C. ELEGANS OMA-1 AND OMA-2 ARE TRANSCRIPTIONAL AND TRANSLATIONAL REPRESSORS OF GERMLINE FATE

APPROVED BY SUPERVISORY COMMITTEE

_____

Mentor: Rueyling Lin, Ph.D.

_____

Committee Chairman: Leon Avery, Ph.D.

_____

Committee Member: James Chen, Ph.D.

_____

Committee Member: Michael Buszczak, Ph.D

DEDICATION

This dissertation is dedicated to my father, Abdi Guven, who was my inspiration during my childhood, to my mother Ummugulsum Guven and to my caring husband Emin Deniz Ozkan, my current inspiration.

C. ELEGANS OMA-1 AND OMA-2 ARE TRANSCRIPTIONAL AND TRANSLATIONAL REPRESSORS OF GERMLINE FATE

by

Tugba Guven Ozkan

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

June, 2009

ACKNOWLEDGEMENTS

I would like to thank my mentor Dr. Rueyling Lin for her full support and excellent guidance during my graduate school. Her enthusiasm to conduct excellent research, motivation to help me improve my experimental and presentation skills and of course, the great projects she gave me, made my graduate school very productive. I would like to also thank my thesis committee members Dr. Leon Avery, Dr. James Chen, Dr. Michael Buszczak and previous committee member Dr. Dennis McKearin for their time, helpful insights and suggestions.

I would like to thank the past and present members of the Lin Lab, for their help and friendship. In particular, Dr. Yuichi Nishi, for the initial work on the TAF-4 project and Dr. Scott Robertson for his help and advice, as well as Dr. Premnath Shetty, Shuyi Huang, Gokalp Celik and Jessica Medina.

I would like to thank worm community for their generosity in reagent sharing. In particular, the *C. elegans* Genetics Center (CGC), Dr. Geraldine Seydoux (Johns Hopkins University), Dr. Keith Blackwell (Harvard Medical School), Dr. James Priess (Fred Hutchinson Cancer Center), Dr. Andy Fire (Stanford University), Dr. Tim Schedl (Washington University), Dr. Zheng Zhou (Baylor College of Medicine), Dr. Yuji Kohara (National Institute of Genetics, Japan), Dr. Susan Strome (University of California, Santa Cruz) and UT Southwestern *C. elegans* community: the Avery Lab and the Cameron Lab.

C. ELEGANS OMA-1 AND OMA-2 ARE TRANSCRIPTIONAL AND TRANSLATIONAL REPRESSORS OF GERMLINE FATE

Publication No. _____

Tugba Guven Ozkan, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2009

Supervising Professor: Rueyling Lin, Ph.D.

The germline is a very specialized cell lineage for the proper transmission of genetic material through many generations, to ensure flawless perpetuation of the species and life cycles. The germline lineage is set aside as early as embryogenesis and kept quiescent until germ cells are needed for adult reproduction. During *C. elegans* germline development global transcription is repressed in specialized, mature diakinetic oocytes of the adult animal and transcription is reactivated as zygotic transcription in the 4-cell stage embryo but only in somatic blastomeres. Global transcription is kept repressed by PIE-1 in germline precursors beginning with 4-cell stage to protect germ lineage from inappropriate somatic differentiation pathways. During my graduate studies, I investigated the redundant roles for two CCCH type RNA binding zinc finger proteins OMA-1 and OMA-2 during *C. elegans* germline development and early embryogenesis.

Previously, OMA proteins were shown to be required for oocyte maturation but they were not assigned any molecular functions. My thesis demonstrates transcriptional repression function of OMA proteins in newly fertilized embryos and translational repression functions during oogenesis. I showed that OMA-1/2 are redundantly required for global transcriptional repression before the onset of zygotic transcription in the 1-cell and 2-cell stages of *C. elegans* embryos by interacting with and sequestering in the cytoplasm TAF-4, a highly conserved essential basal transcription factor. Nuclear enrichment of TAF-4 requires interaction with another transcription factor TAF-12. OMA-1 competes with TAF-12 to interact with and change subcellular localization of TAF-4, in order to displace TAF-4 away from nuclei and prevent transcriptional initiation. I showed that interaction of OMA-1 and TAF-4 is regulated by MBK-2 phosphorylation at oocyte to embryo transition. My data suggest a model in which MBK-2 phosphorylated embryonic OMA-1 can change TAF-4 subcellular localization only in newly fertilized *C. elegans* embryos, not during oogenesis. When properly phosphorylated by MBK-2 kinase, ectopic OMA-1 is sufficient to repress transcription in later embryonic stages. Strikingly, reduction of *oma-1/2* activities not only results in transcriptional derepression in newly fertilized embryos, but also in later germline blastomeres where wild type OMA-1 is normally absent. I show that OMA-1/2 indirectly repress global transcription in later germline blastomeres by preventing premature degradation of PIE-1 during germline development. OMA proteins protect PIE-1 and other CCCH RNA binding proteins from degradation by repressing *zif-1* mRNA translation, the substrate specific binding partner for PIE-1 degradation. A *zif-1* 3'UTR reporter is repressed in the pachytene and proximal regions of the adult *C. elegans*

germline, and expression of the reporter is activated in the 4-cell embryo only in anterior blastomeres, reciprocal to the PIE-1 expression pattern. I show that *zif-1* 3'UTR reporter is repressed in the proximal oocytes and in the pachytene region of the germline by OMA-1/2 and GLD-1 respectively. I further showed that *zif-1* 3'UTR reporter is kept repressed in germline blastomeres of the embryos by POS-1 and SPN-4 and its activation requires anterior cell fate determinants MEX-5/6 during embryogenesis. Contrary to the requirement for MBK-2 phosphorylated OMA-1/2 for embryonic transcriptional repression function, *zif-1* 3'UTR reporter repression by OMA proteins in the oocytes requires un/hypophosphorylated OMA proteins, the version of OMA-1/2 detected in the oocytes.

In summary, my thesis shows that OMA-1/2 are dual function proteins redundantly required for germline development and maintenance of germline identity during oogenesis and embryonic development of *C. elegans.* OMA proteins are critical for the protection of CCCH type maternal proteins during oocyte development by preventing their premature proteasomal degradation through inhibiting translation of *zif-1* mRNA. MBK-2 phosphorylation at the oocyte to embryo transition converts OMA proteins from oocyte translational repressors to embryonic transcriptional repressors. Phosphorylated OMA proteins can interact with TAF-4 in the newly fertilized *C. elegans* embryos and repress global transcription to prevent premature somatic differentiation during early stages of embryogenesis. OMA proteins protect germline identity at the level of both translational and transcriptional repression during the very critical time points of development to regulate a proper oocyte to embryonic transition.

