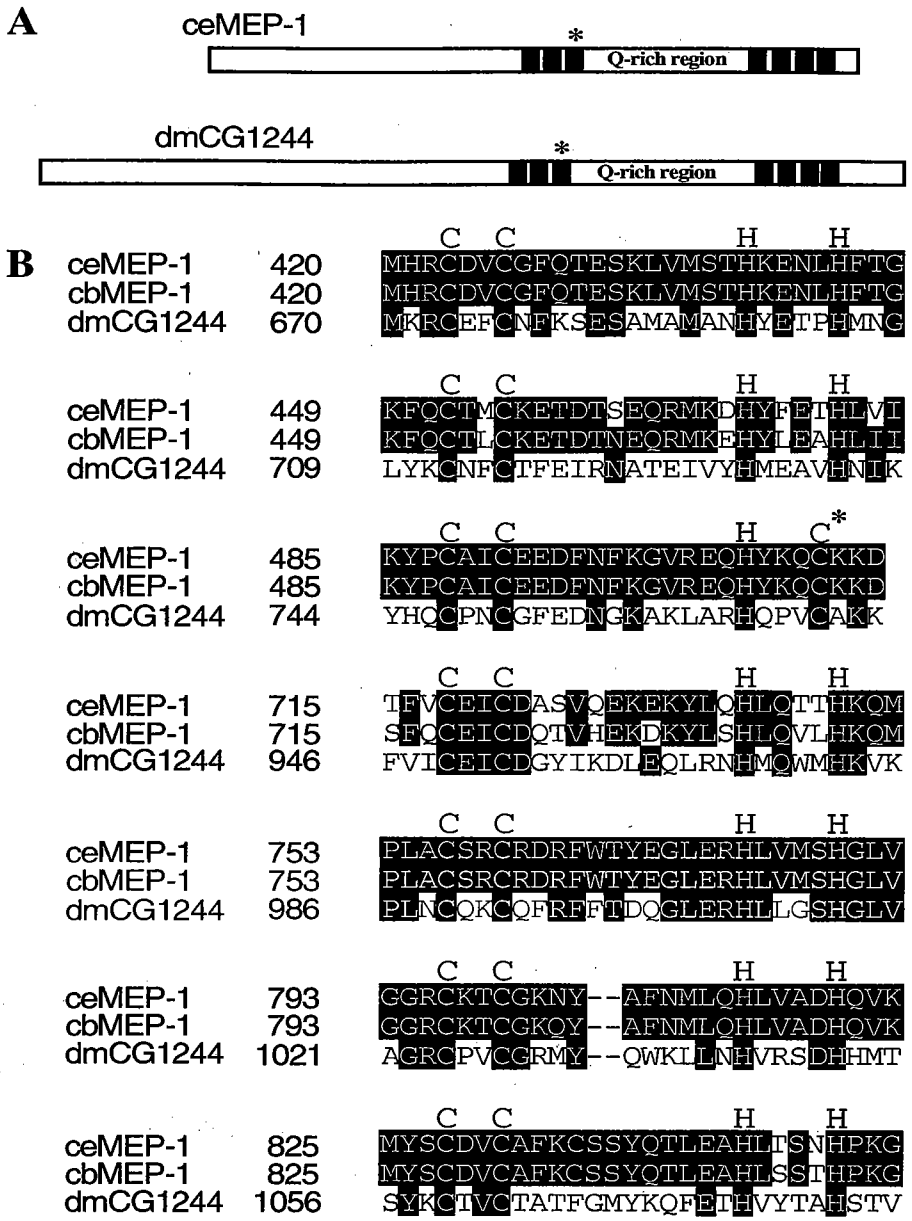

A
ceMEP-1
Q-rich region
dmCG1244
Q-rich region
B
ceMEP-1 420 MHRCDVCGFQTESKLVMSTHKENLHFTG
cbMEP-1 420 MHRCDVCGFQTESKLVMSTHKENLHFTG
dmCG1244 670 MKRCEFCNFKSESAMAMANHYETPHMNG
ceMEP-1 449 KFQCTMCKETDTSEQRMKDHYFETHLVI
cbMEP-1 449 KFQCTLCKETDTNEQRMKEHYLEAHLII
dmCG1244 709 LYKCNFCTFEIRNATEIVYHMEAVHNIK
ceMEP-1 485 KYPCAICEEDFNFKGVREQHYKQCKKD
cbMEP-1 485 KYPCAICEEDFNFKGVREQHYKQCKKD
dmCG1244 744 YHQCPNCGFEDNGKAKLARHQPVCAKK
ceMEP-1 715 TFVCEICDASVQEKEKYLQHLQTTHKQM
cbMEP-1 715 SFQCEICDQTVHEKDKYLSHLQVLHKQM
dmCG1244 946 FVICEICDGYIKDLEQLRNHMQWMHKVK
ceMEP-1 753 PLACSRCRDRFWTYEGLERHLVMSHGLV
cbMEP-1 753 PLACSRCRDRFWTYEGLERHLVMSHGLV
dmCG1244 986 PLNCQKCQFRFFTDQGLERHLLGSHGLV
ceMEP-1 793 GGRCKTCGKNY--AFNMLQHLVADHQVK
cbMEP-1 793 GGRCKTCGKQY--AFNMLQHLVADHQVK
dmCG1244 1021 AGRCPVCGRMY--QWKLLNHVRSDHHMT
ceMEP-1 825 MYSCDVCAFKCSSYQTLEAHLISNHPKG
cbMEP-1 825 MYSCDVCAFKCSSYQTLEAHLSSTHPKG
dmCG1244 1056 SYKCIVCTATFGMYKQFEIHVYTAHSTV

Figure 2

Figure 2. *mep-1* encodes a conserved Zn-finger protein

(A). Diagram of *C. elegans* MEP-1 and a *D. melanogaster* homolog, CG1244. The finger domains are indicated as black boxes flanking a glutamine-rich (Q-rich) region. (B) Alignment of the Zn finger domains of *C.elegans* MEP-1 with the corresponding domains in *C. briggsae,* and *D. melanogaster* MEP-1 homologs. The asterisk (*) indicates the degenerate third Zn fingers in all three proteins.

the enhancer-blocking and the insulator activity of this transposable element (Gostissa et al., 1999; Lukacsovich et al., 2001; Modolell et al., 1983; Rodriguez et al., 1999). Consistent with report that MEP-1 can interact with itself (Belfiore et al., 2002), the C2H2 Zn fingers domain has also been shown to be required for the dimerization of Roaz protein (Tsai and Reed, 1998). However, no other proteins in the current database show significant overall similarity to MEP-1. We have obtained a null allele of *mep-1* (Belfiore et al., 2002) from J. Kimble, A. Pouti and R. Barstead, as a reagent to further examine the phenotype of *mep-1*. *mep-1(q660)* is a deletion strain created via a PCR based deletion screen (Edgley et al., 2002). This particular deletion removes 2183 nucleotides of genomic sequence, including 1915 *mep-1* encoding nucleotides (nt 334 to 2249). The end of the deletion precedes the natural *mep-1* stop codon, resuming a different reading frame and creating a premature stop codon 44 nucleotides downstream of the 3' end of the deletion junction. (Belfiore et al., 2002). *mep-1(q660)/DnTI* segregate zygotic phenotype which is defective in gonadogenesis and oocytes production. To confirm our maternal arrested larvae *mep-1(RNAi)* phenotype, we rescue the *mep-1(q660)* strain with YAC (yeast artificial chromosome) which contains DNA sequences encoding MEP-1::GFP protein. Rescued *mep-1(q660)* segregates arrested L1 worms which lack the MEP-1::GFP protein, due to the loss of the rescuing YAC from the germline of the mother. Similarly, MEP-1 protein, which localizes to the nucleus of every blastomere at all the developmental stages, was undetectable by immunofluorescence staining in all stages of the *mep-1(RNAi)* embryos and arrested larvae (Figure 3, p. 19), indicating that the lack of maternal and zygotic MEP-1 contributed to the arrested L1 phenotype.

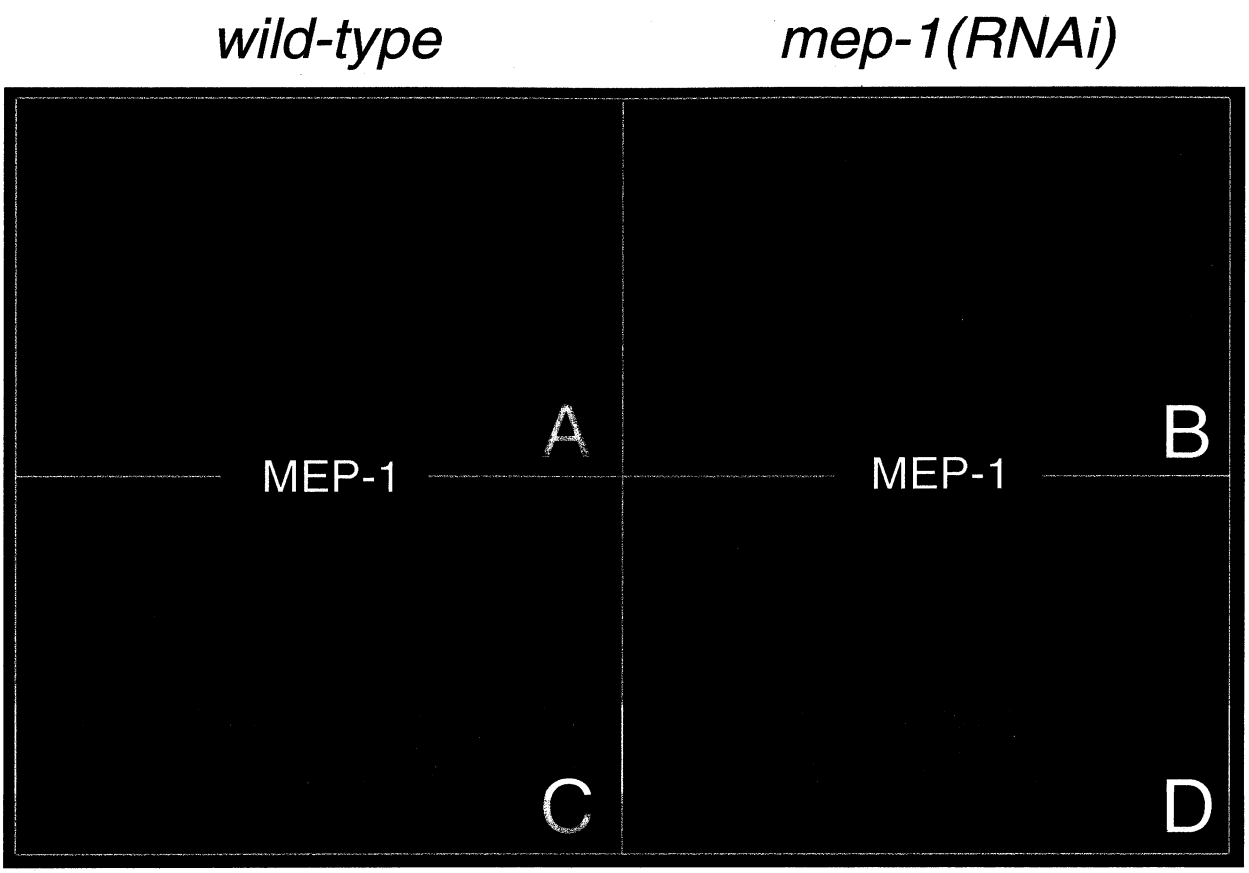


Figure 3

Figure 3. MEP-1 expression is abolished in *mep-1 (RNAi)* embryos.

Comparison of MEP-1 expression in wild-type (A and C) and *mep-1(RNAi)* embryos (B and D) at different embryonic stages (A and B are two-cell embryos, C and D are four-cell embryos). MEP-1 expression is examined by indirect immunofluorescence antibody staining using polyclonal anti-MEP-1.

The *mep-1* L1 arrest phenotype was correlated with 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, and had an overall appearance that resembled early mitotic germline cells (compare figure 4A-B, p. 22). 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 PGL-1 protein is a constitutive component of P granules which are part of the *C. elegans* germ plasm (Belfiore et al., 2002). *pgl-1* gene encodes a novel protein with RGG box motif at the C-terminus. The RGG box motif is found in several RNA-binding proteins (Burd and Dreyfuss, 1994). We found that the *mep-1(RNAi)* arrested larvae express PGL-1 protein ectopically in all intestinal cells and in many lateral hypodermal cells (Figures 4C and D, p.22 and Figure 5-2, p.25)). The ectopic PGL-1 accumulates in perinuclear structures as does PGL-1 in the germline cells of wild-type animals (compare insets in Figures 4C and D, p.22), 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 *Drosophila* germline helicase VASA (Belfiore et al., 2002; Kawasaki et al., 1998). 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 4E-H, p.22). GLH-3 was also visible diffusely in the cytoplasm

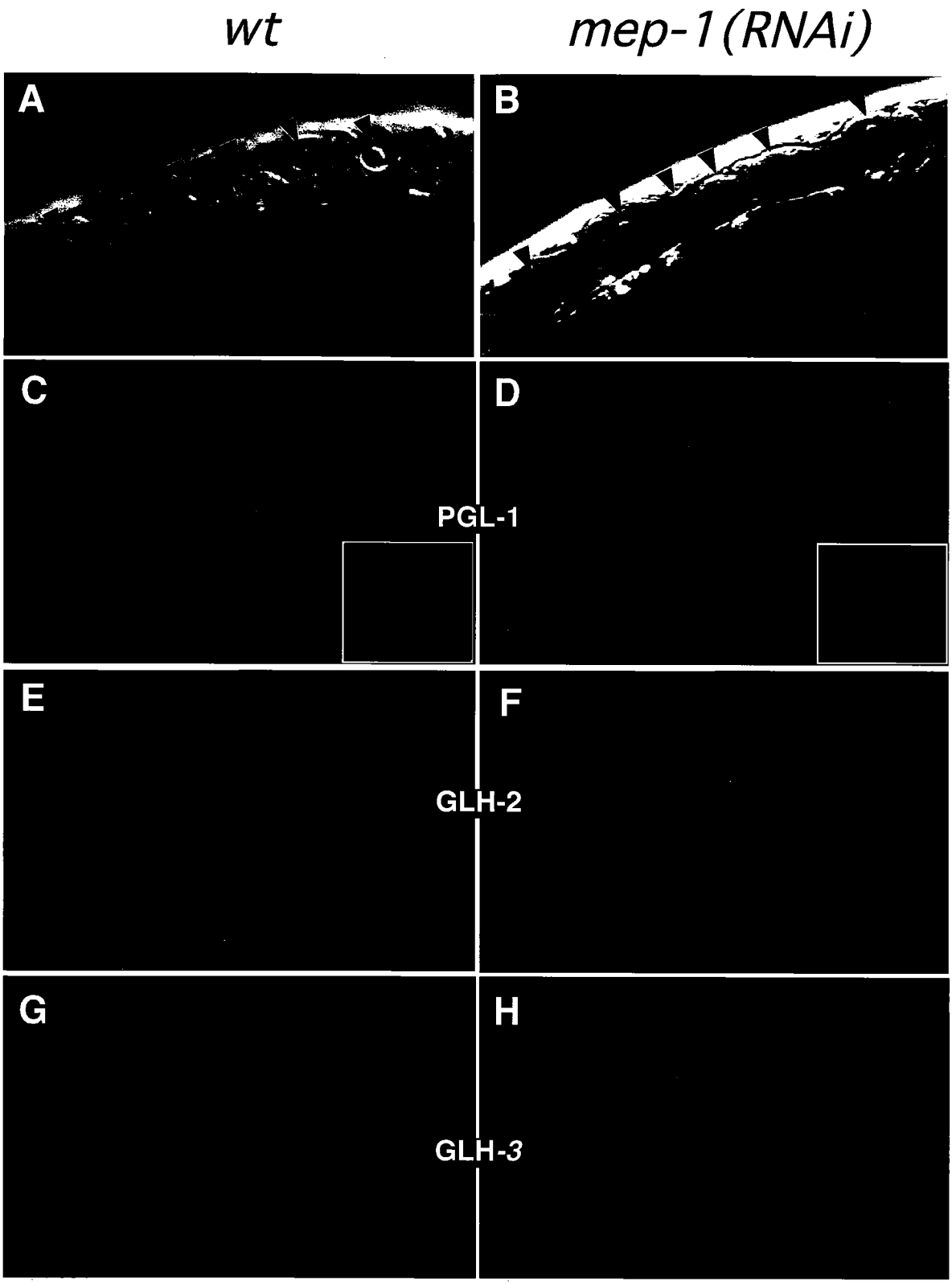


Figure 4

Figure 4. Soma to Germline Transformations in *mep-1(RNAi)* Larvae

Comparison of wild-type L1 larvae (left panels) and *mep-1(RNAi)* L1 larvae (right panels). (A,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) reveals the punctate peri-nuclear distribution of the PGL-1 protein.