TABLE OF CONTENTS

TITLE.....i

DEDICATION.....ii

TITLE…….....iii

ACKNOWLEDGEMENTS.....v

ABSTRACT.....vi

TABLE OF CONTENTS.....ix

PUBLICATIONS.....xv

LIST OF FIGURES.....xvi

LIST OF TABLES.....xx

LIST OF ABBREVIATIONS.....xxi

CHAPTER 1: INTRODUCTION

I. Germline Fate Specification during Embryogenesis.....1

1. Specification of Mouse PGCs.....2

2. Specification of *Drosophila* PGCs.....4

3. Specification of *C. elegans* PGCs.....5

3.1 *C. elegans* Adult Germline.....8

II. Transcription Machinery.....10

1. mRNA Transcription is a regulated, multistep process 10

2. TAF4 is the keystone to main stability of TFIID.....12

3. Transcriptional Regulation by RNAPII CTD Phosphorylations.....14

4. Transcriptional Regulation by Chromatin.....16

III. Transcription is repressed in germ cell precursors during embryogenesis.....
.....17
1. Global transcription is repressed around E8-9 of mouse PGCs.....18
2. Global Transcription is repressed in *Drosophila* pole cells.....19
3. Global Transcription is repressed in *C. elegans* P lineage.....20
IV. Post-transcriptional Control of mRNA during development.....23
1. RNA binding proteins in *C. elegans*.....25
2. Tandem CCCH RNA binding zinc finger proteins in *C. elegans*.....26
2-1. PIE-1 is a bifunctional protein for germline cell fate specification.....
.....27
2-1a. PIE-1 represses transcription by inhibiting the P-TEFb kinase
complex.....27
2-1b. PIE-1 can regulate stability and translation of nos-2 mRNA.....29
2-1c. Asymmetric localization of PIE-1 to P lineage.....29
-Directed Localization of PIE-1 to P lineage before cleavage.....31
-PIE-1 is excluded from soma by ZIF-1 dependent degradation.....32
2-2. MEX-5/6, POS-1 and MEX-1 are other known members of CCCH zinc....
finger proteins in *C. elegans*.....34
2-2a. MEX-5/6 link polarity to cell fate determination.....34
2-2b. POS-1 and MEX-1 are determinants of posterior cell fate.....35
3. KH domain RNA binding proteins.....38
3-1. Loss GLD-1 causes tumorous germline.....39

3-2. MEX-3 is required for anterior fate specification of the embryo.....

.....41

V. OMA-1 and OMA-2 are required for oocyte maturation.....42

1. Regulated expression pattern of OMA-1/2.....43

1-1. MBK-2 marks oocyte proteins for degradation.....44

1-2. MBK-2 primes OMA-1 for GSK-3 phosphorylation.....47

2. OMA-1/2 have redundant roles in 1-cell embryo.....47

CHAPTER 2: OMA-1/2 repress global transcription in germline precursors of *C. elegans* by sequestering TAF-4 in the cytoplasm

Summary.....49

Introduction.....50

Experimental Procedures.....54

Strains.....54

Plasmid Construction.....55

RNA interference (RNAi).....55

HEK293T Cell Transfection Assay).....56

Immunofluorescence.....56

In situ Hybridization.....57

Analysis of embryos, imaging and quantification of cell cycle stage.....57

Yeast Two Hybrid Assay.....58

oma-1; oma-2 Rescue Assay with OMA-1::GFP transgenic lines.....58

Results.....59

I. OMA-1/2 Interact with TAF-4, a key component of the TFIID Complex.....60

II. TAF-4 is nuclearly enriched except 1-cell and early 2-cell stages.....62

III. OMA-1/2 Sequester TAF-4 in the Cytoplasm.....66

IV. OMA1/2 are required for transcriptional repression in the newly fertilized embryos of *C. elegans*.....70

V. OMA-1/2 and TAF-4 interact genetically.....75

VI. OMA-1 interacts with HFD of TAF-4.....76

VII. TAF-4-binding domain of OMA-1 is essential for embryonic viability but not for oocyte maturation.....83

VIII.TAF-4/OMA-1 interaction is regulated by MBK-2 phosphorylation.....87

IX. Properly Phosphorylated Ectopic OMA-1 is sufficient to repress transcription in later stage embryos.....92

Discussion.....97

Repression of somatic genes is evolutionarily conserved in PGCs.....97

Sequential transcriptional repression in *C. elegans* germ cell precursors.....

.....98

TAF-4 as a target for global transcriptional repression.....100

MBK-2 phosphorylation regulates OMA-1/2 and TAF-4 interaction at oocyte to embryo transition.....101

Regulation of OMA-1/2 activities.....101

Activation of zygotic transcription after degradation of OMA proteins.....

.....103

OMA-1/2 are required for the establishment of *C. elegans* germline.....

.....104

CHAPTER 3: OMA1/2 protect CCCH proteins from degradation by repressing *zif-1* translation in adult *C. elegans* germline

Summary.....106

Introduction.....108

Experimental Procedures.....113

Strains.....113

Plasmid Construction.....113

RNA interference (RNAi).....114

Immunofluorescence.....114

Analysis of embryos, imaging.....115

Results.....115

I. OMA-1/2 repress transcription in later germline precursors indirectly by regulating PIE-1 levels.....115

II. Blocking PIE-1 degradation restores the expression in *oma-1/2(-)* animals.....117

III. PIE-1 UTRs are not required for protection by OMA-1/2.....117

IV. Ectopic OMA-1 causes PIE-1 degradation defect in somatic blastomeres.....120

V. *zif-1* 3'UTR Reporter is repressed in the oocytes and activated in anterior blastomeres of 4-cell embryo.....121

VI. OMA-1/2 repress *zif-1* translation in the oocytes.....126

VII. GLD-1 represses *zif-1* 3'UTR reporter in the pachytene region of germline.....

.....128

VIII. MEX-5/6, SPN-4 and POS-1 are required for spatial regulation of *zif-1* 3'UTR reporter during embryogenesis.....129

IX. 120 bp region of 3'UTR is required for translational repression in the germline and activation in the embryo.....132

X. Hypophosphorylated ectopic OMA-1 repress *zif-1* ectopically causing PIE-1 degradation defect.....140

Discussion.....143

Post-transcriptional control of mRNA is critical in the germline and early embryos.....

.....143

Post-transcriptional regulation and disease.....143

OMA-1/2 are CCCH type RNA binding zinc finger proteins required for the establishment of germ lineage.....144

zif-1 translation is regulated spatiotemporally by multiple RNA binding proteins.....

.....145

glp-1 and *zif-1* 3'UTR regulation show parallels.....147

120 nucleotide region of *zif-1* 3'UTR.....148

MBK-2 phosphorylation inhibits *zif-1* 3'UTR repression by OMA-1/2.....150

CHAPTER 4: Conclusions and Future Directions

Dual roles for OMA proteins.....152

OMA-1/2 repress *zif-1* 3'UTR in the oocytes.....153

OMA proteins repress global transcription in embryos.....154

BIBLIOGRAPHY.....157

PUBLICATIONS

Guven-Ozkan T, Nishi Y, Robertson SM, Lin R. (2008). Global transcriptional repression in *C. elegans* germline precursors by regulated sequestration of TAF-4. *Cell* 135, 149-60.

LIST OF FIGURES

Figure 1.1.Mouse PGCs are specified during E6.25-E6.5 of embryogenesis.....3

Figure 1.2. *Drosophila* germline is established by maternal factors during oogenesis.....

.....5

Figure 1.3. *C. elegans* PGCs, Z2 and Z3 are generated through four asymmetric divisions, followed by one equal division.....7

Figure 1.4. *C. elegans* Germline.....9

Figure 1.5. Basal Transcription Machinery.....12

Figure 1.6. Transcriptional Regulation by RNAPII CTD repeat phosphorylations.....

.....15

Figure 1.7. Transcription starts in somatic blastomeres of 4-cell stage embryo.....21

Figure 1.8. PIE-1 protein localizes asymmetrically to P blastomeres.....30

Figure 1.9. Establishment of embryonic polarity in *C. elegans*.....32

Figure 1.10. PIE-1 degradation complex.....33

Figure 1.11. Non overlapping expression patterns of GLD-1 and MEX-3 in adult germline 39

Figure 1.12. OMA-1::GFP live animal.....43

Figure 1.13. Dynamic localization change of MBK-2 at the oocyte to embryo transition....

.....46

Figure 2.1. OMA-1 specifically interacts with TAF-4.....60

Figure 2.2. TAF-4 is nuclearly enriched in oocytes and embryos except 1-cell and early 2-cell embryos.....63

Figure 2.3. TAF-4 has lower nuclear signal in 1-cell embryos.....64

Figure 2.4. Quantification of OMA-1 levels in early embryos.....67

Figure 2.5. *oma-1; oma-2* double RNAi in GFP::TAF-4 embryos.....69

Figure 2.6. OMA-1 and OMA-2 are required for transcriptional repression.....71

Figure 2.7. OMA-1/2 are not required for transcriptional repression in late oocytes.....

.....73

Figure 2.8. OMA-1/2 repress transcription in germline precursors beyond 4-cell stage indirectly by regulating PIE-1 levels.....74

Figure 2.9. OMA-1 N terminal 117 amino acids interacts with TAF-4 HFD.....77

Figure 2.10. TAFH and Histone fold are conserved among TAF4 proteins from different species.....78

Figure 2.11. HFD of TAF-4 and HFD binding partner TAF-12 are required for nuclear localization of TAF-4.....79

Figure 2.12. OMA-1 competes with TAF-12 for TAF-4 interaction.....81

Figure 2.13. HFD is required for changes in subcellular localization of TAF-4.....82

Figure 2.14. TAF-4 interaction domain of OMA-1 resembles to HFD of TAF-12 at secondary structure.....84

Figure 2.15. Lack of TAF-4 interaction domain of OMA-1 causes ectopic transcription and nuclear retention of TAF-4 in 1- and 2-cell embryos.....86

Figure 2.16. MBK-2 is required for cytoplasmic sequesterization of TAF-4 and transcriptional repression.....89

Figure 2.17. *zu405; oma-2 RNAi* 1-cell embryos have higher nuclear GFP::TAF-4 levels.....90

Figure 2.18. Co-expressing MBK-2 enhances GFP::TAF-4 recruitment to cell cortex by myr-OMA-1 FL in HEK293T cells.....92

Figure 2.19. Ectopic hypophosphorylate OMA-1 cannot sequester TAF-4 in the cytoplasm.....93

Figure 2.20. Ectopic OMA-1 when properly phosphorylated can repress transcription... 95

Figure 2.21. Model for transcriptional repression by OMA-1/2.....100

Figure 3.1. Localization of OMA-1/2 and PIE-1 during *C. elegans* germline developmental cycle.....111

Figure 3.2. Loss of *oma-1/2* results in undetectable PIE-1 protein and can be rescued by *zif-1* RNAi.....116

Figure 3.3. OMA-1/2 do not mediate PIE-1 protection through *pie-1* promoter or 3'UTR sequences.....118

Figure 3.4. PIE-1 zinc finger 1 is degraded in anterior blastomeres starting from 4-cell embryo.....119

Figure 3.5. Ectopic OMA-1 causes ectopic PIE-1 in all blastomeres.....121

Figure 3.6.In situ hybridization of *zif-1*.....123

Figure 3.7. 771 bp *zif-1* 3'UTR reporter.....124

Figure 3.8. *zif-1* 3'UTR reporter shows expression pattern reciprocal to PIE-1..... 125

Figure 3.9. *zif-1* 3'UTR reporter is repressed in meiotic germ cells by OMA-1/2 and GLD-1.....127

Figure 3.10. MEX-5/6 are required for activation of *zif-1* 3'UTR reporter in the embryos.....130

Figure 3.11. SPN-4 and POS-1 repress *zif-1* 3'UTR reporter in P blastomeres of the embryo.....131

Figure 3.12. *zif-1* 3'UTR deletion analysis.....133

Figure 3.13. Deletion of nucleotides from 64 to 123 of *zif-1* 3'UTR reporter.....134

Figure 3.14. Deletion of nucleotides from 124 to 183 of *zif-1* 3'UTR reporter.....135

Figure 3.15. 120 bp deletion of *zif-1* 3'UTR reporter causes a stronger derepression in the oocytes.....137

Figure 3.16. 120 bp domain of *zif-1* 3'UTR reporter is sufficient for OMA mediated repression in the oocytes.....139

Figure 3.17. Hypophosphorylated ectopic OMA-1 represses *zif-1* translation and results in PIE-1 degradation defect in soma.....142

Figure 3.18. Putative GLD-1, OMA and POS-1 binding sites in *zif-1* 3'UTR.....149

Figure 3.19. Model: MBK-2 phoshorylation converts OMA-1/2 from oocyte translational regulators to embryonic transcriptional repressors.....151

Figure 4.1. Dual roles for OMA proteins during oogenesis and embryogenesis.....156

LIST OF TABLES

Table 1.1. *C. elegans* embryonic tandem CCCH RNA binding zinc finger proteins.....37

Table 2.1. TAF-4 overexpression causes increased sensitivity to *oma-1/2* reduction.....76

Table 2.2. Quantification of early embryonic stages of OMA-1::GFP strains by DAPI staining.....87

.

LIST OF ABBREVIATIONS

3AT- 3-amino-1,2,4-triazole

Blimp1- B-lymphocyte-induced maturation protein 1

BSA – Bovine Serum Albumin

C terminus- Carboxy Terminus

Cdk- Cyclin Dependent Kinase

CREB- cAMP-responsive element binding protein

CRLs- Cullin-RING Ubiquitin Ligases

CTD- Carboxy Terminal Domain

cul- Cullin

DAPI - 4',6-Diamidino-2-phenylindole

DIC- Differential Interference Contrast

DMEM- Dulbecco's modified Eagle's medium

DMF- Dimethylformamide

dsRNA- double stranded RNA

DYRK- Dual-specificity YAK1 Related Kinase

FBS- Fetal Bovine Serum

FL- Full Length

GFP- Green Fluorescent Protein

GRE- *glp-1* Repression Element

GSK-3 – Glycogen Synthase Kinase 3

HFD- Histone Fold Domain

IPTG - Isopropyl β-D-1-thiogalactopyranoside

KD – Kinase dead

KH- K Homology domain

LB – Luria Broth

LG – Linkage Group

MBP – Maltose Binding Protein

mbk- Minibrain kinase (*Drosophila*) homolog

MSP – Major Sperm Protein

mRNA- messenger RNA

myr- Myristylation

N terminus- Amino terminus

NGM – Nematode Growth Medium

mex- Muscle excess

MTOC- Microtubule Organizing Center

oma- Oocyte maturation defective

ORF- Open Reading Frame

par- Abnormal embryonic PARtitioning of cytoplasm phenotype

PBS- Phosphate-buffered saline

PFA- Paraformaldehyde

PGC- Primordial germ cell

pgl-1- P granule abnormality

pie- pharynx and intestine in excess

pos- Posterior segregation

P-TEFb- Positive Transcription Elongation Factor b

RNAi – RNA interference

RNAPII- RNA Polymerase II

RRM- RNA recognition motif

TAF- TBP Associated Factor

TBP- TATA binding protein

TF- Transcription Factor

TFIID- Transcription Factor IID

TTP- Tristetraprolin

UTR- Untranslated Region

vet-5- very early transcript 5

WT/N2 – Wild Type

zif-1- zinc finger interacting factor

CHAPTER ONE:

Introduction

I. Germline Fate Specification during Embryogenesis

The germline lineage is the key for perpetuation and survival of the species by ensuring proper transmission of genetic material to the next generation. Germline fate is established as early as embryogenesis for the fruitful continuation of the life cycle. In soma, tissue specific transcription factors are activated to restrict the developmental program into certain somatic lineages. Therefore, these transcription factors are inhibited in germ cell precursors to prevent differentiation to somatic fates. Accumulating evidence suggests that transcriptional and post-transcriptional regulation mechanisms acting at different stages of germ cell development are critical to prevent expression of somatic genes in germ cells to prevent their premature differentiation.

Primordial germ cells (PGCs) are the first cells that are restricted to germ cell formation during embryogenesis; PGCs expand by mitotic divisions later during development (Extavour and Akam, 2003). Three model organisms studied extensively: mouse, *Drosophila melanogaster* and *Caenorhabditis elegans*; use different modes to specify their PGCs. Vertebrates use induced germline formation mode in which zygotic extrinsic signaling factors induce germline fate and prevent somatic differentiation. Invertebrates make use of a preformed germline mode, in which maternally inherited messenger RNAs (mRNAs) and proteins are required for the establishment of germline

and for the repression of somatic cell fates (Seydoux and Braun, 2006). Although there are differences in the ways different organisms establish their future germline during embryogenesis, repression of somatic fates is evolutionarily conserved in all animals to protect germline identity and fertility throughout many generations.

Animals specify their PGCs during early embryogenesis before specification of many somatic tissues, to ensure undifferentiated germ cells are set aside early on. Research from mouse, *Drosophila* and *C. elegans* indicates that transcription is shut down during late oogenesis and reactivated as the zygotic transcription program during early embryogenesis. The period of transcriptional quiescence at the maternal to zygotic transition may be a mechanism to provide a smoother transition as the two developmental programs are suggested to be very different from each other (Blackwell and Walker, 2006; Nakamura and Seydoux, 2008).

1. Specification of Mouse PGCs

There is no germ plasm inherited from maternal oocyte during mouse embryogenesis, and PGCs are specified by zygotic factors. At around gastrulation stage E6.25-6.5, four to eight cells from the proximal epiblast are induced to generate germ cell fate by extracellular signaling factors. All embryonic epiblast cells seem to have the potential to develop into PGCs initially (Figure 1.1). Orthotopic and heterotopic transplantation of distal epiblast cells to proximal region induced them to adopt a germline fate. Similarly, when proximal cells were transplanted to distal region they could colonize the neural plate and surface ectoderm (Tam and Zhou, 1996). Plasticity in the commitment to any

lineage suggests that inductive signaling events specific to the site of transplantation are necessary for the commitment of epiblast cell fates.

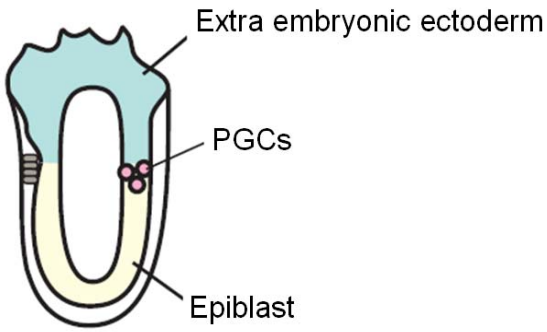


Figure 1.1. Mouse PGCs are specified during E6.25-E6.5 of embryogenesis (Reproduced from Nakamura and Seydoux)
Mouse PGCs are specified during embryonic day E6.25-6.5 by an extracellular signal, BMP (Bone Morphogenetic Protein), secreted from extraembryonic ectoderm, which induces four to eight cells in the proximal epiblast to adopt a germline fate. Concurrent with this induction *Blimp1* (B-lymphocyte-induced maturation protein 1) is expressed in PGCs and epigenetic markers associated with repressed chromatin are detected (Ohinata et al., 2005).

Lack of PGCs in Bmp4, Bmp8b or in Bmp2 knockout mice together with expression of these Bmp family proteins in the extra embryonic ectoderm (Bmp4, Bmp8b) and in the endoderm (Bmp2) of wild type animals suggested that BMP (Bone Morphogenetic Protein) signaling is required for the induction of PGCs and repression of somatic lineages during mouse embryogenesis (Lawson et al., 1999; Saitou et al., 2002; Ying et al., 2000; Ying et al., 2001; Ying and Zhao, 2001). Repression of somatic fates is a hallmark of embryonic germ cell precursors in all organisms. Details of transcriptional

repression in mouse PGCs will be discussed after introducing transcriptional regulation mechanisms.

2. Specification of *Drosophila* PGCs

The *Drosophila* cystoblast divides four times to generate 15 nurse cells and an oocyte. Nurse cells synthesize RNAs and proteins which are transported to the oocyte through cytoplasmic bridges to provide support to the oocyte (Figure 1.2A). Maternal gene products deposited in the oocyte posteriorly are required for the formation the germ plasm, also called pole plasm (Figure 1.2B). A *Drosophila* embryo starts its development by a series of nuclear divisions without cell division, creating a syncytium in which all nuclei share a common cytoplasm. Nuclei in the middle migrate to the surface and form 'syncytial blastoderm'. Plasma membranes on the surface grow inward and invade nuclei to cellularize and form 'the cellular blastoderm' at around 6000 cell stage. Before the onset of zygotic transcription, nuclei located posteriorly on the germ plasm cellularize earlier than the others and form pole cells, which are the germline precursor cells that give rise to the PGCs of the fly (Figure 1.2B). Preformed, maternally inherited factors located in the pole plasm are required for germline fate specification during *Drosophila* embryogenesis (Extavour and Akam, 2003; Seydoux and Braun, 2006).

Genetic screens identified several maternal factors located in the germ plasm that are crucial for the PGC fate. Among the identified factors, Oskar is both necessary and sufficient for the germ cell specification. *osk* mRNA is localized asymmetrically to the posterior of the oocyte by stage 8 oogenesis (Ephrussi and Lehmann, 1992). Posteriorly polarized Oskar protein provides a platform for the recruitment of other downstream

germ plasm factors like Vasa, Tudor, *pgc, nanos, pumilio* and *germ cell-less* (Mahowald, 2001; Tanaka and Nakamura, 2008), which in turn establish the germline identity by both transcriptional and translational regulation of gene expression.