(Figure 4H, p.22). 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 (Burd and Dreyfuss, 1994; Gruidl et al., 1996; Mohler et al., 1998) are expressed at apparently normal levels in *mep-1(RNAi)* embryos (unpublished observation). 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. 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 both the hypodermis and the intestine of the *mep-1(RNAi)* embryos maintain their normal appearance throughout embryogenesis and only began to exhibit morphological defects after hatching. To determine the timing of the onset of developmental abnormalities in more detail, we examined staged *mep-1(RNAi)* embryos for the onset of expression of the germline protein PGL-1, and for the expression of the somatic protein JAM-1, a transmembrane protein expressed at cell adhesion junctions in many hypodermal cells (Francis and Waterston, 1991; Mohler et al., 1998). *mep-1* embryos expressing ectopic PGL-1 exhibited an otherwise wild-type appearance and exhibited a wild-type distribution of the JAM-1::GFP fusion protein throughout morphogenesis (compare Figures 5B to 5A, p.25). About 15-20 hours after the worm

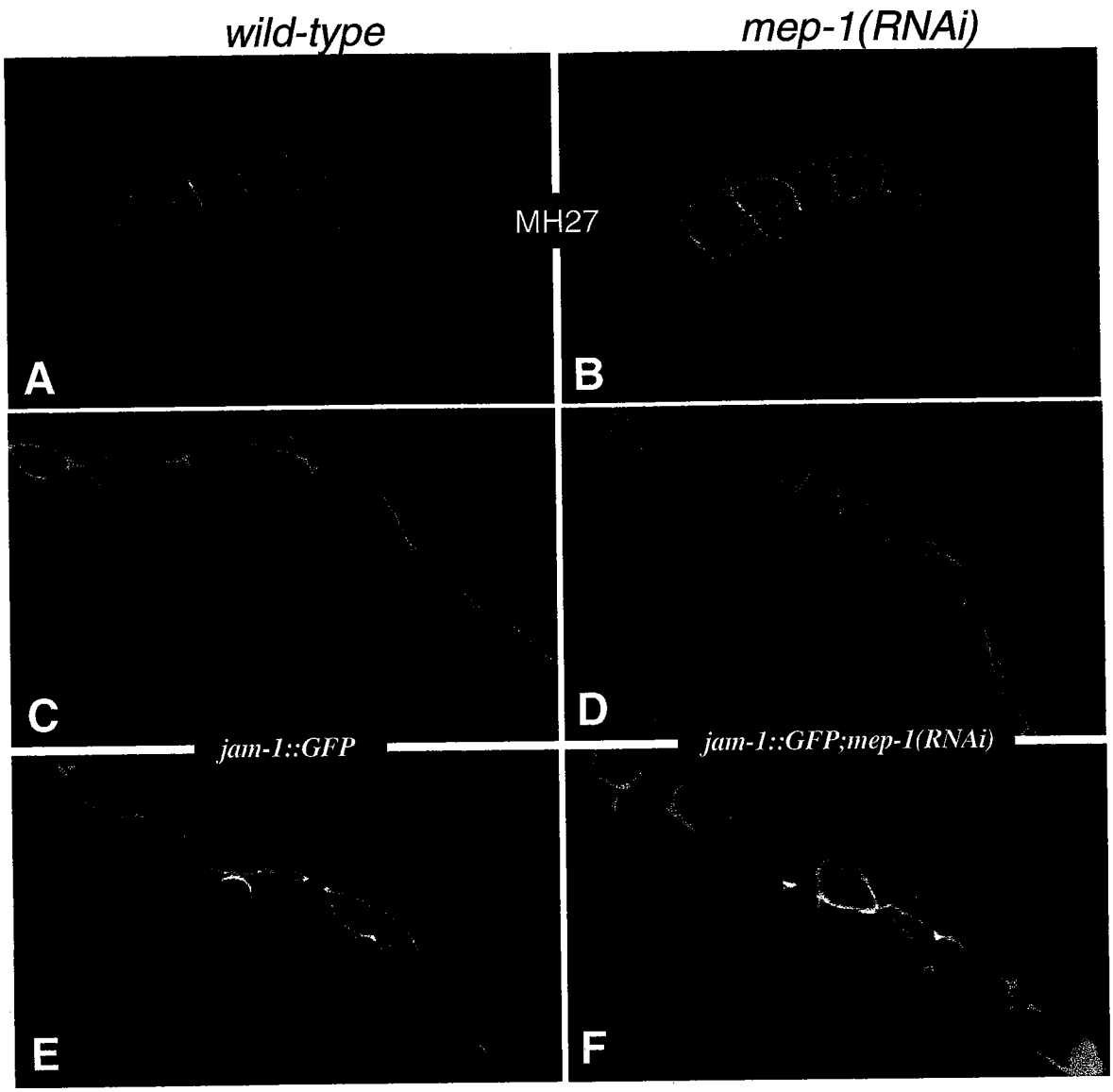


Figure 5-1

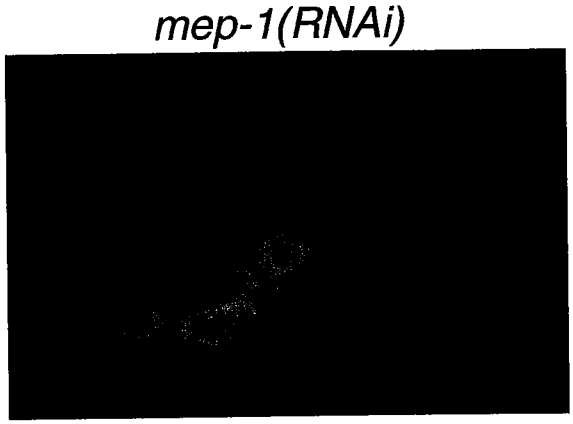


Figure 5-2

Figure 5-1. Somatic Defects in mep-1(RNAi) animal

Comparison of JAM-1::GFP in wild-type L1 larvae (A,C and E) to mep-1(RNAi) L1 larvae (B, D and F) background. JAM-1::GFP is mislocalized in mep-1(RNAi) arrested larvae (D and F) while expressing in a wildtype pattern during embryogenesis (compare A and B).

Figure 5-2. Ectopic PGL-1 in hypodermal cells of *mep-1(RNAi)* larvae

Ectopic PGL-1 localization in the hypodermis of mep-1(RNAi) arrested larvae. PGL-1 is visualized via staining with K76 monoclonal antibody.

hatch, we could observe that the pattern of JAM-1::GFP begins to become irregular. The outline of cellular junction are often disrupted and the general symmetry of the expression pattern is lost (compare Figures 5D and F to 5C and E).

Thus, the appearance of ectopic germline characteristics precedes any visible defects in the morphology or function of somatic cells in *mep-1* deficient animals. There are two simple explanations for the ectopic expression of the P-granules in *mep-1(RNAi)* larvae. The first hypothesis is that the P-granules missegregated during embryogenesis to the intestinal and the hypodermal cells of the arrested larvae. The alternative hypothesis is one in which the ectopic P-granules are made *de novo* in the somatic cells. The second hypothesis is more favorable as we found that the maternally expressed P granules exhibited a wild-type distribution in early *mep-1(RNAi)* embryos (Figure 6-1 A and B, p.28). We found that ectopic PGL-1 expression first became visible in intestinal cells and hypodermal cells at or shortly after the two-fold stage of embryogenesis (Figure 6-1 C and D, p.28), approximately at the same time that zygotic expression of PGL-1 begins in Z2 and Z3 at around the comma stage of embryogenesis in wild-type embryos (Seydoux and Fire, 1994). To examine this *de novo* hypothesis, we used a genetic cross to bring a *pgl-1::gfp* transgene into the embryo via the sperm. The hermaphrodite whose *mep-1* activity is abolished due to *mep-1(RNAi)* does not carry the *pgl-1::gfp* transgenes. We found that PGL-1::GFP was abundantly expressed in somatic cells of the arrested larvae derived from this cross, demonstrating that *de novo* transcription of the paternally provided *pgl-1::gfp* contributed to PGL-1 ectopic expression (compare Figures 6-2A and B, p.28). To further characterize *pgl-1* upregulation in mep-1(RNAi) animal, we used *in*

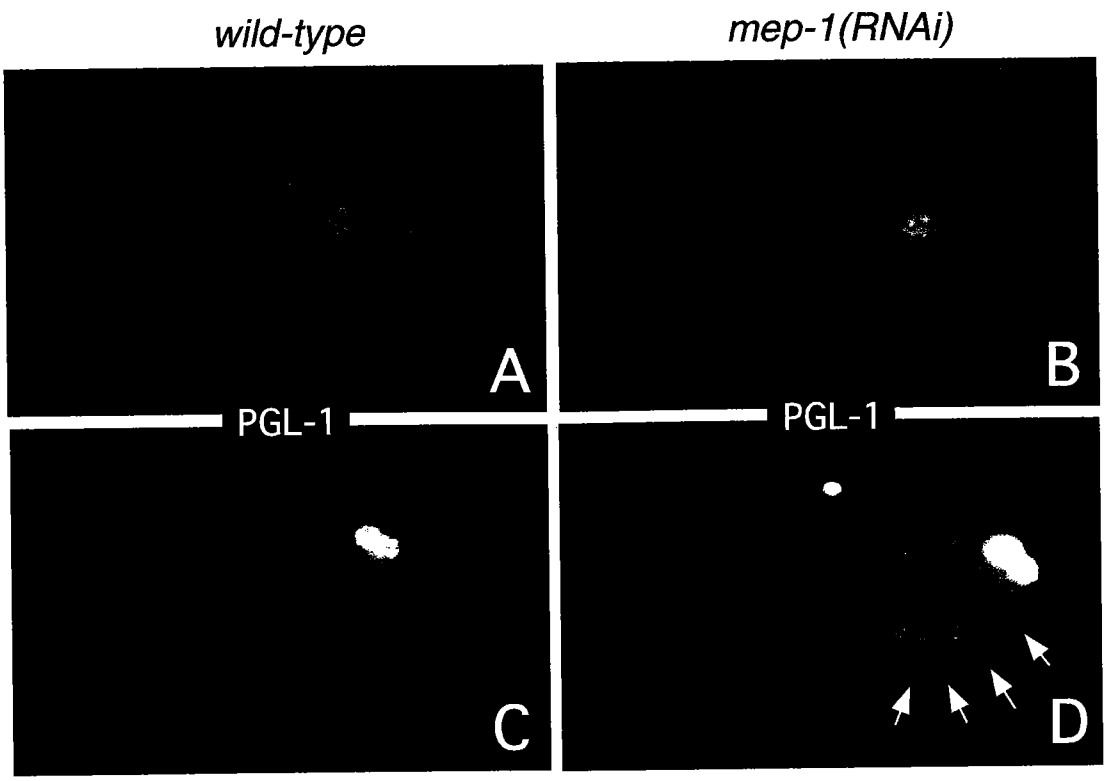
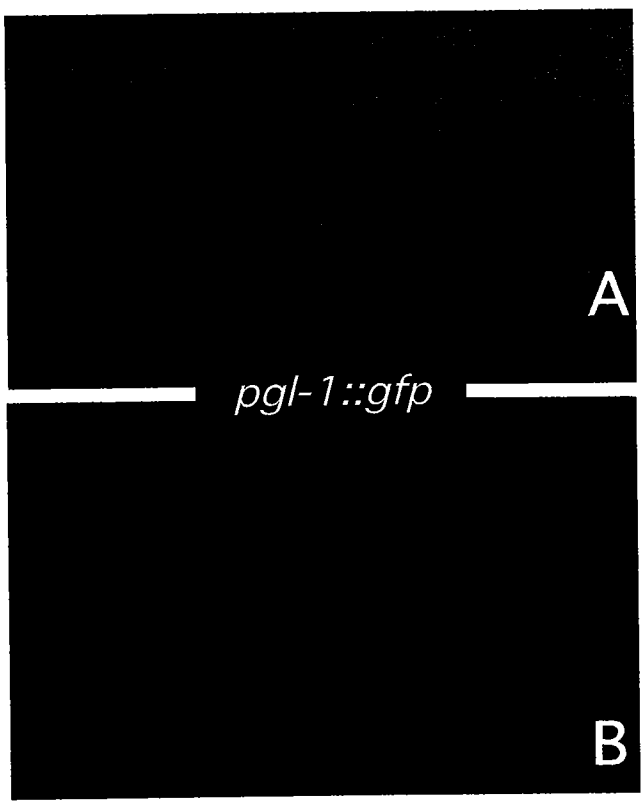


Figure 6-1



mep-1(RNAi)

wild-type

Figure 6-2

Figure 6-1. ectopic PGL-1 expression in *mep-1(RNAi)* embryos

Immunofluorescence visualizing PGL-1 expression by staining embryo samples with K76 antibody. A and B show the bean-stage embryos while C and D show 2-fold embryos. Whereas wild-type embryo shows PGL-1 expression in Z2 and Z3 (A and C), mep-1 (RNAi) embryos show normal PGL-1 expression at the bean stage but start to express PGL-1 ectopically in the intestine at the two fold stage (B and D, respectively. Panel C and D are overexposed to visualize the PGL-1 signal in the *mep-1(RNAi)* embryo.

Figure 6-2. Zygotic Activation of *pgl-1::gfp* in *mep-1(RNAi)* Larvae

(A and B) Light micrographs showing progeny resulting from the crossing of male carrying *pgl-1::gfp* transgene into *mep-1(RNAi)* hermaphrodite (A) or into wild-type control (B).

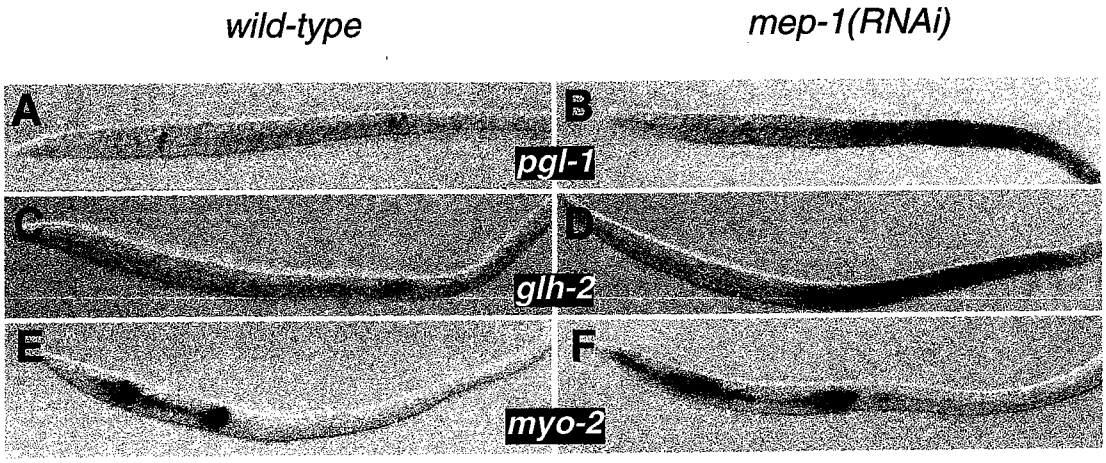


Figure 6-3

Figure 6-3. Accumulation of Germ-specific Messages in *mep-1(RNAi)* larvae

(A-F) *In situ* staining to detect mRNA levels of *pgl-1, glh-2* and the pharyngeal-specific myosin *myo-2* (as indicated).

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 6-3A and B, p.30). It is a possibility that *mep-1(RNAi)* causes a global accumulation of both somatic and germ-specific mRNAs. To address this notion, we probe for *myo-2*, a muscle myosin specific to the pharynx. *In situ* hybridization of *myo-2* exhibited a wild-type pattern of mRNA expression in *mep-1(RNAi)* embryos (Figures 6-3E and F, p.30), suggesting that the accumulation of mRNA in the mep-1 arrested larvae is more specific to the P-granule genes. These observations are consistent with the hypothesis that ectopic P granules in *mep-1(RNAi)* animals may result from transcriptional de-repression during late embryonic and early larval stages.

mep-1(RNAi) appears to cause the ectopic expression of germline genes in somatic cells with the 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 during embryogenesis. Soon after hatching, the somatic cells, most visibly intestinal and hypodermal cells, start to display visible characteristics of germ cell morphology. The function of *mep-1* thus appears to be involved in the maintenance of the repression of P-granule genes. The cause of the arrest of the larvae may be an indirect misexpression of a yet unexamined gene(s) because overexpression of *pgl-1* from a transgene did not kill the animal (data not shown). An approach such as microarray may be utilized to perform a more extensive examination of the genes misregulated in *mep-1(RNAi)* animal. That *mep-1* displays a

zygotic sterile phenotype is indicative of the function of *mep-1* in the germline. *mep-1* sterile animals show both somatic and germline gonad defects. *mep-1* homozygous L4 animals segregated away from a balanced *mep-1* animal exhibit crossed over distal gonad tip as well as gonad tip which does not elongate to a proper length (Belfiore et al., 2002). These defects are usually associated with Z1 and Z4 cells which are the precursor cells for somatic gonad. Consistent with this observation, Belfiore et.al. reports that Z1 and Z4 generated two to five total descendants as opposed to 12 descendant cells generated by the wild type Z1 and Z4 cells (Belfiore et al., 2002). Moreover, the gonad of *mep-1* animal shows gross germline differentiation/proliferation defect. Although the sperm is visible, oogenesis appears to be defective as oocytes are not readily seen. Belfiore et. al. reports that whereas the wild type gonad of the adult hermaphrodites contains 2,400 germ cells, *mep-1* hermaphrodites contains only 771$\pm$88 germ cells, including the sperm (Belfiore et al., 2002).

In summary, *mep-1* appears to be required for different aspects of germ cell development. Our data suggest that the maternal load of *mep-1* is required for the maintenance of the suppression of germ-line gene expression. Consistent with this notion, the *mep-1* arrested-larva phenotype cannot be rescued by sperm carrying a wild type copy of *mep-1*. *mep-1* is also required zygotically for the general somatic gonad and germ line development. The examination of the mechanism of *mep-1* will be presented in the following chapter.

CHAPTER III

ANALYSIS OF *MEP-1* FUNCTION IN THE GERM CELL SPECIFICATION

Introduction

The genomic DNA is packaged into a compact structure called chromatin, comprising of DNA, histones, and other proteins, with nucleosome being the basic building block. The nucleosome is composed of 147 bp of DNA, winding around an octamer of histone proteins (two copies each of histone H2A, H2B, H3 and H4) (Horn and Peterson, 2002). Studies have shown that transcription could be inhibited when the promoter is placed in the context of the nucleosome (Lorch et al., 1992; Workman and Roeder, 1987), suggesting that the organization of DNA packaging can contribute to transcription inhibition. Consistent with this finding, the expression of a transgene carrying the *white* gene in *Drosophila* is hindered when inserted into the heterochomatin, a region of the genome which remains condensed as the cell makes the transition from metaphase to interphase (Wallrath and Elgin, 1995) . In order for the DNA to be accessible for the transcription process, there must be mechanism(s) which can alter the impeding chromatin structure. Recently, two mechanisms have been proposed to accomplish this task. The first mechanism utilizes the energy of ATP to remodel the chromatin (Kadonaga, 1998; Tsukiyama and Wu, 1997; Varga-Weisz and Becker, 1998). The second mechanism involves posttranslational modification, particularly the acetylation of histones (Grunstein, 1997; Struhl, 1998; Wade et al., 1997).

Histone acetylation state and transcriptional control. The acetylation state of the nucleosomal histones has long been correlated with the state of transcription. Histone acetylation is a reversible process. The histone acetylases (HATs) transfer the acetyl group from the Acetyl coenzyme A to the ε-$NH3^+$ group on the highly conserved lysine residue embedded in the N-terminal tail of the core histones. There are several transcriptional coactivators that have been identified as having an intrinsic HAT activities: Gcn5p, p55, p300, PCAF, TIP60, etc., (Kuo and Allis, 1998). The acetyl group neutralizes the overall positive charge of the histone tail, reducing greatly the interaction between the positively-charged histone tail and the negatively-charged DNA. Although acetylation doesn't disturb the overall folding of nucleosomes, acetylated histones tails will, under ionic conditions, reduce the compaction of nucleosomes which can be inhibitory to transcription (Garcia-Ramirez et al., 1995; Hansen and Wolffe, 1992). In a reverse reaction, the histone deacetylases (HDACs) remove the acetyl group from the same residue. The mammalian HDA-1 was identified in a screen for human protein(s) which co-purified with a histone deacetylase inhibitor trapoxin (Taunton et al., 1996), and was found to be similar to *S. cerevisiae* Rpd3p, a protein which is required for full repression and full activation of a subset of genes (Lamping et al., 1994). HDA-1 protein forms a complex comprised of seven subunits; HDAC1 and HDAC2, Sin3(RPD1), RbAp48, RBAp46, SAP30 and SAP18. This complex is referred to as the Sin3 complex, after the Sin3 protein which was identified as a corepressor in *S. cerevisiae*. Since there are no known DNA-binding motifs in either HATs or HDACs subunits, how then do the HATs and HDACs discriminately acetylate/deacetylate

histones of different genes, respectively? Several studies suggested that HATs and HDACs are localized to specific loci via interaction with DNA-binding proteins, namely MAD/MAX, unliganded RXR, and Ume6 (Pazin and Kadonaga, 1997). Thus, transcriptional activators and repressor may be functioning in a concerted manner to regulate the expression of specific genes. Although studies have shown that recruitment of HDACs can result in localized deacetylation of histones and transcriptional repression (Kadosh and Struhl, 1998; Nagy et al., 1997; Rundlett et al., 1998; Zhang et al., 1997b), it is not clear whether the deacetylation is preventing the assembly of stable transcription complex or the deacetylation is inducing a higher order chromatin condensation inhibitory to transcription. Finally, even though a correlation exists between histone deacetylation and transcriptional silencing and *vice versa*, there are exceptions. For example, loss of RPD3 in yeast results in defect of both transcriptional repression and activation (Rundlett et al., 1996; Vidal and Gaber, 1991). In addition, loss of yeast and *Drosophila* RPD3 function results in enhanced heterochromatin silencing (De Rubertis et al., 1996; Vannier et al., 1996; Vidal and Gaber, 1991).