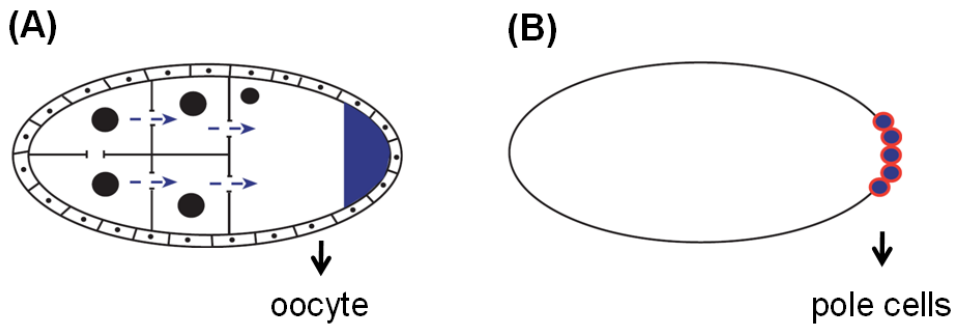


Figure 1.2. *Drosophila* germline is established by maternal factors during oogenesis
(Reproduced from Extavour and Akam et al.,2003)
A. The fly oocyte divides four times to generate one posteriorly localized oocyte and 15 nurse cells that provide support to the oocyte. Nurse cells transport maternal mRNAs and proteins through the cytoplasmic bridges (blue arrows). Transported factors are posteriorly localized (blue) and are required for the formation of future PGCs of the embryo.
B. Posteriorly localized nuclei of the fly syncytium cellularize earlier than other nuclei to form pole cells, which are the germ cell precursors of the *Drosophila* embryo.

3. Specification of *C. elegans* PGCs

C. elegans PGCs are specified through a "preformation mode" like *Drosophila*; however *C. elegans* germ plasm is asymmetrically distributed after fertilization through embryonic polarization initiated by sperm entry, not during oogenesis. *C. elegans* embryogenesis starts with asymmetric divisions of the fertilized embryo without syncytium formation. Fertilization triggers the completion of meiosis I and II, and

microtubules derived from the asters of the sperm pronucleus initiate embryonic polarity formation, the sperm entry point marking the posterior of the embryo (Cowan and Hyman, 2004; Goldstein and Hird, 1996; Wallenfang and Seydoux, 2000).

The *C. elegans* zygote (1-cell embryo) harbors the maternal factors necessary for germ plasm formation like P granules and PIE-1 protein, so the zygote is regarded as the first germ cell precursor of the embryo. The Sperm entry point of *C. elegans* oocyte the marks posterior end, leading to polarity formation. Established embryonic polarity results in unequal cell division of early blastomeres and segregates germ plasm components asymmetrically to a single blastomere: P4, the first germ precursor committed solely to the germline lineage. Finally, P4 divides symmetrically and gives rise to Z2 and Z3 blastomeres, primordial germ cells of the *C. elegans* embryo (Figure 1.3A, B). Maternal germ plasm components are selectively segregated to germ cell precursors, "P blastomeres" by three mechanisms: (1) directed asymmetric segregation towards future P blastomere, (2) localized degradation in somatic blastomeres and (3) translational control of gene expression.

Somatic sisters of the P blastomeres start differentiating into their cell fates after the asymmetric division by turning on transcription factors and by promoting translation of somatic maternal mRNAs. Transcription is globally repressed in the P lineage throughout asymmetric divisions to protect them from premature differentiation. Emerging evidence suggests that translational control of somatic cell fate determinants, in addition to transcriptional repression is crucial to tightly regulate the soma/germline distinction during germline formation and embryonic development of *C. elegans* (Merritt et al., 2008; Nakamura and Seydoux, 2008).

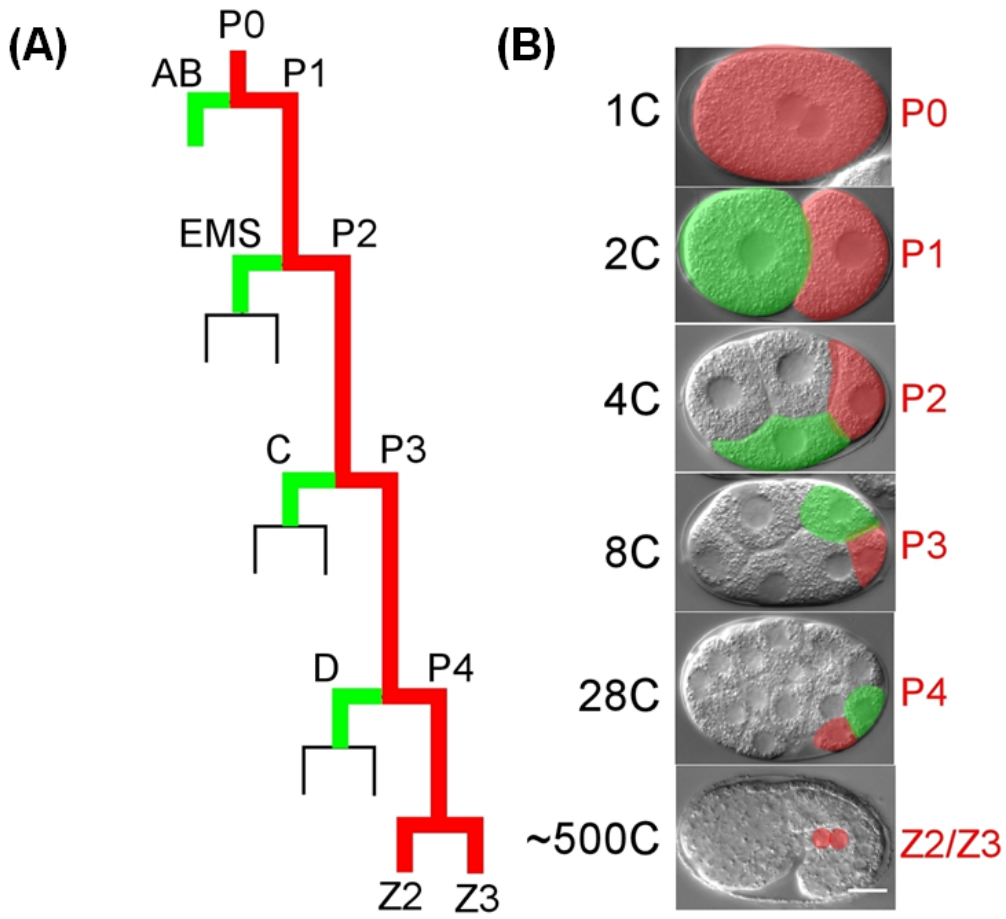


Figure 1.3. *C. elegans* PGCs Z2 and Z3 are generated through four asymmetric divisions, followed by one equal division

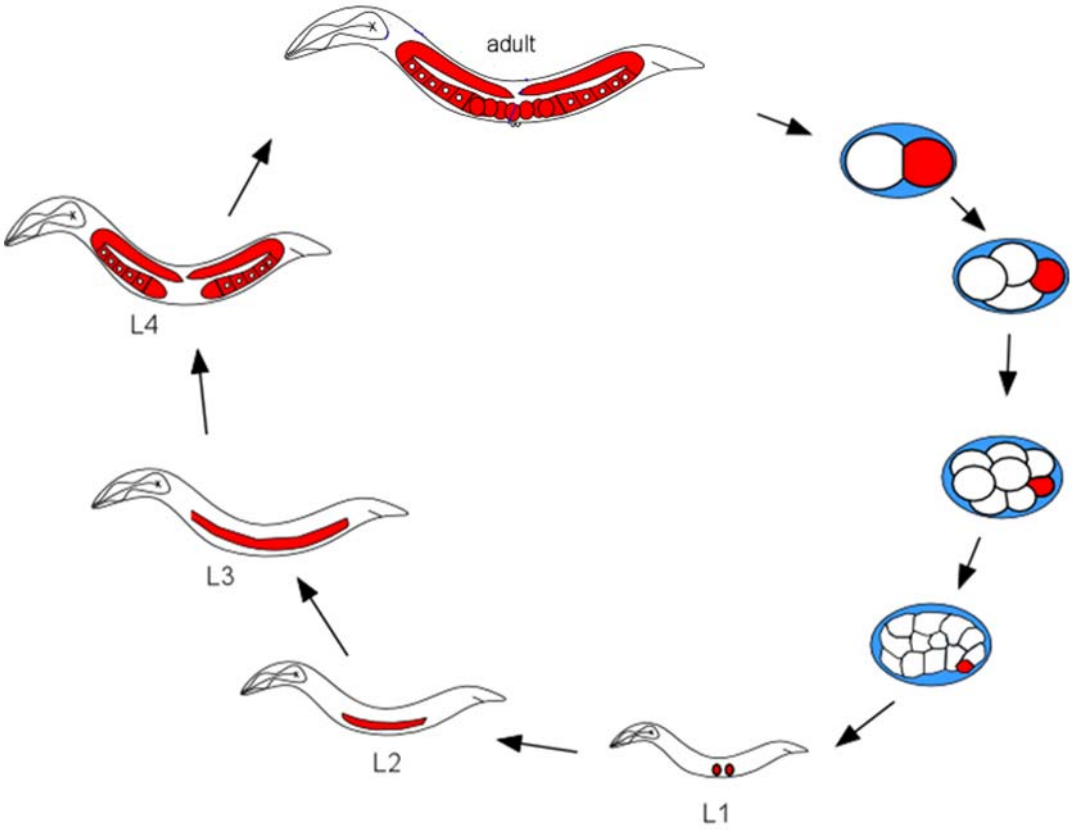
A. Schematic drawing of *C. elegans* germline precursor, P lineage divisions. The 1-cell embryo P0 is the first germline precursor of the *C. elegans* embryo. P0 divides asymmetrically to generate another germline blastomere and a somatic sister blastomere. Four such asymmetric divisions followed by a symmetric division give rise to the PGCs of the *C. elegans* embryo, Z2 and Z3. (Red line represents P lineage and green lines are somatic sister cells generated after asymmetric divisions) (Sulston et al., 1983).

B. Differential interference contrast (DIC) images of *C. elegans* early divisions. P lineage is pseudocolored in red and its somatic sisters are in green. Corresponding embryonic stages are written on the left and stages of the germline precursors on the right of the image.

3-1. *C. elegans* Adult Germline

The PGCs of *C. elegans* embryo, Z2 and Z3, give rise to the entire germ lineage of the adult animal. The *C. elegans* germline consists of two U-shaped gonad loops that are connected to a common uterus (Figure 1.4). Mitotically dividing germline stem cell nuclei are located at the distal end of the gonad loop and give rise to the germline syncytium. *C. elegans* is a hermaphrodite organism that produces sperm during the L4 larval stage and switches to oocyte production during adult gametogenesis (Hirsh et al., 1976). Germ nuclei enter into different stages of meiosis I division as they move along the gonad loop and form oocytes. Maturing oocytes located at the proximal end of the gonad enter diakinesis of meiotic prophase I, cellularize and grow in size. Fully grown oocytes arrest in the diakinesis of meiosis I until MSP (major sperm cytoskeletal protein) secreted from sperm promotes resumption of meiotic divisions and oocyte maturation (Greenstein, 2005; Miller et al., 2001). Fully mature oocytes ovulate into the spermatheca, where they are fertilized by the sperm stores of the hermaphrodite animal to form the zygote and initiate a new life cycle (Figure 1.4) (Hubbard and Greenstein, 2005; McCarter et al., 1999; Ward and Carrel, 1979). Every cycle of oocyte maturation and fertilization occurs fairly quickly in *C. elegans* reproductive system, taking approximately 23 minutes.

(A)


adult
L4
L3
L2
L1

(B)

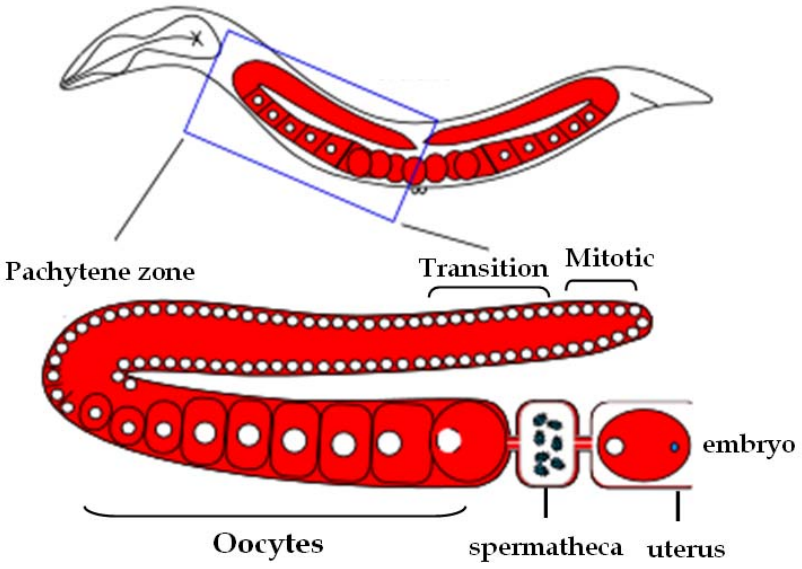

Pachytene zone
Transition
Mitotic
embryo
Oocytes
spermatheca
uterus

Figure 1.4. *C. elegans* Germline
A. Cycle of the *C. elegans* germline development from 2-cell embryo to adult animal. Germline precursor cells during embryogenesis and germline of the different larval stages and adult are shown in red color during the cycle.
B. Close-up image of adult *C. elegans* germline with two gonad loops connected to a common uterus. Bottom picture schematizes one of the gonad loops. Germline stem cell nuclei are located at the distal end of the gonad. As they move along the gonad, they initiate meiotic division steps. Cellularized and grown oocytes are located at the proximal end and they are arrested at prophase I of meiosis. Fertilization in spermatheca induces completion of meiosis I and initiation of asymmetric mitotic divisions to initiate embryogenesis in the uterus.