Chromatin remodeler: SWI/SNF and NuRD complex. The other mechanism used to alter chromatin structure is the machinery which ultilizes ATP hydrolysis to remodel chromatin. Several classes of these multisubunit machines were identified in diverse organisms, which include the yeast SWI/SNF complex, yeast RSC complex, *Drosophila* NURF, *Drosophila* CHRAC, *Drosophila* ACF, *Drosophila* BRM complex, and mammalian BRG1(hbrm)-associated complexes. All the aformentioned complexes share a peptide common in function. This common subunit is homologous to the

SWI2/SNF2 in the SWI/SNF complex which contains the characteristic ATPase/helicase domain (Cairns et al., 1994; Cote et al., 1994; Laurent et al., 1993). The SWI2/SNF2 gene was cloned in a genetic screens as a positive transcriptional regulator. Recombinant SWI2/SNF2 protein shows a DNA-dependent ATPase activity *in vitro* but not helicase activity. The mechanism by which SWI/SNF appears to control transcription is through the organization of the chromatin as shown by the experiment demonstrating that the SUC2 promoter is more resistant to digestion with micrococcal nuclease in the *swi2/snf2* or *snf5* mutant (Hirschhorn et al., 1992; Matallana et al., 1992). Another class of chromatin remodeling complex, called the NuRD complex, was identified in an attempt to identify the polypeptides associated with HDAC1. The NuRD complex is unique because it contains the MI-2 subunit, originally identified as the dermatomyositis-specific autoantigen (Seelig et al., 1995). Mi-2 contains a chromodomain and a motif homologous to the DNA helicase/ATPase domain of SWI/SNF, and the Mi-2-containing complex can execute both remodeling and histone deacetylation of the nucleosome (Zhang et al., 1998). Similar to Sin3 complex, the NuRD complex shares the same core components: HDAC1, HDAC2, RbAp46, and RbAp48. The detailed mechanism of how these remodeling machines disrupt the chromatin is yet unclear.

Histone deacetylases and repression by Polycomb group proteins. In many organisms, transcription factors which are transiently expressed in cells during the early embryogenesis period can determine the fate of the progeny of those cells many cell divisions afterwards, at the time when no such transcription factors are longer present. How the daughter cells are able to remember which loci show be continually expressed

presents us with a unique problem; the *Drosophila polycomb* group proteins provide the solution.

In *Drosophila* and mouse, the body pattern relies on spatially controlling the expression of the HOX genes. The Gap proteins, one of which is Hunchback(Hb), bind directly to the regulatory sequences of HOX genes and repress their transcription in the cells which are not programmed to express HOX. It has been shown that Hunchback is required to initiate, but not maintain, the repression of Hox gene, consistent with the transient expression of Hunchback in early *Drosophila* embryos (Muller and Bienz, 1992; Qian et al., 1991; Shimell et al., 1994; Zhang and Bienz, 1992). The maintenance of the Hox repression is carried out by the polycomb-group (PcG) genes during the development of the fly (McGinnis and Krumlauf, 1992; Struhl, 1981; Struhl and Akam, 1985). PcG proteins maintain silencing of the HOX genes, as well as other developmental control genes, while trithorax-group (trxG) proteins maintain the active states (Brock and van Lohuizen, 2001).

Searching for a molecular link between gap and polycomb-group proteins, Kehle et. al. identified dMi-2, the *Drosophila* homolog of Mi-2, as a yeast two-hybrid interactor of hunchback (Kehle et al., 1998). Consistent with a model in which the recruitment of dMI-2, and possibly the NuRD complex, serves as a link between repression of HOX genes by Hunchback and polycomb, mutations in dMi-2 enhances the defect of hunchback and polycomb HOX gene repression *in vivo* (Kehle et al., 1998). Additional evidence of interaction between HDACs and polycomb proteins came from a study of the mouse EED protein, which is a homologue of the *Drosophila* PcG gene extra sex combs

(ESC). EED and EED-containing complex interact physically with histone deacetylases while the other complexes containing human polycomb homologue HPC2 doesn't (Silva et al., 2003). Thus, the histone deacetylases do not interact indiscriminantly with every polycomb complex. These findings suggest a model in which Hunchback recruits the NuRD complex, which in turn facilitates the binding of polycomb to the target site.

I. Examination of the PIE-1 and MEP-1/LET418 interaction

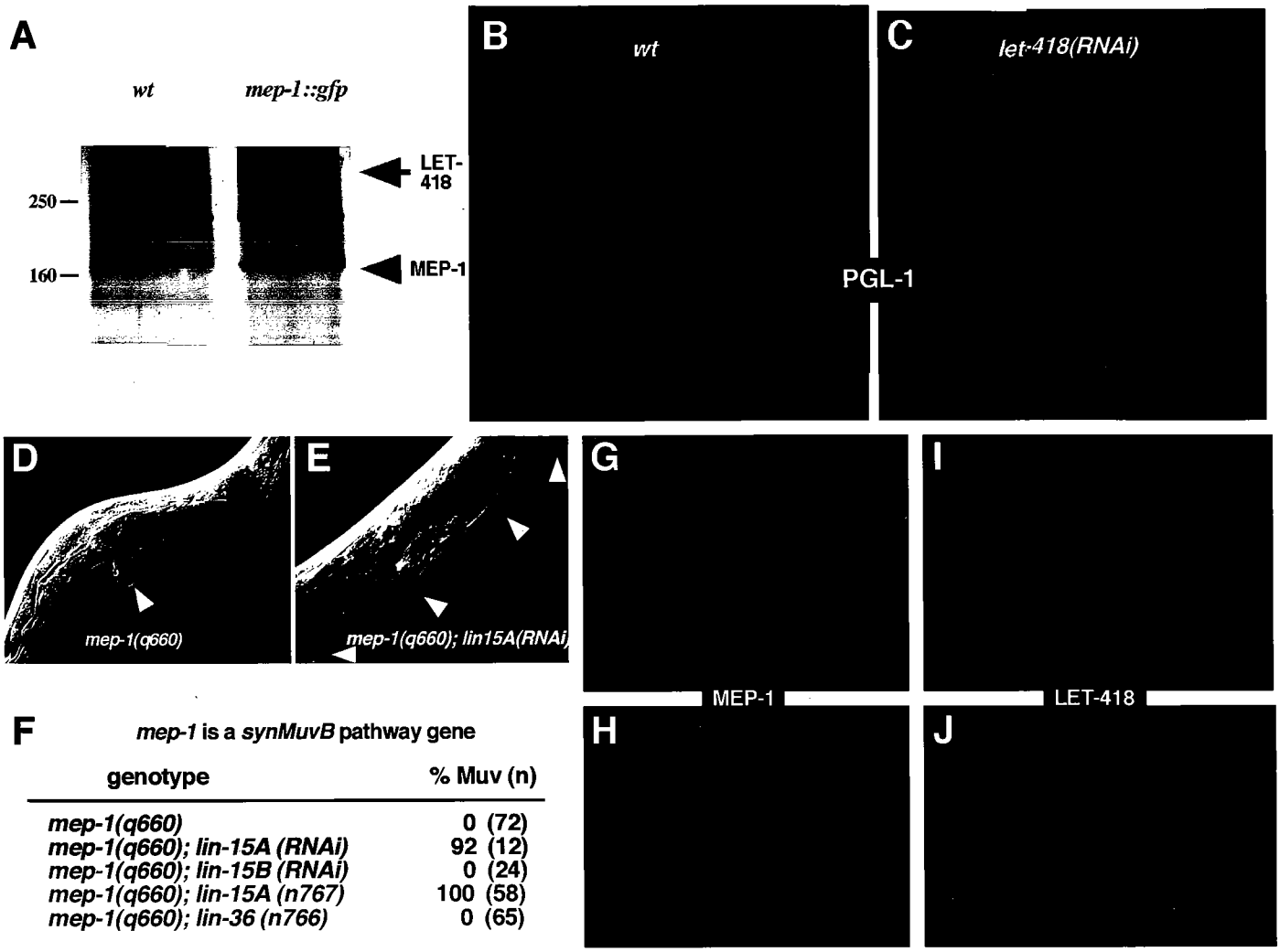
Results

MEP-1 and LET-418 form a stable complex *in vivo* and function together in the maintenance of germline-soma distinctions

In order to gain further insight into MEP-1 function, we expressed an epitope-tagged MEP-1 in the *C. elegans* embryo. MEP-1::GFP is expressed in every cell at all the developmental stages of the *mep-1* rescued animal. Embryonic lysate is made from *mep-1::gfp* rescue strain and MEP-1::GFP is immunoprecipitated from the extracts using antibody recognizing the GFP moiety. Proteins associated with MEP-1 were analyzed using MALDI-TOF mass spectrometry. We found that the MEP-1 immunoprecipitation complex contained roughly 1:1 a ratio of MEP-1 and a second protein of approximately 280 kDa (Figure 7A, p.41). Mass spectrometry analysis identified this protein as the product of the gene *let-418*. LET-418 is a *C. elegans* homolog of MI-2/CHD-3 (von Zelewsky et al., 2000), a core component of the conserved nucleosome remodeling and

histone deacetylase (NURD) complex.

To determine if LET-418 functions along with MEP-1 in development, we analyzed the phenotype induced by *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 while its zygotic loss of function results in sterile animals with incompletely penetrant vulval defects called the Evl (everted vulva) phenotype (Struhl and Akam, 1985). We found that the larval arrest phenotype induced by *let-418(RNAi)* was also similar to the phenotype caused by *mep-1(RNAi)*. The *let-418(RNAi)* arrested larvae contained aberrant cells in both the intestine and the hypodermis that showed partial germ cell-like characteristics. Furthermore, these larvae exhibited ectopic *PGL-1* expression identical to that observed in *mep-1(RNAi)* arrested larvae (Figures 7B and C, p.41) . 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 mutants for either gene produce malformed vulvae and occasional ectopic pseudo-vulvae (similar to Figure 9D, p. 56). The *let-418* gene has been shown to participate in the synMuvB pathway that negatively regulates the induction of the vulval cell fates (Kehle et al., 1998). The synMuvB pathway includes the *lin-53* and *hda-1* genes (Silva et al., 2003), each encoding a *C. elegans* homolog of two additional components of the NURD complex; *lin-53* encodes the mammalian RbAp48, which binds to the RB protein while *hda-1* encodes the homolog of the mammalian histone deacetylase HDA-1 protein (Lu and Horvitz, 1998; von Zelewsky et al., 2000). The synMuvB pathway acts redundantly with a second



F *mep-1* is a *synMuvB* pathway gene

genotype	% Muv (n)
mep-1(q660)	0 (72)
mep-1(q660); lin-15A (RNAi)	92 (12)
mep-1(q660); lin-15B (RNAi)	0 (24)
mep-1(q660); lin-15A (n767)	100 (58)
mep-1(q660); lin-36 (n766)	0 (65)

Figure 7

Figure 7. 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 a *mep-1::gfp* transgenic strain were placed over a column of sepharose beads linked to a GFP-specific monoclonal antibody. Proteins bound to the column were eluted and 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,C) *let-418(RNAi)* induces soma to germline transformations. Immunofuoresence 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,E) Light micrographs of an adult *mep-1(q660)* homozygote (D) and of an adult *mep-1(q660);lin-15A(RNAi)* animal (E). (D,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,I) and of comma-stage embryos (H,J) stained with antibodies raised against MEP-1 and LET-418, as indicated.

genetic pathway defined by the synMuvA genes (Zhang et al., 1998). Any combination of mutations in the synMuvA and synMuvB genetic pathways results in a penetrant expression of a multivulva (synthetic Muv or synMuv) phenotype (reviewed in Taunton et al., 1996) . 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 7D-F, p.41). These genetic interactions are similar to those previously reported for *let-418* and suggest that MEP-1 functions 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 7G-J, p.41). This ubiquitous nuclear staining was abolished by RNAi targeting each corresponding gene, supporting the conclusion that each antibody specifically recognizes the corresponding MEP-1 (Figure 3. p.19) and LET-418 proteins (data not shown). Finally, we examined a potential genetic interaction between *mep-1* and *chd-3*, a gene encoding a second *C. elegans* homolog of Mi-2/CHD3 (Qian et al., 1993). Although a putative null mutation of this gene, *chd- 3(eh4),* shows no apparent phenotype on its own, this mutation enhances the *let-418(RNAi)* phenotype leading to a late embryonic rather than larval arrest (Ferguson and Horvitz, 1989). Thus *chd-3* and *let-418* appear to act redundantly in an essential developmental process during embryogenesis. In contrast, we found that the

genotype	phenotype
eh4; let 418 (RNAi)	100% (26) dead embryos
eh4; mep-1 (RNAi)	100% (75) arrested larvae
eh4; let 418 (RNAi); mep-1(RNAi)	100% (43) dead embryos
mep-1(RNAi); let-418 (RNAi)	100% (56) arrested larvae

Table.2 interaction between *mep-1*, *let-418* and *chd3*

severity of the *mep-1(RNAi)* phenotype was unchanged in the *chd-3(eh4)* mutant background (Table 1, p.44). Thus, LET-418 and MEP-1 differ with respect to their interactions with *chd-3*, suggesting that MEP-1 is not an obligatory partner in all of the activities of LET-418.

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 (P4G5) was used for immunoprecipitation on extracts prepared from early *C. elegans* embryos prepared from populations of early *C. elegans* embryos (Figure 8-1A, P.46). Antibodies raised against MEP-1, LET-418 and HDA-1 were then used to probe Western blots for coimmunoprecipitation with PIE-1 from early embryo extracts. We found that all three proteins co-immunoprecipitate with PIE-1 from early embryonic extracts (Figure 8-1A, lane 4, P.46). Identical immunoprecipitation assays on extracts prepared from mixed-stage animals where PIE-1 protein itself is undetectable failed to recover detectable quantities of MEP-1 and LET-418, 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 8-1A, lane 3, p.46) and could reflect a background of non-specific immunoprecipitation under these conditions or

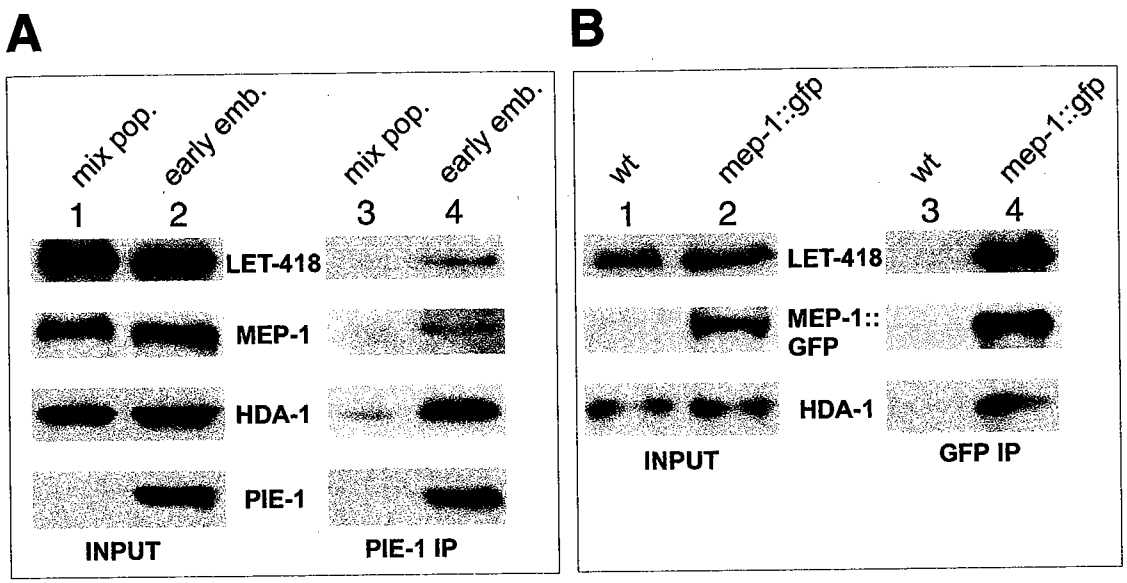

A
mix pop.
early emb.
mix pop.
early emb.
1
2
3
4
LET-418
MEP-1
HDA-1
PIE-1
INPUT
PIE-1 IP
B
wt
mep-1::gfp
wt
mep-1::gfp
1
2
3
4
LET-418
MEP-1::
GFP
HDA-1
INPUT
GFP IP

Figure 8-1

Figure 8-1. PIE-1 forms a complex with LET-418, MEP-1, and HDA-1 in C. elegans extract

(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 non-transgenic (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.

might reflect co-precipitation 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 found in an average of one cell (the germ cell) in each early embryo while the other proteins are found in all cells. The MEP-1, LET-418 and HDA-1 sera were not suitable for immunoprecipitation assays. In order to perform reciprocal immunoprecipitation, we made embryonic lysate from *mep-1* rescued strain. By using anti-GFP antisera to immunoprecipitated MEP-1::GFP, western blots confirmed that both HDA-1 and LET-418 interact with MEP-1::GFP *in vivo* (Figure 8-1B, lane 4, p.46). It was not possible to analyze the GFP precipitates for PIE-1 protein due to incompatibility of the anti-PIE-1 and anti-GFP monoclonal sera (See Experimental Procedures).

In order to confirm direct interaction between PIE-1 and MEP-1, we carried out an *in vitro* protein-binding assay. Several truncated forms of PIE-1 fused to HA tag was constructed and translated *in vitro*. By incubating these PIE-1 construct with GST::MEP-1 purified from *E. coli*, we found that *in vitro* translated PIE-1 can interact with a GST-MEP-1 protein and that this interaction requires a C-terminal proline-rich region of PIE-1. This proline-rich region contains a heptapeptide motif YAPMAPT, which has previously been shown to confer transcription repression of reporter genes in HeLa cells (Batchelder et al., 1999). The GST pull-down assays identified a region between 240-268 amino acids adjacent to, but not including the YAPMAPT motif, as a minimal MEP-

1 binding region (Figure 8-2A, p.50). In a reciprocal set of experiments, we made mutiple constructs of truncated and full length MEP-1 fused to GST and asked which domain is required for the interaction with full length PIE-1 fused to HA tag. Our results show that the N-terminal 200 amino acid of MEP-1 is dispensable for its interaction with PIE-1 (Figure 8-2B, p. 50). This reciprocal assay also identified at least two separate regions in MEP-1 as major sites of PIE-1 interaction. The first region consists of Zn-fingers II and III, and the second region consists of a conserved glutamine-rich stretch of 130 amino acids containing 40% glutamines. *in vitro* protein binding experiment was conducted to further examine the interaction between the Zn fingers/Q rich region of MEP-1 and the Proline rich region of PIE-1. In Figure 8-2B, p. 50, the result suggests that the proline-rich domain of PIE-1 interacts primarily with the second and third Zn-fingers of MEP-1. That MEP-1 binding site in PIE-1 does not overlap with the YAPMAPT region, a region implicated in the silencing of reporter gene (Batchelder et al., 1999), suggests that there may be a bifurcation of the PIE-1 function.