Like those of most animals, *C. elegans* oocytes arrest during meiotic prophase I. Both meiosis I and II divisions are completed after fertilization, and second meiotic arrest at metaphase II of meiosis that normally occurs in vertebrates does not exist in *C. elegans* (Miller et al., 2001; Ward and Carrel, 1979). After completion of meiosis, oocyte and sperm pronuclei decondense and fuse to generate the zygote, which then proceeds with the first mitotic division.

II. Transcription Machinery

1. mRNA Transcription is a regulated, multistep process

Flawless regulation of mRNA transcription is central to many biological events like tissue and organ growth, response to different environmental stimuli and development of an entire new organism. Eukaryotic mRNA transcription by RNA Polymerase II (RNAPII) is a multistep process involving multisubunit complexes, and it is regulated at

different levels to manage many different aspects of biological events and development of different structures (Lemon and Tjian, 2000).

Initial biochemical experiments using purified RNAPII suggested that RNAPII itself cannot recognize core promoter elements or initiate transcription, requiring association with General Transcription Factors (GTFs): TFIIA (Transcription factor IIA), B, D, E, F, and H. GTFs assemble into a large multiprotein complex and bind to the core promoter in a stepwise manner before RNAPII can join to the complex (Matsui et al., 1980; Samuels et al., 1982).

Assembly of the transcription initiation complex starts with binding of TFIID (Transcription factor IID) to core promoter elements, which is primarily a TA dinucleotide rich sequence called the 'TATA-box', and then TFIID serves as a platform for the assembly of other GTFs and RNAPII, to coordinate this complicated process (Buratowski et al., 1989; Nakajima et al., 1988). TFIID consists of TATA-binding protein (TBP) and 8 to 12 TBP associated factors (TAFs). TBP itself is required and sufficient for the nucleation of the initiation complex (Peterson et al., 1990). However, in vitro reconstitution experiments suggested that TBP alone is not sufficient to activate transcription, requiring coactivator functions of TAFs to bridge transcription activators to basal transcription machinery (Figure 1.5). TBP and TAF proteins from different species demonstrate high similarity and TAF proteins possess highly conserved domains like WD repeats or histone fold domains, critical for their functions (Albright and Tjian, 2000).

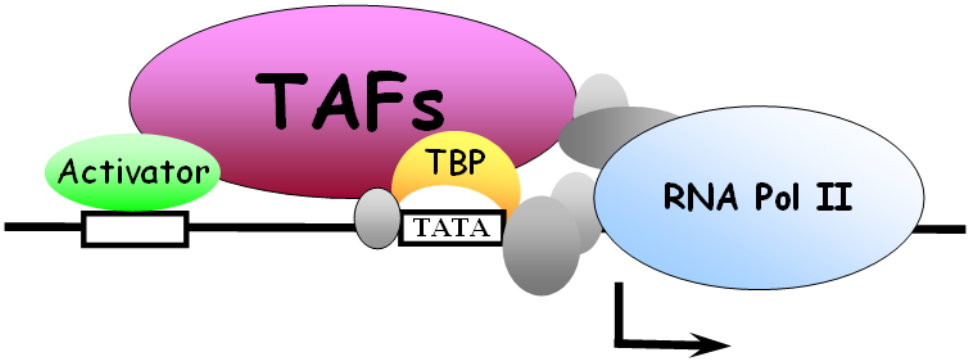


Figure 1.5. Basal Transcription Machinery
RNA Polymerase II does not have the ability to initiate or elongate mRNA transcription without accessory factors: TFII (Transcription factor II) complexes. TBP binds to the TATA-box promoter element and recruits TAFs to the promoter. TAFs connect upstream activators to basal transcription machinery located at the transcription initiation site, which in turn promotes joining of RNAPII to the transcription complex and initiation of mRNA transcription. (Gray factors represent General Transcription Factors that are required for transcription)

2. TAF4 is the keystone to maintain stability of TFIID

Most studies of the TFIID complex involved in vitro reconstitution experiments; therefore it is not very clear how TBP and TAFs are assembled to form the multisubunit complex in vivo. RNAi knockdown of individual TAFs in *Drosophila* tissue culture cells demonstrated that TAF4 is the most crucial component to maintain stability of TFIID in vivo, suggesting that TAF4 is in the core of the basal transcription complex and it is required to nucleate and maintain TFIID stability. TAF4 carboxy (C) terminus and TAF6 amino (N) terminus domains are sufficient to restore TFIID stability. Both of these contain histone fold domains (HFD). The same analysis also indicated that a stable sub-complex includes TAF4, TAF5, TAF6, TAF9, and TAF12, which are important for the

stability of the TFIID complex. TBP, TAF1, TAF2 and TAF11 are likely peripheral subunits of TFIID (Wright et al., 2006).

Sequence comparisons and structural studies suggest that more than half of the TAFs have a histone fold domain. TAF6 and TAF9 have histone folds similar to H4 (Histone 4) and H3 (Histone 3) respectively, forming a H3-H4 like heterotetramer, the result of interaction between two heterodimers (Hoffmann et al., 1996; Xie et al., 1996). TAF4 and TAF12 have histone folds similar to H2A (Histone 2A) and H2B (Histone 2B) respectively, interacting in homodimers (Gangloff et al., 2000). It has been suggested that these four TAFs might form a histone-like octamer to nucleate the multisubunit TAF complex (Gangloff et al., 2000; Hoffmann et al., 1996). *Drosophila* RNAi experiments discussed above support this idea, because TAF4, TAF6, TAF9 and TAF12 are all important for the stability of TFIID (Gangloff et al., 2000; Hoffmann et al., 1996; Wright et al., 2006).

C. elegans provides a good model system to study function of essential genes during embryogenesis by using RNAi depletion of mRNAs of interest, which results in reduction of both maternal and zygotic messages. RNAi depletion of TAF protein functions in vivo showed that TAF-1, TAF-2, TAF-5, TAF-9 and TAF-10 are dispensible for transcription of some developmental genes, and these TAFs are restricted to certain lineages, suggesting that they demonstrate tissue-specific expression during embryogenesis. In contrast, TAF-4 seems to be essential for global zygotic transcription since knockdown of *taf-4* by RNAi results in arrest of embryos around the 100-cell stage, a phenotype similar to RNAPII large component (*ama-1*) knockdown. In addition, *taf-4(RNAi)* embryos did not express active transcription markers (phophorylation of

RNAPII carboxy terminal domain, more details in the next pages) either in germline, or in somatic blastomeres, again demonstrating that there is a block of all zygotic transcription. The transcriptional requirement for TAF-4 was even more general than requirement for the TBP (Walker and Blackwell, 2003; Walker et al., 2001; Walker et al., 2004).

In vivo data from both *Drosophila* and *C. elegans* suggest that TAF4 is essential for TFIID structural integrity, as well as for the activation of global transcription (Furukawa and Tanese, 2000).