Ectopic expression of PIE-1 in somatic cells mimics *mep-1, let-418 and hda-1* phenotypes

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

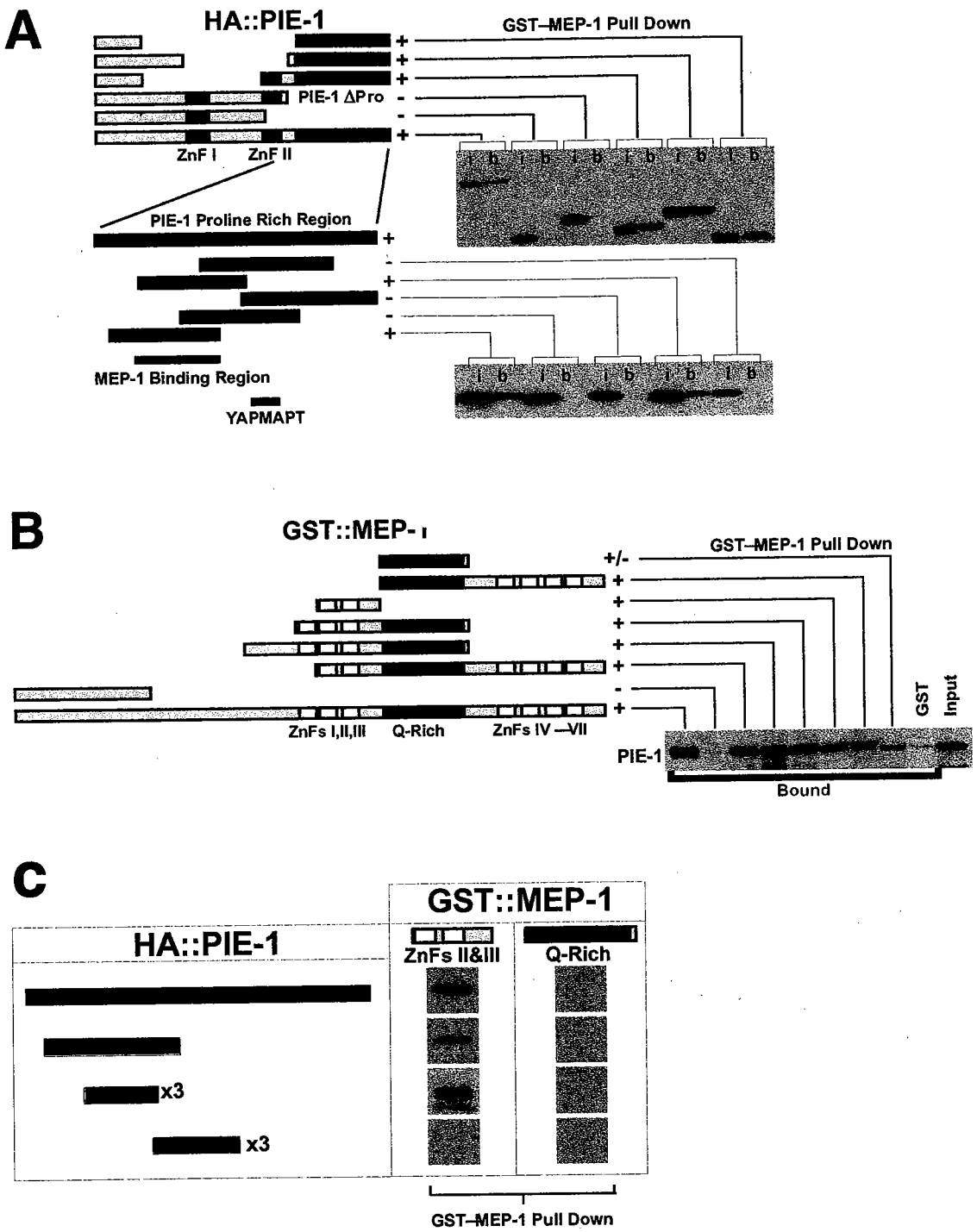
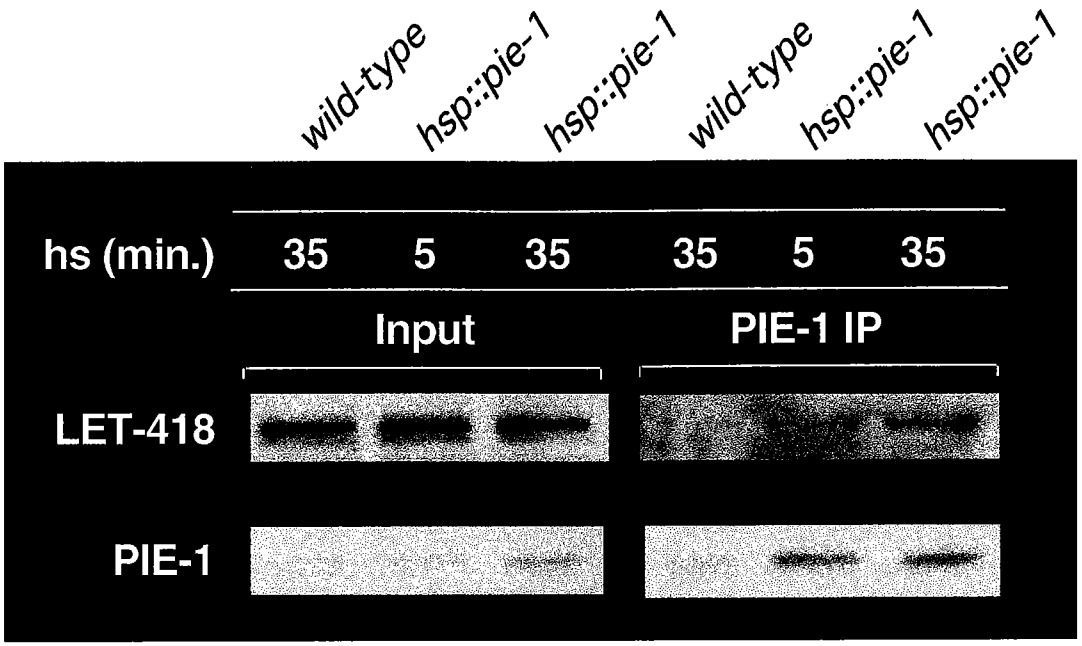

A
HA::PIE-1
GST–MEP-1 Pull Down
PIE-1 ΔPro
ZnF I
ZnF II
PIE-1 Proline Rich Region
MEP-1 Binding Region
YAPMAPT
B
GST::MEP-1
GST–MEP-1 Pull Down
ZnFs I,II,III
Q-Rich
ZnFs IV —VII
GST
Input
PIE-1
Bound
C
GST::MEP-1
HA::PIE-1
ZnFs II&III
Q-Rich
x3
x3
GST–MEP-1 Pull Down

Figure 8-2

Figure 8-2. *In vitro* binding, and domain analysis of the PIE-1, MEP-1 interaction. (A) 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). (B) MEP-1 Zinc Fingers II and III and a Glutamine-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 (A). (C) The proline-rich domain of PIE-1 interacts with Zinc Fingers II and III of MEP-1. The GST-fused fragments of MEP-1 comprising fingers II and III and the glutamine-rich region of MEP-1 (Q-Rich) were tested for interactions with proline-rich fragments of PIE-1 (as illustrated), including constructs that contain 3 tandem copies of small segments of the PIE-1 protein.

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(zu177)* 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 ectopic 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. The earlier requirement of PIE-1 during embryogenesis, as well as the opposite phenotype between *pie-1* and *mep-1*, suggest a possibility that *pie-1* may be inhibiting the function of *mep-1*. We therefore decided to ask if the forced expression of PIE-1 in somatic cells could induce functional consequences consistent with inactivation of MEP-1. We first confirmed that PIE-1 protein expressed in somatic cells via the heat-shock promoter *hsp16-1* (Seydoux et al., 1996) could interact *in vivo* in co-immunoprecipitation assays with LET-418 and MEP-1 (Figure 8-3, p.54). 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 (Seydoux et al., 1996). However, we found that induction of PIE-1 for two hours 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 9A and B, p.54), 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 (von Zelewsky et al., 2000). 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


wild-type
hsp::pie-1
hsp::pie-1
wild-type
hsp::pie-1
hsp::pie-1
hs (min.)
35
5
35
35
5
35
Input
PIE-1 IP
LET-418
PIE-1

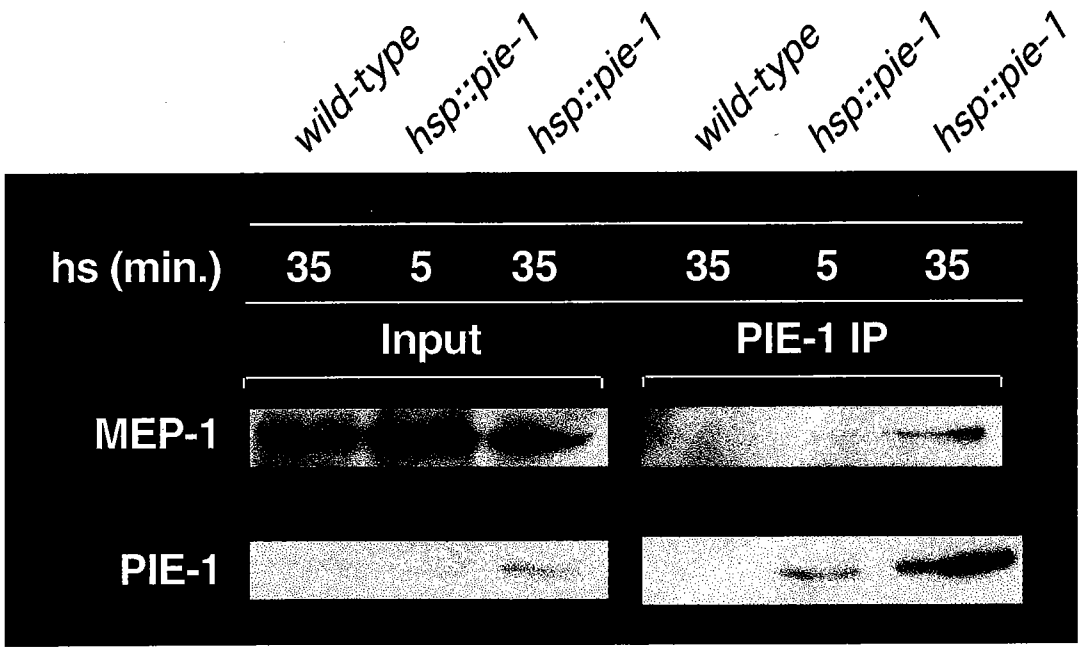
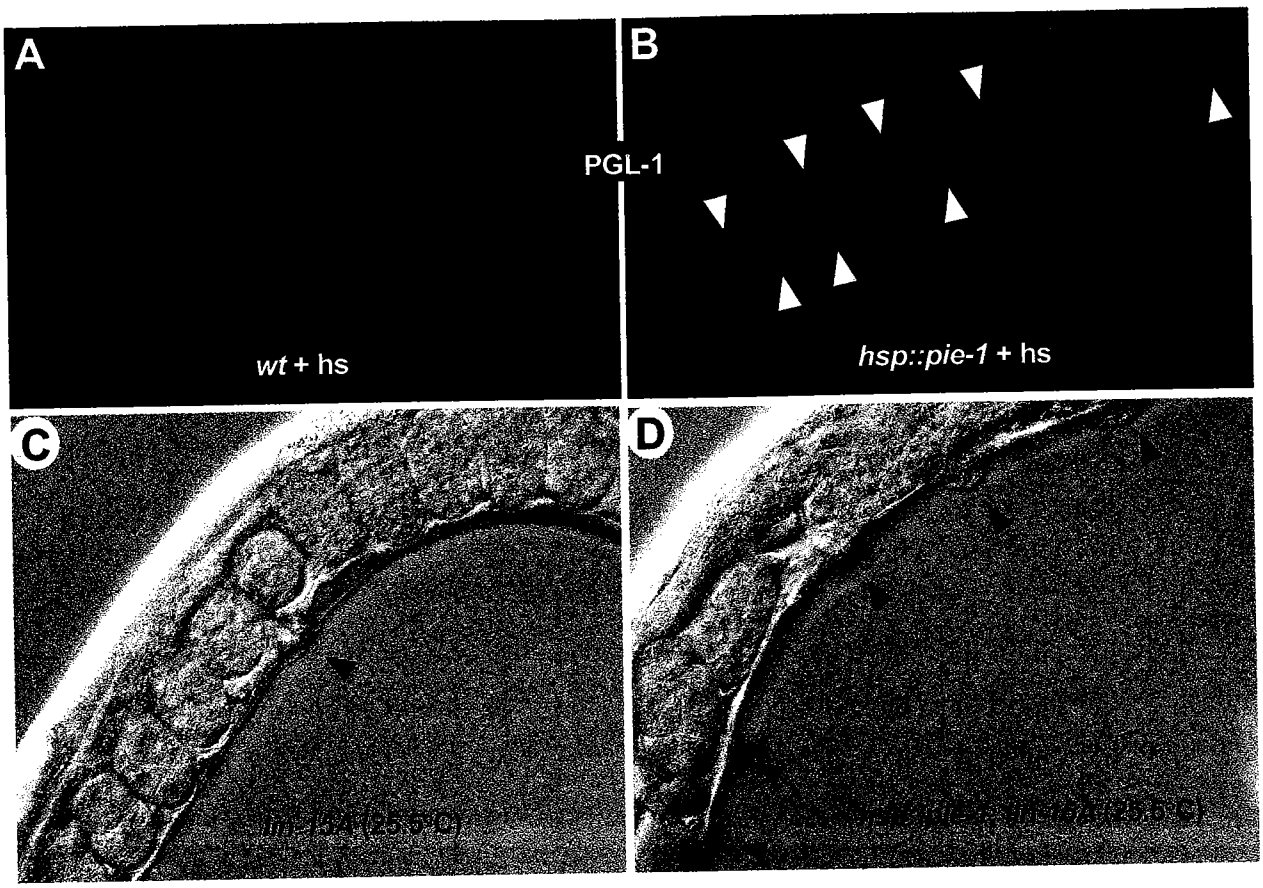

wild-type
hsp::pie-1
hsp::pie-1
wild-type
hsp::pie-1
hsp::pie-1
hs (min.)
35
5
35
35
5
35
Input
PIE-1 IP
MEP-1
PIE-1

Figure 8-3

Figure 8-3. PIE-1 interaction with synMuv B components.

LET-418 and MEP-1 coimmunoprecipitated with PIE-1 from *hs-pie-1* extract made from heatshocked mixed stages embryos and not from wild-type extract (A and B, respectively).



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 9

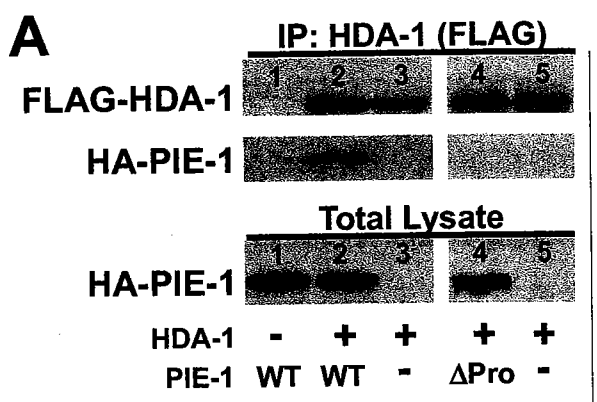
Figure 9. Somatic expression of PIE-1 induces the expression of PGL-1 protein and a SynMuv B phenotype.

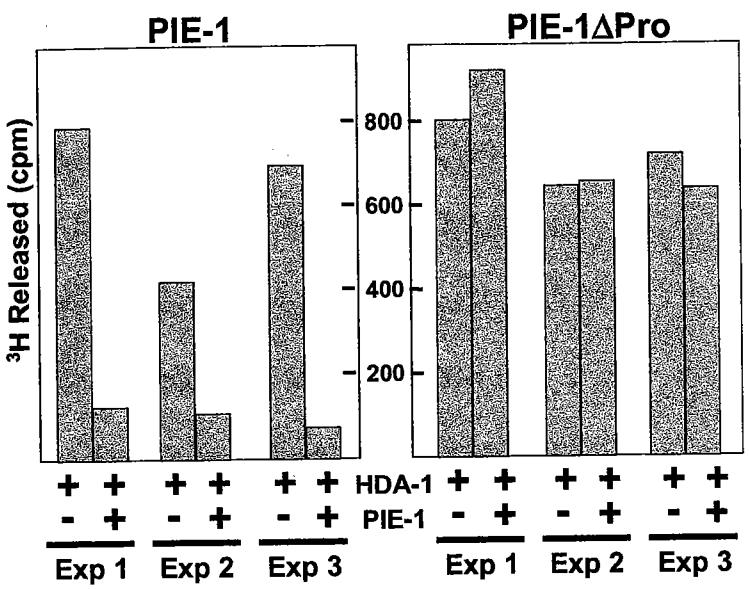
(A, B) Immunofluorescence micrographs of wild-type L1 larvae (A) and *hs::pie-1* L1 larvae (B) stained with the anti-PGL-1 antibody (k76) to detect PGL-1 protein expression (arrowheads). (C-E) Ectopic expression of PIE-1 induces a synMuv phenotype. (C and D) Light micrographs of a *lin-15A(n767)* adult (C) and of a *hs::pie-1;lin-15A(n767)* adult animal (D). Regions of vulva development in (C,D) are indicated with arrowheads. (E) Tabular representation of the data depicted in (D).

expression of PIE-1 can induce the synMuvB phenotype characteristic of MEP-1 and LET-418 inhibition. To do this, we constructed *lin-15A(n767)* mutant strains that carry either a full-length *hs::pie-1* transgene or a *pie-1* transgene bearing a deletion of the domain required for MEP-1 binding (*hs::pie-1ΔPro*, see Figure 8-2A,p.50). Strikingly, we found that even with mild heat shock (continuous culture at 23 to 26°C), a large fraction of the *lin-15A* animals bearing the full-length *pie-1* transgene exhibited a multi-vulva phenotype (Figures 9C-E, p.56). In contrast, although PIE-1ΔPro and full-length PIE-1 were expressed at similar levels in somatic nuclei of the transgenic animals (data not shown) we did not observe multi-vulva animals bearing the *hsp::pie-1ΔPro* strain (Figure 9C, p.56). 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 expression inhibits the activity rather than the expression of MEP-1 and LET-418. Under the conditions that induce 99% synMuv animals, strains carrying the hsp::*pie-1* gene produces 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, LET-418 and by extension the NURD complex. In order to further examine this model we asked whether PIE-1 can inhibit the histone deacetylase 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 immunoprecipitated via the epitope tag. We found that PIE-1 co-immunoprecipitated with HDA-1 in these assays, indicating that PIE-1 can form a complex with HDA-1 in mammalian cells (Figure 10A, lane 2, p.58). PIE-1 was not detected in the contol lanes where either HDA or PIE-1 alone was expressed. 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 co-immunoprecipitate with HDA-1 in these assays (Figure 10A, lane 4, p.60). In order to analyze the histone deacetylase activity of HDA-1, we used immunoblotting to estimate the amounts of HDA-1 protein present in each immunoprecipitation sample, and subjected approximately equal amounts of the 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 immunoprecipitated HDA-1 exhibits sodium butyrate-sensitive deacetylase activity when prepared from extracts containing no PIE-1 (Figure 10B, p.60).





C

Percentage Deacetylase Activity	
HDA-1	100%
HDA-1+PIE-1	18.7±7.5% (n=3)
HDA-1+PIE-1ΔC	102±1.2% (n=3)

Figure 10

Figure 10. 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 co precipitation of HA::PIE-1 (as indicated). (B) PIE-1 inhibits HDA-1 histone deacetylase activity. Equal amounts of HDA-1 protein precipitated from the transfected COS-7 cells obtained in three independent experiments were subjected to a histone deacetylase assay using a radio-labeled (^{3}H) acetylated synthetic peptide corresponding to the N-terminus of histone H4. 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 calculate from the data shown graphically in (B).

In contrast, samples immunoprecipitated in the presence of the full-length PIE-1 protein showed significantly reduced deacetylase activity (Figure 10B and C, p.60), indicating that PIE-1 inhibits the histone deacetylase activity of HDA-1. The C-terminally truncated form of PIE-1 (PIE-1ΔPro), which does not co-immunoprecipitate with HDA-1, had no affect on HDA-1 histone deacetylase activity (Figure 10B and C, p.60), 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 the HDA-1 protein or of endogenous β-actin in COS-7 cells (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 non-expressing 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 NURD complex activity.

II. MEP-1 antagonize the activities of the MES proteins

Results

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 wondered if the *mes* genes might also be required to promote ectopic germline-like development in the somatic cells of *mep-1(RNAi)* arrested larvae. Consistent with this idea, we found that the removal of each *mes(+)* activity, either by RNAi or by mutation, resulted in partial suppression of ectopic *PGL-1* expression and a partial rescue of *mep-1* and *let-418* larval lethality (Figure 11, p.62 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 11 and B, p.64). The *mes-2(bn11)* mutant induced only a slight (but significant) suppression of the L1 arrest, resulting in 4 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 11C, p.64).

The MES proteins are present in somatic cells at the stage immediately preceding the first ectopic expression of PGL-1 protein in *mep-1* and *let-418* depleted embryos (Capowski et al., 1991; Garvin et al., 1998; Seydoux and Strome, 1999). Therefore, MEP-1 and LET-418 may function to inactivate the MES proteins in wild-type somatic cells, or instead may function to prevent the continued somatic expression of the corresponding *mes* genes. Consistent with the former possibility, we found that the MES-2, MES-3, MES-4 and MES-6 proteins each exhibited a wild-type abundance and

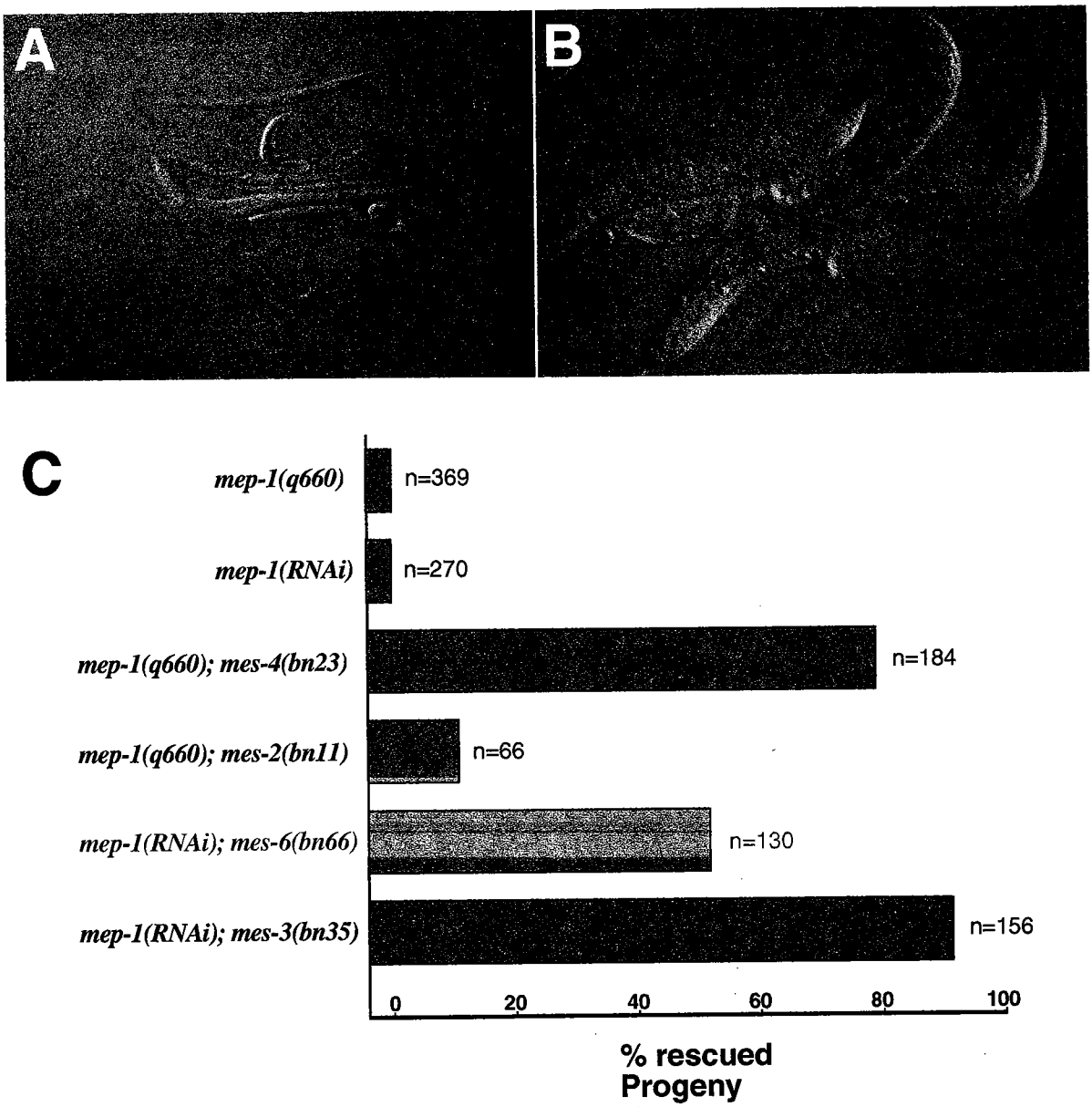

A
B
C
mep-1(q660)
n=369
mep-1(RNAi)
n=270
mep-1(q660); mes-4(bn23)
n=184
mep-1(q660); mes-2(bn11)
n=66
mep-1(RNAi); mes-6(bn66)
n=130
mep-1(RNAi); mes-3(bn35)
n=156
0
20
40
60
80
100
% rescued
Progeny

Figure 11

Figure 11. The *mes-2, mes-3, mes-4* and *mes-6* genes interact genetically with *mep-1*. (A,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 4 *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 (see Experimental Procedures), were identified as larvae and were monitored for development to the adult stage (% Adult Progeny).

localization in *mep-1(RNAi)* animals (data not shown). Thus, MEP-1 and LET-418 appear to antagonize the activity rather than the expression of the MES proteins. A previous study has reported that MES-2 and MES-6 are expressed at low levels in intestinal nuclei at later stages. Perhaps the particularly intense activation of germline-specific genes in intestinal cells in *mep-1(RNAi)* arrested larvae is driven, in part, by the higher levels of MES-2 and MES-6 that are found in these cells relative to other somatic cells.

CHAPTER IV

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. Somatic cells differentiate properly in embryos depleted of maternal MEP-1 protein, but after embryogenesis, the somatic cells appear to lose their differentiated state and begin to express gene products normally restricted to the germline. We have shown that MEP-1 forms a complex *in vivo* 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 (Korf et al., 1998).

In *C. elegans* embryos, MEP-1 and LET-418 are required to prevent germline development in the somatic tissues. This repressive function antagonizes positive inputs from MES-2, MES-3, MES-4 and MES-6. The *mes-2* and *mes-6* genes appear to be *C. elegans* orthologs of the PcG genes, *Enhancer of zeste* and *extra sex comb* (Holdeman et al., 1998; Paulsen et al., 1995). The *mes-4* gene does not have clear orthologs in other animals but encodes two conserved domains, a PHD domain and a SET domain. The PHD domain is found in proteins with roles in regulating transcription via modification

of chromatin structure (Holdeman et al., 1998; Korf et al., 1998; Zhang et al., 1998). The SET domain is found in a variety of transcriptional regulators, including Enhancer of Zeste/MES-2, Trithorax, Suppressor of Variegation 3-9, NSD1 and SET1p (Aasland et al., 1995; Jeanmougin et al., 1997; Winston and Allis, 1999). These include newly characterized histone methyltransferases, which can mediate histone H3 methylation, transcriptional repression, position-dependent variegation and heterochromatin formation in diverse species (Briggs et al., 2001; Jenuwein, 2001; Jenuwein et al., 1998; Nakayama et al., 2001). SET domains of the aformentioned proteins have been shown to be required in chromatin association, gene silencing, and histone methylation (Briggs et al., 2001; Chinwalla et al., 1995; Peters et al., 2001; Petruk et al., 2001; Rea et al., 2000). The PcG and TrxG proteins in *Drosophila* are the best characterized protein families known to participate in the maintenance of transcriptional states in animal development. Although these two groups of proteins have genetically distinct activities and include a variety of distinct types of proteins, both PcG and TrxG protein groups are thought to function in maintenance of transcriptional repression or activation through direct interactions with chromatin (reviewed in Ivanova et al., 1998; Nislow et al., 1997).

In *C. elegans*, the MES proteins, in addition to their role in germline maintenance, have been implicated in transcriptional silencing of high-copy number transgenes (Chan et al., 1994), suggesting that they may function to maintain heterochromatic transcriptional repression essential for germline maintenance. Our finding that the MES proteins promote the expression of germline-specific genes raises the interesting possibility that in addition to repressive functions, these proteins can also stabilize

transcriptionally active chromatin domains. Consistent with this idea, MES-4 protein is found associated with the transcriptionally active autosomes, but not with the repressed X chromosome during early embryogenesis in *C. elegans* or with silenced repetitive germline transgenes (Fong et al., 2002). In this light, it is noteworthy that although PcG proteins are generally associated with the repression of gene expression, the phenotypes of some PcG mutations, including that of the *Drosophila* MES-2 homolog, *En(z)*, are also consistent with their positive functions in transcription, at least with respect to certain target genes (Brock and van Lohuizen, 2001; Kelly and Fire, 1998; LaJeunesse and Shearn, 1996; Milne et al., 1999; Pirrotta, 1998; Sinclair et al., 1992).

Regulation of stage-specific transcription by MEP-1, LET-418 and the MES proteins

The findings described above suggest an intriguing model for the interactions between MEP-1, LET-418, and the MES proteins (Figure 12, p.70). According to this model, stage-specific patterns of chromatin organization are established sequentially within each cell lineage in the developing animal. Once established, these chromatin domains define transcriptional competency but need not be actively transcribed. Maintenance of these domains is controlled at least in part through the action of PcG- and SET domain-related proteins, including the MES proteins, which appear to exert their functions primarily in germline cells and perhaps in a limited number of their somatic descendants. These maintenance factors may be of special importance when founder

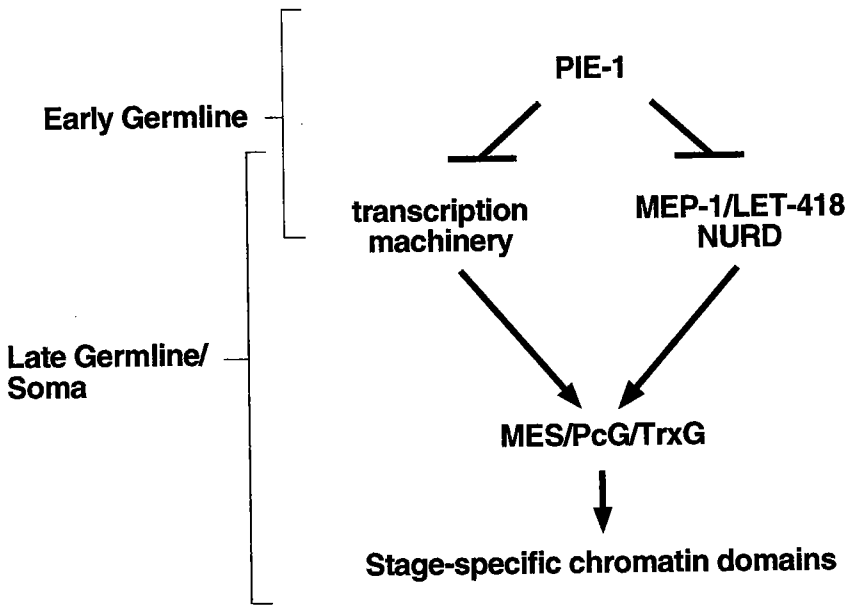

Early Germline
Late Germline/
Soma
PIE-1
transcription
machinery
MEP-1/LET-418
NURD
MES/PcG/TrxG
Stage-specific chromatin domains

Figure 12

Figure 12. 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 somatic cells, and after PIE-1 is gone in the germline, the concerted action of the transcriptional machinery and the NuRD complex modify chromatin, and concomitantly modify the distribution of chromatin associated proteins, including the MES proteins and 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.

cells are specified many hours before they initiate terminal cell-fate-specific differentiation, as is true for the germline precursors and the vulval precursors (see below). The MEP-1 and LET-418 proteins function 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 12, p.70). Thus chromatin remodeling by MEP-1 and LET-418, and by extension the NuRD complex, could function through PcG and TrxG related proteins to sequentially erase and establish new differentiation competent chromatin domains throughout development. Consistent with this idea, genetic analysis in *Drosophila* has shown that dMi-2 participates in Poly-comb mediated repression of HOX gene expression (Brock and van Lohuizen, 2001).

This model also suggests an explanation for the role of MEP-1 and LET-418 in regulating the competency of vulval precursor cells (VPCs) to respond to the vulval induction signal. During the first larval stage, six VPCs (named P3.p through P8.p) are selected from a group of twelve lateral ectodermal cells (Akasaka et al., 2001; Gildea et al., 2000). This selection process depends at least in part on the action of the *C. elegans* homeobox protein LIN-39, whose activity in these cells prevents them from fusing with the surrounding hypodermal syncitium (Kehle et al., 1998; Sulston and Horvitz, 1977; Sulston and White, 1980). Once selected, these six cells migrate to the ventral side of the larva. Approximately 36 hours later, during the mid L3 stage, vulval inductive signal from the anchor cell of the somatic gonad initiates vulval development in the three most

proximal VPCs, while the remaining three VPCs terminally differentiate and fuse with the hypodermal syncitium (reviewed in Maloof and Kenyon, 1998). The synMuvA and B pathways act redundantly during the larval stages to prevent the VPCs from responding inappropriately to the developmental signals for vulva differentiation (Clandinin et al., 1997). In mutants defective in both of the synMuv pathways, all six of the VPCs undergo vulval development in response to the vulval inductive signal. While little is known about the molecular function of synMuvA genes, the known components of the synMuvB pathway encode homologs of highly conserved transcriptional regulators. These include *C. elegans* homologs of E2F, its binding partner Dp, the Retinoblastoma protein Rb, the histone deacetylase HDAC/HDA-1, Mi-2/LET-418 and other components of the NuRD complex (Clark et al., 1993).

We propose that when the VPCs are specified during the L1 stage, chromatin-associated proteins, perhaps encoded by some of the numerous PcG and TrxG related proteins in *C. elegans* (Chamberlin and Thomas, 2000; Ferguson and Horvitz, 1989; Solari and Ahringer, 2000; Wang and Sternberg, 2001), establish a stable chromatin conformation that is competent to respond to vulval induction and to execute vulval differentiation in later development. When the gonad signals the VPCs to initiate vulval differentiation, SynMuvB components including MEP-1, LET-418, and HDA-1 are either downregulated 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 syncitium. 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 genes, are required during larval development for VPCs to stably inactivate the potential to undergo vulval differentiation.

A role for PIE-1 in regulating MEP-1, LET-418 and HDA-1

The observation that PIE-1 and MEP-1 interact in yeast two hybrid and appear to have nearly opposite functions in promoting or preventing germline development, respectively, suggests a model in which PIE-1 binds to and inactivates MEP-1 in the early embryonic germline. Consistent with this view, our data show that endogenous PIE-1 protein interacts with a MEP-1 protein complex *in vivo* through coimmunoprecipitation assays using early *C. elegans* embryo lysate. We also show that PIE-1 also interacts directly with MEP-1 in *in vitro* binding assays. Because the PIE-1 protein has additional functions in preventing the transformation of early germline blastomeres into somatic cells (Mello et al., 1992; 1996) it was not possible to examine the genetic relationship between these factors during germline development. For example, though a *pie-1;mep-1* dead embryo shows the *pie-1* phenotype, it also exhibits increased accumulation of P-granules in the somatic tissue. Instead, we decided to investigate the consequences of the ectopic expression of PIE-1 at later developmental times. Consistent with a negative regulatory relationship between PIE-1 and MEP-1, we

found that the inhibition of MEP-1 activity 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, our data show that an enzymatic component of the NURD complex, HDA-1, interacts with both PIE-1 and MEP-1 in *C. elegans* embryos, and furthermore, we have shown that PIE-1 significantly reduces the histone-deacetylase activity of HDA-1 when both proteins are co-expressed in vertebrate cells.

Taken together, the above findings are consistent with a simple model, in which PIE-1 transiently binds to and inactivates a complex that contains MEP-1, LET-418 and HDA-1 in the nucleus of early germline cells. By inhibiting the MEP-1 complex, PIE-1 may prevent chromatin remodeling in early germline cells, which could take place even in the absence of mRNA transcription. This remodeling in absence of transcription may possibly be due to the recruitment of chromatin remodeling factors to the chromatin by soma-specific transcription factors present in the nucleus of the germ cell. This activity of PIE-1 might thus ensure that germline cells maintain a chromatin confirmation that is poised to initiate germline development after the initial rapid phase of somatic differentiation is completed. Thus, we propose that in addition to its previously described role in blocking Pol II-dependent transcription (Xu and Strome, 2001), PIE-1 has a second role in promoting later germline-specific transcription by preventing chromatin remodeling at germline-specific loci during early embryogenesis.

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, at later times in the germline, MEP-1 and LET-418 may be required for chromatin remodeling that underlies developmental transitions from mitosis to meiosis, and from spermatogenesis to oogenesis. Consistent with this idea a recent report suggests that the X chromosome undergoes changes in histone modification consistent with chromatin remodeling during early to mid pachytene of meiosis in 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. 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. For example, *pie-1* is required for the efficient expression nos-2 protein (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*; and posttranscriptional and transcriptional silencing phenomena are clearly connected to one another in both plants and animals. 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 genes-silencing pathways.

The function of NuRD/SIN3 complex in development and the conservation of Mi-2 function in maintaining germline-soma distinctions

It is becoming clear that histone deacetylase complexes have specific functions in development as opposed to a global one. Recent works have shown that NuRD and SIN3 complex are involved in development and various repression mechanisms such as DNA-methylation, polycomb protein-dependent repression, and transcription repressors (Ahringer, 2000). In *Drosophila*, components of the NuRD and SIN3 complex are required for embryonic segmentation, embryonic/germ cell viability, and *hox* gene repression (Mannervik and Levine, 1999) (Kehle et al., 1998). In *C.elegans*, these components are required for embryonic lethality and vulval development, whereas in *Arabidopsis* they are required for the repression of embryo and meristem genes (Ogas et al., 1999; von Zelewsky et al., 2000).

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* (Kehle et al., 1998; von Zelewsky et al., 2000). *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., 1999). Thus, in

Arabidopsis, Mi-2-related proteins appear to function not only in the maintenance of somatic differentiation but indeed in the suppression of totipotency, the potential to make a whole new organism. Conceivably, the germline-like somatic cells in *mep-1(RNAi)* 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 mean for preventing stem cells from adopting differentiated fates or perhaps for resetting cells to more immature stem-cell fates.

A long-standing question in development biology centers on where the specificity lies for differences in the cell-type-specific or stage-specific outcomes of developmental signaling pathway. 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.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Since the identification of the *pie-1* mutant in a screen for maternal effect mutations that have defects in the specification of blastomere fates in *C. elegans*, much effort has been put into the elucidation of the mechanism of PIE-1 function in the specification of the germ cell fate. The majority of studies have focused on the role of PIE-1 in transcriptional regulation of germ cell precursors. We demonstrated that PIE-1 is in a complex with MEP-1 and LET-418, and that the overexpression of PIE-1 can mimic *mep-1/let-418* phenotype. Our data support a model in which PIE-1 has dual functions in the germ cell: 1.) the inhibition of RNA PolII-dependent transcription, and 2.) the inhibition of chromatin remodeling activity. The work done in this thesis has shown, for the first time in *C. elegans*, that the specification of germ cells utilizes a chromatin-based mechanism, and that this mechanism is conserved in other organism. Mutations in *arabidopsis Mi-2* exhibit similar phenotype to that of *C. elegans Mi-2(let-418)*, in which somatic cells partially transform into germ cell-like cells. This transformation suggests that the reorganization of the chromatin can regulate the totipotency of the somatic blastomeres.

During the course of this work, several questions have been raised. Although the phenotypical analysis of *mep-1* and *pie-1*, as well as the biochemical analysis, has pointed out a model in which PIE-1 inhibits the function of MEP-1/NuRD complex, the

exact mechanism of this inhibition is still unclear. A clue toward elucidating this interaction may lie in an interactor of PIE-1, CeUbc9, identified through a yeast two-hybrid screen. CeUbc9 is able to catalyze the sumoylation of MEP-1 in a PIE-1 dependent manner (Shin and Mello, unpublished data). Consistent with the model that MEP-1/LET-418 are regulated by sumoylation, a growing body of evidence show that transcriptional activators are inhibited by sumoylation (Verger et al., 2003). More over, SUMO-1/smt3C, a member of the SUMO family, interacts with the CHD3/ZFH Zn-finger-containing helicase that is present in histone deacetylase complexes (Minty et al., 2000). The significance of MEP-1 sumoylation will required further study.

The next question concerns the molecular basis of the genetic interaction between MEP-1/NuRD complex and the *mes* genes. Several studies have shown that chromatin remodeling and PcG/TrxG-dependent chromatin maintenance are converging on gene silencing at the chromatin level. Trithorax-containing complex has histone acetyltransferase activity (Petruk et al., 2001) while the PcG proteins, in contrast, are associated with HDAC-containing complexes (Kehle et al., 1998; Nakamura et al., 2002; Nakayama et al., 2001; Silva et al., 2003). The significance of the interaction between polycomb-related proteins and histone deacetylase complex in development is emphasized by our results showing that the *mes* genes are required for *mep-1*-dependent repression of germline gene expression in the soma. However, the mechanism of how MES proteins function to facilitate MEP-1/NuRD complex is not examined in this thesis. Previous studies on interactions between polycomb-group proteins and histone deacetylase complexes (Kehle et al., 1998; Lachner et al., 2001) suggest a model in

which MEP-1/NuRD complex recruits MES proteins which then mark the chromatin expression domains, perhaps via DNA modification such as methylation, and transcriptionally represses that chromatin domain. This model can contribute toward the explanation of the suppression of the loss of *mep-1* phenotype by mutation in the *mes* genes. According to work by Fong et. al., MES-4 localization to the active autosomes, as oppose to the generally inactive X chromosome in the C. elegans hermaphrodite, required MES-2, 3 and 6 (Fong et al., 2002); MES-2 and MES-6 are homologs of polycomb-group proteins while MES-3 is a novel protein (Seydoux and Strome, 1999). Thus, MES-2,3,and 6 may exclusively function to localize MES-4, which then act as a positive regulator of germ-specific transcription program. This explanation depicts MES-4 as functionally resemblance to trxG in which MES-4 is require for the activation of germ-specific genes. How then is the NuRD complex able to specifically erase the mes-dependent, transcriptionally active germ-specific chromatin loci, as shown in this work? MES-2,3,and 6 may function to localize the NuRD complex to erase the germ-specific chromatin domain activated by MES-4. This antagonistic action between NuRD and MES-4 is predicted to involve the regulation of histone methylation/acetylation state. The examination of the histone methylation/acetylation state of MEP-1/NuRD target genes in wild type and *mes* background may shed light on this problem.

After the work from this thesis was published, additional interactions between *mes/polycomb* genes and synMuv pathway genes are being discovered, further solidifying the significance of our data on genetic interactions between *mes* genes and *mep-1*. Genetic interaction between *mes-4* and *lin-35(Rb)*, a component of the synMuvB

pathway, has been reported (Suh and Strome, unpublished data). In addition, mutations in the ISW-1, a C. elegans homolog of the component of Drosophila remodeling complex ISWI, can suppress the synMuv phenotype (Anderson and Horvitz, unpublished data).

Finally, MEP-1 was also identified as an interactor of MOG proteins through yeast two hybrid (Belfiore et al., 2002). MOG-1,4 and 5, have been shown to be required for the repression of the 3'UTR of *fem-3*, a sex-determination gene (Gallegos et al., 1998). The MEP-1/MOG interaction raises the question of whether MEP-1 also is required in translational control (see discussion). Based upon the nuclear localization of MEP-1, it is unlikely that MEP-1 directly regulates *fem-3* because MEP-1 does not bind to the regulatory region of *fem-3* 3'UTR (Puoti, unpublished data). The *C.elegans* FBF protein, a homolog of *Drosophila* PUMILIO, binds to this regulatory region of *fem-3* 3'UTR and regulates *fem-3* expression (Zhang et al., 1997a). It is possible that MEP-1 regulates an intermediate component which is required for *fem-3* 3'UTR regulation. We also cannot rule out the possibility that *mep-1/mog* gene products may act independently to regulate *fem-3* RNA. Addressing these possibilities will be necessary to elucidate the mechanism of the MEP-1/MOG interaction.

The ability of the germ cell to differentiate into many cell types, termed pluripotency, is evident in many organisms. mouse genital ridges transplanted into ectopic sites can transform into tumors that differentiate into many cell types (Stevens, 1968; Stevens, 1970). Pluripotential cell lines can be derived from murine diploid PGCs in culture (Matsui et al., 1991; Resnick et al., 1992). Migrating PGCs from Xenopus, which were extracted and transplanted into the early embryo, can differentiate into other

cell types (Wylie, 1999). These findings suggest that the germ cell has the potential to differentiate into many types of cells. If there is a conservation of Mi-2 function in germ/soma distinction, our findings suggests a valuable source of pluripotent cell in which the somatic cell may be made pluripotent by transient inhibition of the Mi-2. This notion will have great implications in the field of organ transplantation/tissue regeneration. 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.

pgl-1 and deficiency in RNAi inheritance

Through the course of *mep-1* research, we serendipitously came across an uncharacterized phenotype of the *pgl-1* mutant; we found that the *pgl-1* mutant is deficient in the inheritance of RNAi.

Introduction. RNA interference (RNAi) is a term used to describe a dsRNA-induced, sequence-specific, gene-silencing phenomenon. RNAi was first observed in *C. elegans* although similar homology-dependent silencing phenomenon have been seen in various eukaryotes (Hannon, 2002). In *C. elegans*, introduction of dsRNA of gene-encoding sequences conferred instability of mRNA of the corresponding genes while sequences in the promoter and intronic region were generally ineffective, suggesting that RNAi was operating at the post-transcriptional levels (Fire et al., 1998). In plant, pigment-encoding transgenes introduced into petunias resulted in variegated

pigmentation, with some petunias losing all of the pigment (Napoli et al., 1990; van der Krol et al., 1990). In contrast to worms, introduction of the dsRNA containing sequences of the promoter regions could induce transcriptional silencing in plants as well as the methylation of the genomic sequence homologous to the trigger (Mette et al., 2000; Wassenegger et al., 1994). To add more pieces to the puzzle, components of the RNAi machinery may also function at the translational level in *C. elegans* (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001). Other evidence also suggests that chromatin modification is correlated with RNAi activity in yeast (Hall et al., 2003; Schramke and Allshire, 2003; Volpe et al., 2002). These observations suggest that RNAi machinery is utilized in several gene regulation processes in diverse organisms.

To this end, a simple model for the mechanism of RNAi is emerging in which the RNAi process is grouped into the initiation step and the effector step. Introduced dsRNA trigger has shown to be processed into 25 nucleotide long dsRNAs, termed small interfering RNAs (siRNAs) (Hammond et al., 2000; Yang et al., 2000; Zamore et al., 2000), in the initiation step. This dsRNA has been shown to mediate RNAi (Elbashir et al., 2001) and is processed by the enzyme called DICER, a protein containing an RNase III catalytic domain, as well as additional helicase and PAZ motifs (Bernstein et al., 2001). Next comes the effector step in which the siRNAs are paired up with the homologous target mRNA substrates via the RISC complex (RNA-induced silencing complex), which was also identified using the *Drosophila* extract (Hammond et al., 2000).

In *C.elegans*, Mello and colleagues have identified a collection of *rde (RNAi deficient)* mutants through a genetic screen for mutations which confer insensitivity to RNAi (Tabara et al., 1999b). Among the *rde* mutants identified are *rde*-1 and *rde-4*; RDE-1 is a homolog of the Argonaute protein family whose member was later found to copurify along with the RISC complex (Hammond et al., 2001) while RDE-4 encodes a dsRNA binding protein (Tabara et al., 1999b; Tabara et al., 2002). *rde-1* and *rde-4* function in the initiation step of RNAi while *rde-2* and *mut-7* is required in the effector step, as defined genetically by Grishok et. al. (Grishok et al., 2000). How *rde-2* and *mut-7* function in the effector step is not known. However, RDE-1 and RDE-4 has been shown to exist as a complex with the *C. elegans* Dicer (Tabara et al., 2002). RDE-4 may function in the recognizing of the dsRNA and deliver it to DICER for processing, while RDE-1 may function downstream of DICER as a bridge between DICER and RISC complex. Consistent with this model, siRNA, while greatly reduced in RDE-4 mutant, is not altered in RDE-1 mutant (Parrish and Fire, 2001).

Exactly how Dicer recognizes the dsRNA, how the RISC complex is activated, and how the complex induces RNAi are not yet clear. Thus far, small dsRNA appears to be the conserved aspect in the RNAi processes of several model organisms although several differences also exist within those model organisms. Whereas *Drosophila* and mammal model exhibit cell-autonomous, non-inheritable gene silencing, C. elegans exhibits systemic and inheritable one. The nature of silencing in plant is a hybrid of the two previous examples in which the silencing is systemic but the inheritability of the silencing has not yet been shown. Thus, germline transmission of RNAi in *C. elegans*,

which can last up to two generations (Grishok et al., 2000), present us with a very unique question. What are the factors required for the inheritance of RNAi and how is the inheritance carried out? Here we describe the requirement of a unique component of the germplasm, the P-granules proteins PGL, for the inheritance of RNAi in *C. elegans*.

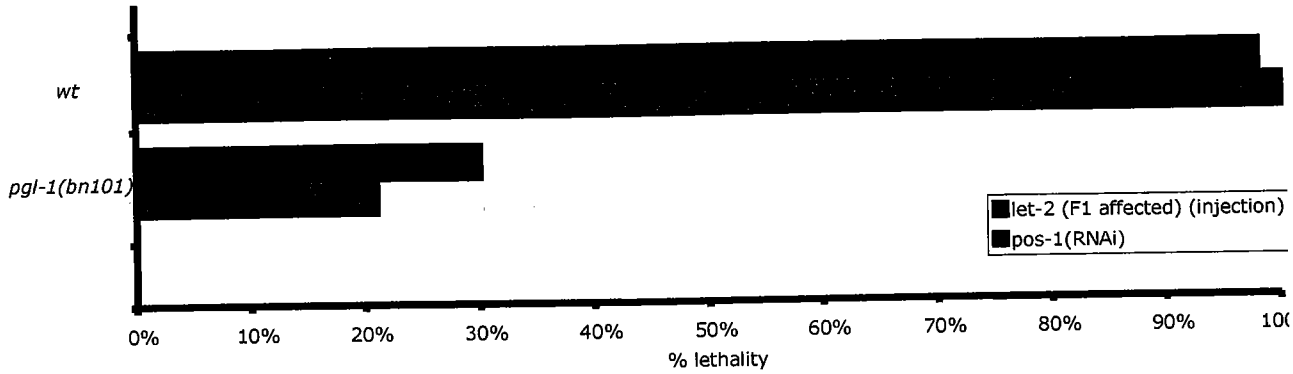
Results and discussion. *mep-1* mutation results in arrested larvae with ectopic P-granule expression, including PGL-1 epitope. To find out whether the ectopic expression of PGL-1 is the cause of the arrested-larva phenotype, we performed *mep-1(RNAi)* in *pgl-1 (bn101)* animal. A small percentage of *pgl-1(bn101);mep-1(RNAi)* animals escaped the larval arrest and developed into adult worm, in strike contrast to the 100% L1-stage arrested *mep-1(RNAi)* animal. This observation suggests that either *pgl-1* is required for *mep-1* phenotype or that *pgl-1* may be required for RNAi. Subsequent injection into *pgl-1* with control dsRNA such as *mex-5* (a maternal gene required for the anterior blastomere suppression of germline genes, see above for details) and *unc-22* (a muscle-specific gene) showed a small percentage of unaffected progeny to either injection, indicating the partial resistance of *pgl-1* mutant to RNAi (Mello, unpublished observations).

Interestingly, although the progeny of *pgl-1* is partially resistant to *mex-5* and *unc-22 (RNAi)*, Po generation was not sensitive to the RNAi of *unc-22* (Mello lab, unpublished observation). To distinguish whether *pgl-1* is required for RNAi in Po or F1 generation, we subjected wild type and *pgl-1(bn101)* worms to RNAi of different genes via injection or feeding (see Figure 13, p.88,89). Results show that *pgl-1* is partially resistant to a number of somatic and germline genes in the F1 generation. Progenies of

pgl-1 animal were approximately 70% viable on *let-2* food compared to 0% viability in wild-type (Figure 13A, p.88). *pos-1* RNAi feeding of *pgl-1* animal resulted in embryonic lethality of all of the progeny, with 30% of the dead embryos making gut in contrast to wild type worms fed to pos-1 dsRNA, which resulted in 100% dead embryos which made no gut, a hallmark of *pos-1* phenotype. Injection of *unc-22* dsRNA caused F1 progeny to weakly twitch in levamisole while wild-type control animals twitch more severely (Figure 13B, p.88). *sqt-3* RNAi resulted in variety of phenotypes, ranging from embryonic lethality to viable dumpy animal. To further distinguish the resistance of *pgl-1* mutant to *sqt-3* dsRNA, we titrated the *sqt-3* dsRNA concentration. The result showed that with lower concentration of *sqt-3* dsRNA, various *sqt-3* phenotypes became less severe in wild-type worms while *pgl-1* mutants exhibited a still less severe *sqt-3* phenotype than the wild-type worms at both concentration of *sqt-3* dsRNA injected (Figure 13D, p.89). On the contrary to the observation that *pgl-1* is partially resistance to RNAi at the F1 generation, *pgl-1* is completely sensitive to RNAi in the P0 generation as wild-type worms (Figure 13C) while L1 staged *pgl-1* and wild-type worms cultured on *let-2* food exhibited *let-2* phenotype of sterile and bursting worm at 100%. *unc-22* dsRNA injection into *pgl-1* caused vigorous twitching in P0 generation, comparable to wild-type (Figure 13B, p.88). These preliminary results indicate that *pgl-1* mutation confers RNAi resistance in the F1 generation.

How do P-granules confer RNAi resistance in F1 and not the P0 generation? Since P-granule components are present only in the germline and are inherited from the

A

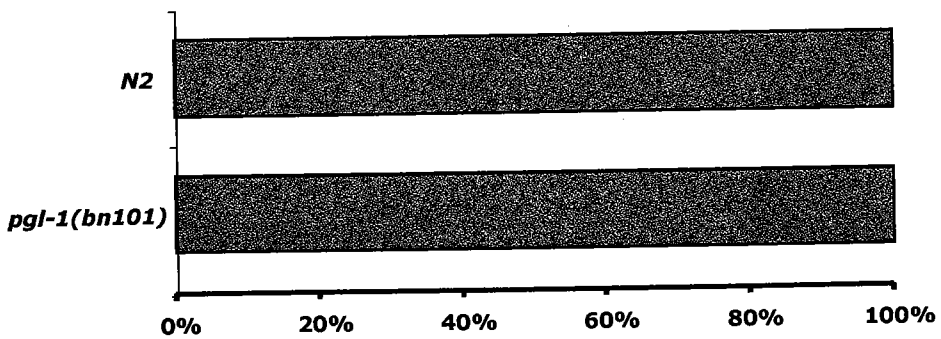


B

***unc-22* dsRNA feeding**

	Po twitching	**F1 twitching in1mM levamisole**
wt	+++	+++
pgl-1(bn101)	+++	+

C



% Lethality on *let-2(RN* food

Figure 13 A-C

D

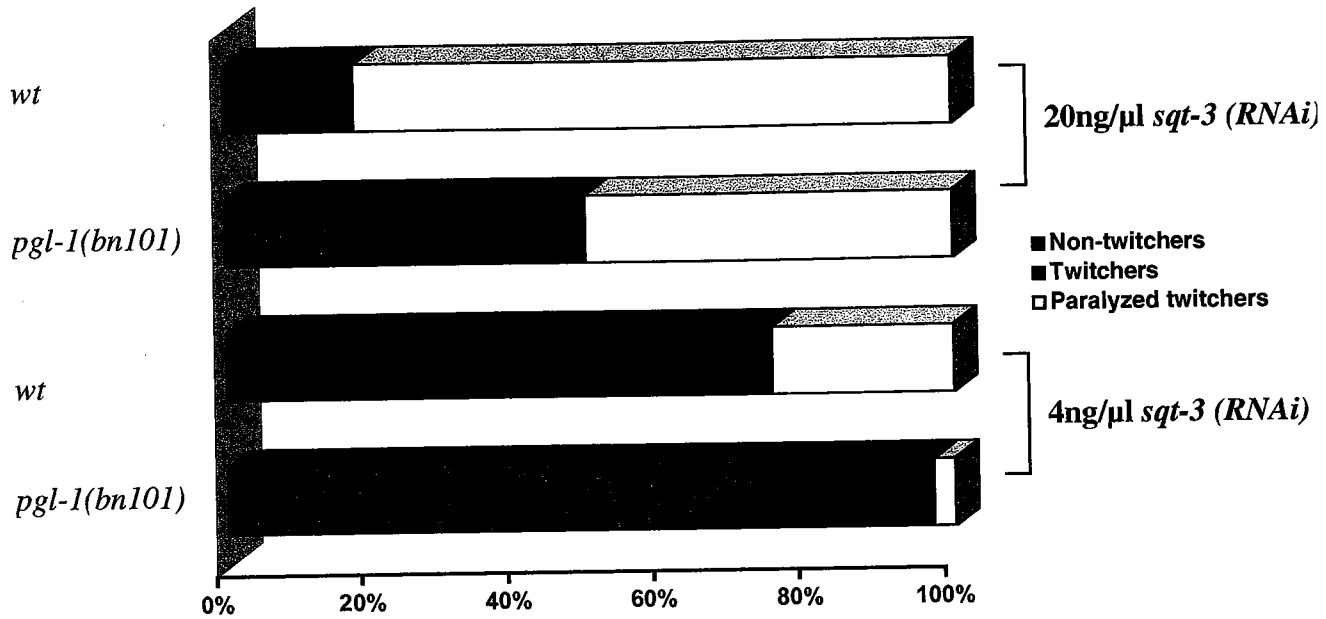

wt
pgl-1(bn101)
wt
pgl-1(bn101)
20ng/µl sqt-3 (RNAi)
4ng/µl sqt-3 (RNAi)
Non-twitchers
Twitchers
Paralyzed twitchers
0%
20%
40%
60%
80%
100%

Figure 13D

Figure 13. *pgl-1* is partially resistant to RNAi in the F1 generation.

(A) Bar graphs showing *let-2* and *pos-2 (RNAi)* in *pgl-1* (bn101) and wildtype. *let-2 (RNAi)* is depicted in red bars and pos-1(RNAi) is depicted in pink bars. (B) Table showing *unc-22* dsRNA injection in *pgl-1 (bn101)* and wild-type. For F1 analysis, 1mM levamisole is utilized to optimize the scoring of otherwise weak twitching of F1 *unc-22(RNAi)* animal. (C) Bar graphs of *let-2(RNAi)* phenotype exhibited by P0 of *wild-type* and *pgl-1 (bn101)*. L1 animals from each background were cultured on *let-2* RNAi food. (D) Bar graphs despicting the quantification of *sqt-3* phenotypes of *sqt-3 (RNAi)* animals in wild-type and *pgl-1 (bn101)* background, using two different concentration of *sqt-3* dsRNA.

previous generation, it is very probable that P-granules may be required for the inheritance of RNAi of the genes tested above. RNAs of various genes have been observed to localized to P-granules (Schisa et al., 2001). Thus, P-granules may serve as a reservoir of dsRNA which causes RNAi in the next generation. This interesting possibility predicts that we may detect 25nt dsRNA associated with P-granules and that these small dsRNAs will be reduced in *pgl-1* mutant.

The observation that *pgl-1* is resistant to RNAi, as well as *pgl-1* sterile phenotype which is present in both permissive (15°) and nonpermissive temperature(25°), is explainable by the fact that there exist *pgl-1* homologs within the worm genome, namely *pgl-2* and *pgl-3*. These homolog could redundantly function with *pgl-1*, explaining the *pgl-1* partial resistance of RNAi. Moreover, there are also other P-granule components such as the GLH proteins (Roussell and Bennett, 1993) which can further contribute to the functional redundancy of the P-granule components. *pgl-1* partial resistance to the inheritance of RNAi may also implicates RNAi as a process that is required for the proper development of the *C. elegans* germline. It will be important to address whether PGL-1 homologs, as well as the GLH proteins, can synergistically affect the RNAi resistance of *pgl-1*.

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),hsp::pie-1, and lin-15AB((e1763) were kindly provided by A. Puoti (University of Fribourg), Geraldine Seydoux, 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 (Roussell and Bennett, 1993). 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 ~1kb 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 transform 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 200µg/ml yeast DNA and 100µg/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 (Lachner et al., 2001), 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 twenty 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 (Belfiore et al., 2002).

The L1 arrest phenotype induced by RNAi was compared to the phenotype of *mep-1(q660).* Although *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 kD and 280 kD in size, respectively. Affinity purified sera were used for immunofluorescence staining at a 1:100 dilution. Embryos and larvae were made permeable for staining by a "freeze-crack" method and fixed in methanol/acetone according to the standard procedures (Gallegos et al., 1998). Mouse monoclonal anti-PGL-1 antibodies (Rocheleau et al., 1999) and Chicken anti-GLH-2 and anti-GLH-3 antibodies (James 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 Motohashi et al. (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 0.1M triethanolamine (pH 8.0) for 2 min, then in 0.15% acetic anhydride, 0.1M 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 were homogenized in the buffer containing 25mM HEPES-NaOH pH 7.4, 150mM NaCl, 1mM 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., 1997). Approximately 1mg of protein was 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.

BIBLIOGRAPHY

Aasland, R., Gibson, T. J., and Stewart, A. F. (1995). The PHD finger: implications for chromatin-mediated transcriptional regulation. Trends Biochem Sci *20*, 56-59.

Ahringer, J. (2000). NuRD and SIN3 histone deacetylase complexes in development. Trends Genet *16*, 351-356.

Akasaka, T., van Lohuizen, M., van der Lugt, N., Mizutani-Koseki, Y., Kanno, M., Taniguchi, M., Vidal, M., Alkema, M., Berns, A., and Koseki, H. (2001). Mice doubly deficient for the Polycomb Group genes Mel18 and Bmi1 reveal synergy and requirement for maintenance but not initiation of Hox gene expression. Development *128*, 1587-1597.

Asaoka, M., Sano, H., Obara, Y., and Kobayashi, S. (1998). Maternal Nanos regulates zygotic gene expression in germline progenitors of Drosophila melanogaster. Mech Dev *78*, 153-158.

Asaoka-Taguchi, M., Yamada, M., Nakamura, A., Hanyu, K., and Kobayashi, S. (1999). Maternal Pumilio acts together with Nanos in germline development in Drosophila embryos. Nat Cell Biol *1*, 431-437.

Barabino, S. M., Hubner, W., Jenny, A., Minvielle-Sebastia, L., and Keller, W. (1997). The 30-kD subunit of mammalian cleavage and polyadenylation specificity factor and its yeast homolog are RNA-binding zinc finger proteins. Genes Dev *11*, 1703-1716.

Batchelder, C., Dunn, M. A., Choy, B., Suh, Y., Cassie, C., Shim, E. Y., Shin, T. H., Mello, C., Seydoux, G., and Blackwell, T. K. (1999). Transcriptional repression by the Caenorhabditis elegans germ-line protein PIE-1. Genes Dev *13*, 202-212.

Beams, H. W., and Kessel, R. G. (1974). The problem of germ cell determinants. Int Rev Cytol *39*, 413-479.

Belfiore, M., Mathies, L. D., Pugnale, P., Moulder, G., Barstead, R., Kimble, J., and Puoti, A. (2002). The MEP-1 zinc-finger protein acts with MOG DEAH box proteins to control gene expression via the fem-3 3' untranslated region in Caenorhabditis elegans. Rna *8*, 725-739.

Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409*, 363-366.

Bowerman, B., Eaton, B. A., and Priess, J. R. (1992). skn-1, a maternally expressed gene required to specify the fate of ventral blastomeres in the early C. elegans embryo. Cell *68*, 1061-1075.

Briggs, S. D., Bryk, M., Strahl, B. D., Cheung, W. L., Davie, J. K., Dent, S. Y., Winston, F., and Allis, C. D. (2001). Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in Saccharomyces cerevisiae. Genes Dev *15*, 3286-3295.

Brock, H. W., and van Lohuizen, M. (2001). The Polycomb group--no longer an exclusive club? Curr Opin Genet Dev *11*, 175-181.

Burd, C. G., and Dreyfuss, G. (1994). Conserved structures and diversity of functions of RNA-binding proteins. Science *265*, 615-621.

Cairns, B. R., Kim, Y. J., Sayre, M. H., Laurent, B. C., and Kornberg, R. D. (1994). A multisubunit complex containing the SWI1/ADR6, SWI2/SNF2, SWI3, SNF5, and SNF6 gene products isolated from yeast. Proc Natl Acad Sci U S A *91*, 1950-1954.

Campbell, K. H., McWhir, J., Ritchie, W. A., and Wilmut, I. (1996). Sheep cloned by nuclear transfer from a cultured cell line. Nature *380*, 64-66.

Capowski, E. E., Martin, P., Garvin, C., and Strome, S. (1991). Identification of grandchildless loci whose products are required for normal germ-line development in the nematode Caenorhabditis elegans. Genetics *129*, 1061-1072.

Chamberlin, H. M., and Thomas, J. H. (2000). The bromodomain protein LIN-49 and trithorax-related protein LIN-59 affect development and gene expression in Caenorhabditis elegans. Development *127*, 713-723.

Chan, C. S., Rastelli, L., and Pirrotta, V. (1994). A Polycomb response element in the Ubx gene that determines an epigenetically inherited state of repression. Embo J *13*, 2553-2564.

Chen, T., Damaj, B. B., Herrera, C., Lasko, P., and Richard, S. (1997). Self-association of the single-KH-domain family members Sam68, GRP33, GLD-1, and Qk1: role of the KH domain. Mol Cell Biol *17*, 5707-5718.

Chinwalla, V., Jane, E. P., and Harte, P. J. (1995). The Drosophila trithorax protein binds to specific chromosomal sites and is co-localized with Polycomb at many sites. Embo J *14*, 2056-2065.

Clandinin, T. R., Katz, W. S., and Sternberg, P. W. (1997). Caenorhabditis elegans HOM-C genes regulate the response of vulval precursor cells to inductive signal. Dev Biol *182*, 150-161.

Clark, S. G., Chisholm, A. D., and Horvitz, H. R. (1993). Control of cell fates in the central body region of C. elegans by the homeobox gene lin-39. Cell *74*, 43-55.

Cote, J., Quinn, J., Workman, J. L., and Peterson, C. L. (1994). Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex. Science *265*, 53-60.

Crittenden, S. L., Rudel, D., Binder, J., Evans, T. C., and Kimble, J. (1997). Genes required for GLP-1 asymmetry in the early Caenorhabditis elegans embryo. Dev Biol *181*, 36-46.

Dahmus, M. E. (1996). Reversible phosphorylation of the C-terminal domain of RNA polymerase II. J Biol Chem *271*, 19009-19012.

De Rubertis, F., Kadosh, D., Henchoz, S., Pauli, D., Reuter, G., Struhl, K., and Spierer, P. (1996). The histone deacetylase RPD3 counteracts genomic silencing in Drosophila and yeast. Nature *384*, 589-591.

Deshpande, G., Calhoun, G., Yanowitz, J. L., and Schedl, P. D. (1999). Novel functions of nanos in downregulating mitosis and transcription during the development of the Drosophila germline. Cell *99*, 271-281.

Draper, B. W., Mello, C. C., Bowerman, B., Hardin, J., and Priess, J. R. (1996). MEX-3 is a KH domain protein that regulates blastomere identity in early C. elegans embryos. Cell *87*, 205-216.

Eckmann, C. R., Kraemer, B., Wickens, M., and Kimble, J. (2002). GLD-3, a bicaudal-C homolog that inhibits FBF to control germline sex determination in C. elegans. Dev Cell *3*, 697-710.

Eddy, E. M. (1975). Germ plasm and the differentiation of the germ cell line. Int Rev Cytol *43*, 229-280.

Edgar, L. G., Wolf, N., and Wood, W. B. (1994). Early transcription in Caenorhabditis elegans embryos. Development *120*, 443-451.

Edgley, M., D'Souza, A., Moulder, G., McKay, S., Shen, B., Gilchrist, E., Moerman, D., and Barstead, R. (2002). Improved detection of small deletions in complex pools of DNA. Nucleic Acids Res *30*, e52.

Elbashir, S. M., Lendeckel, W., and Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev *15*, 188-200.

Ephrussi, A., Dickinson, L. K., and Lehmann, R. (1991). Oskar organizes the germ plasm and directs localization of the posterior determinant nanos. Cell *66*, 37-50.

Ephrussi, A., and Lehmann, R. (1992). Induction of germ cell formation by oskar. Nature *358*, 387-392.

Ferguson, E. L., and Horvitz, H. R. (1989). The multivulva phenotype of certain Caenorhabditis elegans mutants results from defects in two functionally redundant pathways. Genetics *123*, 109-121.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806-811.

Fong, Y., Bender, L., Wang, W., and Strome, S. (2002). Regulation of the different chromatin states of autosomes and X chromosomes in the germ line of C. elegans. Science *296*, 2235-2238.

Francis, R., and Waterston, R. H. (1991). Muscle cell attachment in Caenorhabditis elegans. J Cell Biol *114*, 465-479.

Gallegos, M., Ahringer, J., Crittenden, S., and Kimble, J. (1998). Repression by the 3' UTR of fem-3, a sex-determining gene, relies on a ubiquitous mog-dependent control in Caenorhabditis elegans. Embo J *17*, 6337-6347.

Garcia-Ramirez, M., Rocchini, C., and Ausio, J. (1995). Modulation of chromatin folding by histone acetylation. J Biol Chem *270*, 17923-17928.

Gardner, R. L., and Rossant, J. (1979). Investigation of the fate of 4-5 day post-coitum mouse inner cell mass cells by blastocyst injection. J Embryol Exp Morphol *52*, 141-152.

Garvin, C., Holdeman, R., and Strome, S. (1998). The phenotype of mes-2, mes-3, mes-4 and mes-6, maternal-effect genes required for survival of the germline in Caenorhabditis elegans, is sensitive to chromosome dosage. Genetics *148*, 167-185.

Gildea, J. J., Lopez, R., and Shearn, A. (2000). A screen for new trithorax group genes identified little imaginal discs, the Drosophila melanogaster homologue of human retinoblastoma binding protein 2. Genetics *156*, 645-663.

Ginsburg, M., Snow, M. H., and McLaren, A. (1990). Primordial germ cells in the mouse embryo during gastrulation. Development *110*, 521-528.

Gostissa, M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S. E., Scheffner, M., and Del Sal, G. (1999). Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1. Embo J *18*, 6462-6471.

Gotta, M., and Ahringer, J. (2001). Axis determination in C. elegans: initiating and transducing polarity. Curr Opin Genet Dev *11*, 367-373.

Grishok, A., Pasquinelli, A. E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D. L., Fire, A., Ruvkun, G., and Mello, C. C. (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell *106*, 23-34.

Grishok, A., Tabara, H., and Mello, C. C. (2000). Genetic requirements for inheritance of RNAi in C. elegans. Science *287*, 2494-2497.

Gruidl, M. E., Smith, P. A., Kuznicki, K. A., McCrone, J. S., Kirchner, J., Roussell, D. L., Strome, S., and Bennett, K. L. (1996). Multiple potential germ-line helicases are components of the germ-line-specific P granules of Caenorhabditis elegans. Proc Natl Acad Sci U S A *93*, 13837-13842.

Grunstein, M. (1997). Histone acetylation in chromatin structure and transcription. Nature *389*, 349-352.

Guedes, S., and Priess, J. R. (1997). The C. elegans MEX-1 protein is present in germline blastomeres and is a P granule component. Development *124*, 731-739.

Gurdon, J. (1974). The control of gene expression in animal develpment (Oxford, Oxford University Press).

Hall, I. M., Noma, K., and Grewal, S. I. (2003). RNA interference machinery regulates chromosome dynamics during mitosis and meiosis in fission yeast. Proc Natl Acad Sci U S A *100*, 193-198.

Hammond, S. M., Bernstein, E., Beach, D., and Hannon, G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature *404*, 293-296.

Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R., and Hannon, G. J. (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. Science *293*, 1146-1150.

Hannon, G. J. (2002). RNA interference. Nature *418*, 244-251.

Hansen, J. C., and Wolffe, A. P. (1992). Influence of chromatin folding on transcription initiation and elongation by RNA polymerase III. Biochemistry *31*, 7977-7988.

Hirschhorn, J. N., Brown, S. A., Clark, C. D., and Winston, F. (1992). Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure. Genes Dev *6*, 2288-2298.

Holdeman, R., Nehrt, S., and Strome, S. (1998). MES-2, a maternal protein essential for viability of the germline in Caenorhabditis elegans, is homologous to a Drosophila Polycomb group protein. Development *125*, 2457-2467.

Horn, P. J., and Peterson, C. L. (2002). Molecular biology. Chromatin higher order folding--wrapping up transcription. Science *297*, 1824-1827.

Hutvagner, G., McLachlan, J., Pasquinelli, A. E., Balint, E., Tuschl, T., and Zamore, P. D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science *293*, 834-838.

Ikenishi, K. (1987). Functional gametes derived from explants of single blastomeres containing the "germ plasm" in Xenopus laevis: a genetic marker study. Dev Biol *122*, 35-38.

Illmensee, K., and Mahowald, A. P. (1974). Transplantation of posterior polar plasm in Drosophila. Induction of germ cells at the anterior pole of the egg. Proc Natl Acad Sci U S A *71*, 1016-1020.

Ivanova, A. V., Bonaduce, M. J., Ivanov, S. V., and Klar, A. J. (1998). The chromo and SET domains of the Clr4 protein are essential for silencing in fission yeast. Nat Genet *19*, 192-195.

James, P., Halladay, J., and Craig, E. A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics *144*, 1425-1436.

Jan, E., Motzny, C. K., Graves, L. E., and Goodwin, E. B. (1999). The STAR protein, GLD-1, is a translational regulator of sexual identity in Caenorhabditis elegans. Embo J *18*, 258-269.

Jeanmougin, F., Wurtz, J. M., Le Douarin, B., Chambon, P., and Losson, R. (1997). The bromodomain revisited. Trends Biochem Sci *22*, 151-153.

Jenuwein, T. (2001). Re-SET-ting heterochromatin by histone methyltransferases. Trends Cell Biol *11*, 266-273.

Jenuwein, T., Laible, G., Dorn, R., and Reuter, G. (1998). SET domain proteins modulate chromatin domains in eu- and heterochromatin. Cell Mol Life Sci *54*, 80-93.

Kadonaga, J. T. (1998). Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines. Cell *92*, 307-313.

Kadosh, D., and Struhl, K. (1998). Targeted recruitment of the Sin3-Rpd3 histone deacetylase complex generates a highly localized domain of repressed chromatin in vivo. Mol Cell Biol *18*, 5121-5127.

Kawasaki, I., Shim, Y. H., Kirchner, J., Kaminker, J., Wood, W. B., and Strome, S. (1998). PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in C. elegans. Cell *94*, 635-645.

Kehle, J., Beuchle, D., Treuheit, S., Christen, B., Kennison, J. A., Bienz, M., and Muller, J. (1998). dMi-2, a hunchback-interacting protein that functions in polycomb repression. Science *282*, 1897-1900.

Kelly, W. G., and Fire, A. (1998). Chromatin silencing and the maintenance of a functional germline in Caenorhabditis elegans. Development *125*, 2451-2456.
Kemphues, K. (2000). PARsing embryonic polarity. Cell *101*, 345-348.

Ketting, R. F., Fischer, S. E., Bernstein, E., Sijen, T., Hannon, G. J., and Plasterk, R. H. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev *15*, 2654-2659.

Kobayashi, S., Yamada, M., Asaoka, M., and Kitamura, T. (1996). Essential role of the posterior morphogen nanos for germline development in Drosophila. Nature *380*, 708-711.

Korf, I., Fan, Y., and Strome, S. (1998). The Polycomb group in Caenorhabditis elegans and maternal control of germline development. Development *125*, 2469-2478.

Kuo, M. H., and Allis, C. D. (1998). Roles of histone acetyltransferases and deacetylases in gene regulation. Bioessays *20*, 615-626.

Kuznicki, K. A., Smith, P. A., Leung-Chiu, W. M., Estevez, A. O., Scott, H. C., and Bennett, K. L. (2000). Combinatorial RNA interference indicates GLH-4 can compensate for GLH-1; these two P granule components are critical for fertility in C. elegans. Development *127*, 2907-2916.

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature *410*, 116-120.

LaJeunesse, D., and Shearn, A. (1996). E(z): a polycomb group gene or a trithorax group gene? Development *122*, 2189-2197.

Lamb, M. M., and Laird, C. D. (1976). Increase in nuclear poly(A)-containing RNA at syncytial blastoderm in Drosophila melanogaster embryos. Dev Biol *52*, 31-42.

Lamping, E., Luckl, J., Paltauf, F., Henry, S. A., and Kohlwein, S. D. (1994). Isolation and characterization of a mutant of Saccharomyces cerevisiae with pleiotropic deficiencies in transcriptional activation and repression. Genetics *137*, 55-65.

Laufer, J. S., Bazzicalupo, P., and Wood, W. B. (1980). Segregation of developmental potential in early embryos of Caenorhabditis elegans. Cell *19*, 569-577.

Laurent, B. C., Treich, I., and Carlson, M. (1993). The yeast SNF2/SWI2 protein has DNA-stimulated ATPase activity required for transcriptional activation. Genes Dev *7*, 583-591.

Lawson, K. A., and Hage, W. J. (1994). Clonal analysis of the origin of primordial germ cells in the mouse. Ciba Found Symp *182*, 68-84; discussion 84-91.

Leatherman, J. L., Levin, L., Boero, J., and Jongens, T. A. (2002). germ cell-less acts to repress transcription during the establishment of the Drosophila germ cell lineage. Curr Biol *12*, 1681-1685.

Lehmann, R., and Ephrussi, A. (1994). Germ plasm formation and germ cell determination in Drosophila. Ciba Found Symp *182*, 282-296; discussion 296-300.

Lehmann, R., and Nusslein-Volhard, C. (1986). Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of oskar, a maternal gene in Drosophila. Cell *47*, 141-152.

Lorch, Y., LaPointe, J. W., and Kornberg, R. D. (1992). Initiation on chromatin templates in a yeast RNA polymerase II transcription system. Genes Dev *6*, 2282-2287.

Lu, X., and Horvitz, H. R. (1998). lin-35 and lin-53, two genes that antagonize a C. elegans Ras pathway, encode proteins similar to Rb and its binding protein RbAp48. Cell *95*, 981-991.

Lukacsovich, T., Asztalos, Z., Awano, W., Baba, K., Kondo, S., Niwa, S., and Yamamoto, D. (2001). Dual-tagging gene trap of novel genes in Drosophila melanogaster. Genetics *157*, 727-742.

Mahone, M., Saffman, E. E., and Lasko, P. F. (1995). Localized Bicaudal-C RNA encodes a protein containing a KH domain, the RNA binding motif of FMR1. Embo J *14*, 2043-2055.

Maloof, J. N., and Kenyon, C. (1998). The Hox gene lin-39 is required during C. elegans vulval induction to select the outcome of Ras signaling. Development *125*, 181-190.

Mannervik, M., and Levine, M. (1999). The Rpd3 histone deacetylase is required for segmentation of the Drosophila embryo. Proc Natl Acad Sci U S A *96*, 6797-6801.

Matallana, E., Franco, L., and Perez-Ortin, J. E. (1992). Chromatin structure of the yeast SUC2 promoter in regulatory mutants. Mol Gen Genet *231*, 395-400.

Matsui, Y., Toksoz, D., Nishikawa, S., Williams, D., Zsebo, K., and Hogan, B. L. (1991). Effect of Steel factor and leukaemia inhibitory factor on murine primordial germ cells in culture. Nature *353*, 750-752.

McGinnis, W., and Krumlauf, R. (1992). Homeobox genes and axial patterning. Cell *68*, 283-302.

Mello, C. C., Draper, B. W., Krause, M., Weintraub, H., and Priess, J. R. (1992). The pie-1 and mex-1 genes and maternal control of blastomere identity in early C. elegans embryos. Cell *70*, 163-176.

Mello, C. C., Schubert, C., Draper, B., Zhang, W., Lobel, R., and Priess, J. R. (1996). The PIE-1 protein and germline specification in C. elegans embryos. Nature *382*, 710-712.

Mette, M. F., Aufsatz, W., van der Winden, J., Matzke, M. A., and Matzke, A. J. (2000). Transcriptional silencing and promoter methylation triggered by double-stranded RNA. Embo J *19*, 5194-5201.

Milne, T. A., Sinclair, D. A., and Brock, H. W. (1999). The Additional sex combs gene of Drosophila is required for activation and repression of homeotic loci, and interacts specifically with Polycomb and super sex combs. Mol Gen Genet *261*, 753-761.

Minty, A., Dumont, X., Kaghad, M., and Caput, D. (2000). Covalent modification of p73alpha by SUMO-1. Two-hybrid screening with p73 identifies novel SUMO-1-interacting proteins and a SUMO-1 interaction motif. J Biol Chem *275*, 36316-36323.

Modolell, J., Bender, W., and Meselson, M. (1983). Drosophila melanogaster mutations suppressible by the suppressor of Hairy-wing are insertions of a 7.3-kilobase mobile element. Proc Natl Acad Sci U S A *80*, 1678-1682.

Mohler, W. A., Simske, J. S., Williams-Masson, E. M., Hardin, J. D., and White, J. G. (1998). Dynamics and ultrastructure of developmental cell fusions in the Caenorhabditis elegans hypodermis. Curr Biol *8*, 1087-1090.

Muller, J., and Bienz, M. (1992). Sharp anterior boundary of homeotic gene expression conferred by the fushi tarazu protein. Embo J *11*, 3653-3661.

Murray, M. V., Turnage, M. A., Williamson, K. J., Steinhauer, W. R., and Searles, L. L. (1997). The Drosophila suppressor of sable protein binds to RNA and associates with a subset of polytene chromosome bands. Mol Cell Biol *17*, 2291-2300.

Nagy, L., Kao, H. Y., Chakravarti, D., Lin, R. J., Hassig, C. A., Ayer, D. E., Schreiber, S. L., and Evans, R. M. (1997). Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell *89*, 373-380.

Nakamura, T., Mori, T., Tada, S., Krajewski, W., Rozovskaia, T., Wassell, R., Dubois, G., Mazo, A., Croce, C. M., and Canaani, E. (2002). ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. Mol Cell *10*, 1119-1128.

Nakayama, J., Rice, J. C., Strahl, B. D., Allis, C. D., and Grewal, S. I. (2001). Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science *292*, 110-113.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. Plant Cell *2*, 279-289.

Nislow, C., Ray, E., and Pillus, L. (1997). SET1, a yeast member of the trithorax family, functions in transcriptional silencing and diverse cellular processes. Mol Biol Cell *8*, 2421-2436.

Niwa, H., Miyazaki, J., and Smith, A. G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet *24*, 372-376.

Ogas, J., Kaufmann, S., Henderson, J., and Somerville, C. (1999). PICKLE is a CHD3 chromatin-remodeling factor that regulates the transition from embryonic to vegetative development in Arabidopsis. Proc Natl Acad Sci U S A *96*, 13839-13844.

Ogura, K., Kishimoto, N., Mitani, S., Gengyo-Ando, K., and Kohara, Y. (2003). Translational control of maternal glp-1 mRNA by POS-1 and its interacting protein SPN-4 in Caenorhabditis elegans. Development *130*, 2495-2503.

Parrish, S., and Fire, A. (2001). Distinct roles for RDE-1 and RDE-4 during RNA interference in Caenorhabditis elegans. Rna *7*, 1397-1402.

Paulsen, J. E., Capowski, E. E., and Strome, S. (1995). Phenotypic and molecular analysis of mes-3, a maternal-effect gene required for proliferation and viability of the germ line in C. elegans. Genetics *141*, 1383-1398.

Pazin, M. J., and Kadonaga, J. T. (1997). What's up and down with histone deacetylation and transcription? Cell *89*, 325-328.

Peters, A. H., O'Carroll, D., Scherthan, H., Mechtler, K., Sauer, S., Schofer, C., Weipoltshammer, K., Pagani, M., Lachner, M., Kohlmaier, A., *et al.* (2001). Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. Cell *107*, 323-337.

Petruk, S., Sedkov, Y., Smith, S., Tillib, S., Kraevski, V., Nakamura, T., Canaani, E., Croce, C. M., and Mazo, A. (2001). Trithorax and dCBP acting in a complex to maintain expression of a homeotic gene. Science *294*, 1331-1334.

Pirrotta, V. (1998). Polycombing the genome: PcG, trxG, and chromatin silencing. Cell *93*, 333-336.

Pitt, J. N., Schisa, J. A., and Priess, J. R. (2000). P granules in the germ cells of Caenorhabditis elegans adults are associated with clusters of nuclear pores and contain RNA. Dev Biol *219*, 315-333.

Priess, J. R. (1994). Establishment of initial asymmetry in early Caenorhabditis elegans embryos. Curr Opin Genet Dev *4*, 563-568.

Qian, S., Capovilla, M., and Pirrotta, V. (1991). The bx region enhancer, a distant cis-control element of the Drosophila Ubx gene and its regulation by hunchback and other segmentation genes. Embo J *10*, 1415-1425.

Qian, Y. W., Wang, Y. C., Hollingsworth, R. E., Jr., Jones, D., Ling, N., and Lee, E. Y. (1993). A retinoblastoma-binding protein related to a negative regulator of Ras in yeast. Nature *364*, 648-652.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B. D., Sun, Z. W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C. P., Allis, C. D., and Jenuwein, T. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature *406*, 593-599.

Resnick, J. L., Bixler, L. S., Cheng, L., and Donovan, P. J. (1992). Long-term proliferation of mouse primordial germ cells in culture. Nature *359*, 550-551.

Riddle, L. D., Blumenthal T., Meyer, B.J., Priess, J.R., ed. (1997). *C. elegans* II (Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press).

Rocheleau, C. E., Downs, W. D., Lin, R., Wittmann, C., Bei, Y., Cha, Y. H., Ali, M., Priess, J. R., and Mello, C. C. (1997). Wnt signaling and an APC-related gene specify endoderm in early C. elegans embryos. Cell *90*, 707-716.

Rocheleau, C. E., Yasuda, J., Shin, T. H., Lin, R., Sawa, H., Okano, H., Priess, J. R., Davis, R. J., and Mello, C. C. (1999). WRM-1 activates the LIT-1 protein kinase to transduce anterior/posterior polarity signals in C. elegans. Cell *97*, 717-726.

Rodriguez, M. S., Desterro, J. M., Lain, S., Midgley, C. A., Lane, D. P., and Hay, R. T. (1999). SUMO-1 modification activates the transcriptional response of p53. Embo J *18*, 6455-6461.

Rongo, C., and Lehmann, R. (1996). Regulated synthesis, transport and assembly of the Drosophila germ plasm. Trends Genet *12*, 102-109.

Roussell, D. L., and Bennett, K. L. (1993). glh-1, a germ-line putative RNA helicase from Caenorhabditis, has four zinc fingers. Proc Natl Acad Sci U S A *90*, 9300-9304.

Rudner, D. Z., Breger, K. S., and Rio, D. C. (1998). Molecular genetic analysis of the heterodimeric splicing factor U2AF: the RS domain on either the large or small Drosophila subunit is dispensable in vivo. Genes Dev *12*, 1010-1021.

Rundlett, S. E., Carmen, A. A., Kobayashi, R., Bavykin, S., Turner, B. M., and Grunstein, M. (1996). HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. Proc Natl Acad Sci U S A *93*, 14503-14508.

Rundlett, S. E., Carmen, A. A., Suka, N., Turner, B. M., and Grunstein, M. (1998). Transcriptional repression by UME6 involves deacetylation of lysine 5 of histone H4 by RPD3. Nature *392*, 831-835.

Saffman, E. E., and Lasko, P. (1999). Germline development in vertebrates and invertebrates. Cell Mol Life Sci *55*, 1141-1163.

Saitoh, H., Sparrow, D. B., Shiomi, T., Pu, R. T., Nishimoto, T., Mohun, T. J., and Dasso, M. (1998). Ubc9p and the conjugation of SUMO-1 to RanGAP1 and RanBP2. Curr Biol *8*, 121-124.

Schisa, J. A., Pitt, J. N., and Priess, J. R. (2001). Analysis of RNA associated with P granules in germ cells of C. elegans adults. Development *128*, 1287-1298.

Schramke, V., and Allshire, R. (2003). Hairpin RNAs and retrotransposon LTRs effect RNAi and chromatin-based gene silencing. Science *301*, 1069-1074.

Schubert, C. M., Lin, R., de Vries, C. J., Plasterk, R. H., and Priess, J. R. (2000). MEX-5 and MEX-6 function to establish soma/germline asymmetry in early C. elegans embryos. Mol Cell *5*, 671-682.

Seelig, H. P., Moosbrugger, I., Ehrfeld, H., Fink, T., Renz, M., and Genth, E. (1995). The major dermatomyositis-specific Mi-2 autoantigen is a presumed helicase involved in transcriptional activation. Arthritis Rheum *38*, 1389-1399.

Seydoux, G., and Dunn, M. A. (1997). Transcriptionally repressed germ cells lack a subpopulation of phosphorylated RNA polymerase II in early embryos of Caenorhabditis elegans and Drosophila melanogaster. Development *124*, 2191-2201.

Seydoux, G., and Fire, A. (1994). Soma-germline asymmetry in the distributions of embryonic RNAs in Caenorhabditis elegans. Development *120*, 2823-2834.

Seydoux, G., Mello, C. C., Pettitt, J., Wood, W. B., Priess, J. R., and Fire, A. (1996). Repression of gene expression in the embryonic germ lineage of C. elegans. Nature *382*, 713-716.

Seydoux, G., and Strome, S. (1999). Launching the germline in Caenorhabditis elegans: regulation of gene expression in early germ cells. Development *126*, 3275-3283.

Shimell, M. J., Simon, J., Bender, W., and O'Connor, M. B. (1994). Enhancer point mutation results in a homeotic transformation in Drosophila. Science *264*, 968-971.

Silva, J., Mak, W., Zvetkova, I., Appanah, R., Nesterova, T. B., Webster, Z., Peters, A. H., Jenuwein, T., Otte, A. P., and Brockdorff, N. (2003). Establishment of histone h3 methylation on the inactive x chromosome requires transient recruitment of eed-enx1 polycomb group complexes. Dev Cell *4*, 481-495.

Sinclair, D. A., Campbell, R. B., Nicholls, F., Slade, E., and Brock, H. W. (1992). Genetic analysis of the additional sex combs locus of Drosophila melanogaster. Genetics *130*, 817-825.

Smith, J. L., Wilson, J. E., and Macdonald, P. M. (1992). Overexpression of oskar directs ectopic activation of nanos and presumptive pole cell formation in Drosophila embryos. Cell *70*, 849-859.

Snow, M. H. (1981). Autonomous development of parts isolated from primitive-streak-stage mouse embryos. Is development clonal? J Embryol Exp Morphol *65 Suppl*, 269-287.

Solari, F., and Ahringer, J. (2000). NURD-complex genes antagonise Ras-induced vulval development in Caenorhabditis elegans. Curr Biol *10*, 223-226.

Sonnenblick, B. P. (1950). The early embryology of *Drosophila melanogaster*. In: Biology of *Drosophila* (New York, Wiley).

Stevens, L. C. (1968). The development of teratomas from intratesticular grafts of tubal mouse eggs. J Embryol Exp Morphol *20*, 329-341.

Stevens, L. C. (1970). The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos. Dev Biol *21*, 364-382.

Strome, S., and Wood, W. B. (1983). Generation of asymmetry and segregation of germ-line granules in early C. elegans embryos. Cell *35*, 15-25.

Struhl, G. (1981). A gene product required for correct initiation of segmental determination in Drosophila. Nature *293*, 36-41.

Struhl, G., and Akam, M. (1985). Altered distributions of Ultrabithorax transcripts in extra sex combs mutant embryos of Drosophila. Embo J *4*, 3259-3264.

Struhl, K. (1998). Histone acetylation and transcriptional regulatory mechanisms. Genes Dev *12*, 599-606.

Sulston, J. E., and Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev Biol *56*, 110-156.

Sulston, J. E., and White, J. G. (1980). Regulation and cell autonomy during postembryonic development of Caenorhabditis elegans. Dev Biol *78*, 577-597.

Tabara, H., Hill, R. J., Mello, C. C., Priess, J. R., and Kohara, Y. (1999a). pos-1 encodes a cytoplasmic zinc-finger protein essential for germline specification in C. elegans. Development *126*, 1-11.

Tabara, H., Sarkissian, M., Kelly, W. G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., and Mello, C. C. (1999b). The rde-1 gene, RNA interference, and transposon silencing in C. elegans. Cell *99*, 123-132.

Tabara, H., Yigit, E., Siomi, H., and Mello, C. C. (2002). The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in C. elegans. Cell *109*, 861-871.

Tam, P. P., and Zhou, S. X. (1996). The allocation of epiblast cells to ectodermal and germ-line lineages is influenced by the position of the cells in the gastrulating mouse embryo. Dev Biol *178*, 124-132.

Taunton, J., Hassig, C. A., and Schreiber, S. L. (1996). A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science *272*, 408-411.

Tenenhaus, C., Subramaniam, K., Dunn, M. A., and Seydoux, G. (2001). PIE-1 is a bifunctional protein that regulates maternal and zygotic gene expression in the embryonic germ line of Caenorhabditis elegans. Genes Dev *15*, 1031-1040.

Tsai, R. Y., and Reed, R. R. (1998). Identification of DNA recognition sequences and protein interaction domains of the multiple-Zn-finger protein Roaz. Mol Cell Biol *18*, 6447-6456.

Tsukiyama, T., and Wu, C. (1997). Chromatin remodeling and transcription. Curr Opin Genet Dev *7*, 182-191.

van der Krol, A. R., Mur, L. A., de Lange, P., Mol, J. N., and Stuitje, A. R. (1990). Inhibition of flower pigmentation by antisense CHS genes: promoter and minimal sequence requirements for the antisense effect. Plant Mol Biol *14*, 457-466.

Van Doren, M., Williamson, A. L., and Lehmann, R. (1998). Regulation of zygotic gene expression in Drosophila primordial germ cells. Curr Biol *8*, 243-246.

Vannier, D., Balderes, D., and Shore, D. (1996). Evidence that the transcriptional regulators SIN3 and RPD3, and a novel gene (SDS3) with similar functions, are involved in transcriptional silencing in S. cerevisiae. Genetics *144*, 1343-1353.

Varga-Weisz, P. D., and Becker, P. B. (1998). Chromatin-remodeling factors: machines that regulate? Curr Opin Cell Biol *10*, 346-353.

Verger, A., Perdomo, J., and Crossley, M. (2003). Modification with SUMO. EMBO Rep *4*, 137-142.

Vidal, M., and Gaber, R. F. (1991). RPD3 encodes a second factor required to achieve maximum positive and negative transcriptional states in Saccharomyces cerevisiae. Mol Cell Biol *11*, 6317-6327.

Volpe, T. A., Kidner, C., Hall, I. M., Teng, G., Grewal, S. I., and Martienssen, R. A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science *297*, 1833-1837.

von Zelewsky, T., Palladino, F., Brunschwig, K., Tobler, H., Hajnal, A., and Muller, F. (2000). The C. elegans Mi-2 chromatin-remodelling proteins function in vulval cell fate determination. Development *127*, 5277-5284.

Wade, P. A., Pruss, D., and Wolffe, A. P. (1997). Histone acetylation: chromatin in action. Trends Biochem Sci *22*, 128-132.

Wakayama, T., Perry, A. C., Zuccotti, M., Johnson, K. R., and Yanagimachi, R. (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature *394*, 369-374.

Wallrath, L. L., and Elgin, S. C. (1995). Position effect variegation in Drosophila is associated with an altered chromatin structure. Genes Dev *9*, 1263-1277.

Wang, M., and Sternberg, P. W. (2001). Pattern formation during C. elegans vulval induction. Curr Top Dev Biol *51*, 189-220.

Wassenegger, M., Heimes, S., Riedel, L., and Sanger, H. L. (1994). RNA-directed de novo methylation of genomic sequences in plants. Cell *76*, 567-576.

Whitington, P. M., and Dixon, K. E. (1975). Quantitative studies of germ plasm and germ cells during early embryogenesis of Xenopus laevis. J Embryol Exp Morphol *33*, 57-74.

Wilsch-Brauninger, M., Schwarz, H., and Nusslein-Volhard, C. (1997). A sponge-like structure involved in the association and transport of maternal products during Drosophila oogenesis. J Cell Biol *139*, 817-829.

Winston, F., and Allis, C. D. (1999). The bromodomain: a chromatin-targeting module? Nat Struct Biol *6*, 601-604.

Workman, J. L., and Roeder, R. G. (1987). Binding of transcription factor TFIID to the major late promoter during in vitro nucleosome assembly potentiates subsequent initiation by RNA polymerase II. Cell *51*, 613-622.

Wu-Scharf, D., Jeong, B., Zhang, C., and Cerutti, H. (2000). Transgene and transposon silencing in Chlamydomonas reinhardtii by a DEAH-box RNA helicase. Science *290*, 1159-1162.

Wylie, C. (1999). Germ cells. Cell *96*, 165-174.

Xu, L., and Strome, S. (2001). Depletion of a novel SET-domain protein enhances the sterility of mes-3 and mes-4 mutants of Caenorhabditis elegans. Genetics *159*, 1019-1029.

Yang, D., Lu, H., and Erickson, J. W. (2000). Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in Drosophila embryos. Curr Biol *10*, 1191-1200.

Zalokar, M. (1976). Autoradiographic study of protein and RNA formation during early development of Drosophila eggs. Dev Biol *49*, 425-437.

Zamore, P. D., Tuschl, T., Sharp, P. A., and Bartel, D. P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell *101*, 25-33.

Zhang, B., Gallegos, M., Puoti, A., Durkin, E., Fields, S., Kimble, J., and Wickens, M. P. (1997a). A conserved RNA-binding protein that regulates sexual fates in the C. elegans hermaphrodite germ line. Nature *390*, 477-484.

Zhang, C. C., and Bienz, M. (1992). Segmental determination in Drosophila conferred by hunchback (hb), a repressor of the homeotic gene Ultrabithorax (Ubx). Proc Natl Acad Sci U S A *89*, 7511-7515.

Zhang, F., Barboric, M., Blackwell, T. K., and Peterlin, B. M. (2003). A model of repression: CTD analogs and PIE-1 inhibit transcriptional elongation by P-TEFb. Genes Dev *17*, 748-758.

Zhang, Y., Iratni, R., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (1997b). Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex. Cell *89*, 357-364.

Zhang, Y., LeRoy, G., Seelig, H. P., Lane, W. S., and Reinberg, D. (1998). The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. Cell *95*, 279-289.