3. Transcriptional Regulation by RNAPII CTD Phosphorylations

After recruitment of all the factors required for the RNAPII basal transcription machinery, RNAPII can begin elongating the mRNA. A key step for the initiation and elongation of RNAPII transcription is extensive phosphorylation of the RNAPII large subunit tail, called the CTD (carboxy terminal domain). The CTD has seven amino acid YSPTSPS sequence repeats (38 repeats in *C. elegans* and 52 in humans) with two serine residues per repeat, at positions 2 and 5, that can be phosphorylated to regulate RNAPII activity. Cdk7, the kinase component of TFIIH, phosphorylates serine 5 for transcriptional initiation. TFIIH is the general transcription factor with kinase and DNA helicase activities. After this first phosphorylation, RNAPII can disengage from the general transcription factors and its CTD repeats are phosphorylated at serine 2 by Cdk9, the kinase component of the P-TEFb (positive transcription elongation factor b) complex. This second phosphorylation results in a conformational change that tightens RNAPII interaction with DNA and starts transcriptional elongation, the step in which a new set of

proteins is recruited to the complex to transcribe long distances of DNA without dissociation (Figure 1.6) (Blackwell and Walker, 2006; Shim et al., 2002). Once mRNA elongation begins, most of the general transcription factors are released from the complex to initiate new rounds of transcription using other RNAPII molecules. Eukaryotic mRNA elongation and RNAPII CTD phosphorylations are dynamically coupled to 5' capping, 3' polyadenylation and removal of intronic sequences by a process called mRNA splicing (Bentley, 2005; Blackwell and Walker, 2006; Buratowski et al., 1989).

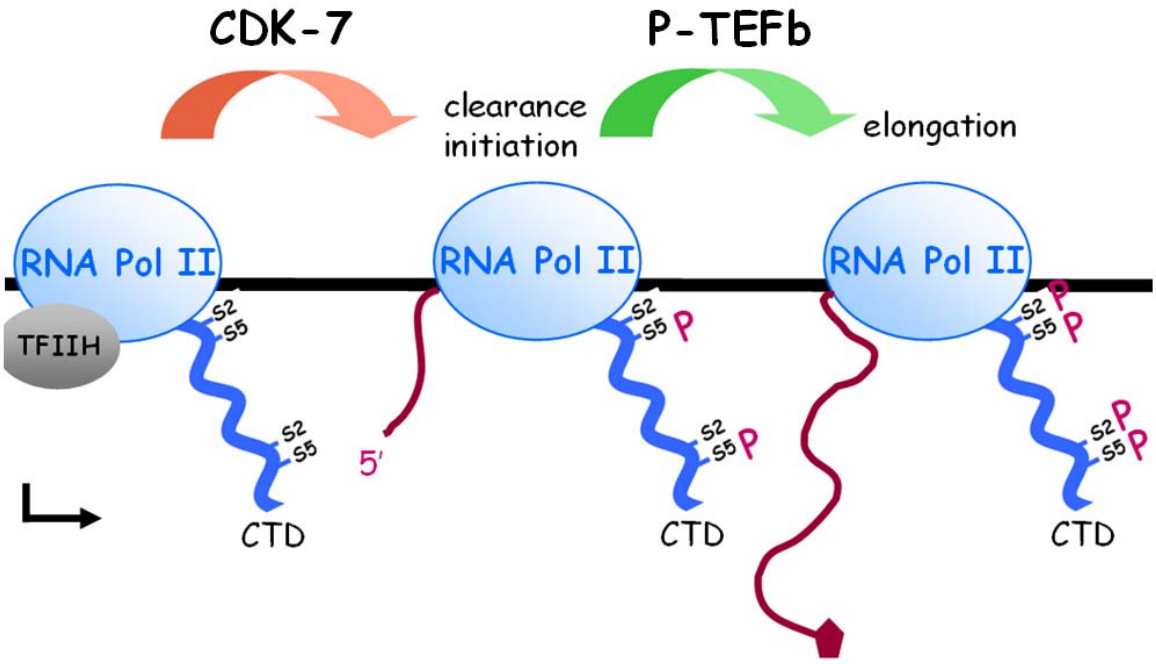


Figure 1.6. Transcriptional Regulation by RNAPII CTD repeat phosphorylation (Reproduced from Blackwell and Walker *et al,* 2006)
RNAPII carboxy terminal domain (CTD) plays an active role in the regulation of transcription initiation and elongation, coupling them to processing of pre-mRNA. After general transcription factors and RNAPII assemble onto the promoter, a component of the TFIIH DNA helicase complex, CDK-7, phosphorylates RNAPII CTD tail at serine 5 repeats promoting the initiation of mRNA transcription, which is coupled to 5' capping. CDK-9, the kinase component of P-TEFb, phosphorylates the CTD tail at serine 2 repeats for elongation of the transcript, which is coupled to further processing of the message by 3' polyadenylation and mRNA splicing.

Monoclonal antibodies that specifically recognize phosphorylated serine 2 or serine 5 of RNAPII CTD hepta repeats are available and they are commonly used to characterize the phosphorylation status of RNAPII CTD (Patturajan et al., 1998). RNAPII CTD phospho antibodies enable researchers to study global transcriptional events in vivo in different developmental contexts, in addition to other available tools like UTP incorporation and in situ hybridization (Bentley, 2005; Blackwell and Walker, 2006; Buratowski et al., 1989; Deshpande et al., 2004; Kim et al., 1997; Seydoux and Dunn, 1997).

4. Transcriptional Regulation by Chromatin

Transcription is induced by recruitment of gene specific transcription factors to promoters and can be regulated by interactions with or modifications to basal transcription machinery components. In addition, transcription can also be regulated by changes to chromatin. Eukaryotic DNA is wrapped around histone octamers (H2A, H2B, H3 and H4) to form nucleosomes, which are the fundamental organized units of chromatin. Post translational modifications like methylation, acetylation, phosphorylation and ubiquitylation to histone protein N-terminal tails that protrude out of the nucleosomes result in changes of chromatin structure causing, DNA to be either more or less accessible to transcription factors (Peterson and Laniel, 2004; Schaner and Kelly, 2006). Regulation of transcription at the chromatin level is an increasingly convoluted subject; for the purpose of this study only some most general histone modifications will be given as examples.

In general, acetylation of lysines on H3 and H4 N terminal tails correlates with looser wrapped DNA and is associated with transcriptionally competent euchromatin (Grunstein, 1997). Histone methylation can correlate with both silenced and active chromatin. Methylation of lysine 4 and 36 of H3 (H3K4me, H3K36me) is correlated with transcriptionally active euchromatin (Kouzarides, 2002); on the other hand methylation of lysine 9 and 27 of H3 (H3K9me, H3K27me) represents transcriptionally repressed chromatin (Peterson and Laniel, 2004).

III. Transcription is repressed in germ cell precursors during embryogenesis

Vertebrates and invertebrates have different modes of germ cell specification. Maternal factors contribute more during embryonic events of invertebrates, like *Drosophila* and *C. elegans.* Most of the early developmental events are carried out by maternal mRNAs and proteins in invertebrates, which enables these animals to initiate zygotic transcription later, after fertilization. This delay in zygotic transcription might be a mechanism to properly regulate the switch from maternal transcription to zygotic. When zygotic transcription is activated in the somatic cells, transcription is still globally repressed in germ line precursors of invertebrates.

Mouse germ cells are specified through extracellular BMP signaling around E6.5 of embryonic development, and, unlike in invertebrates, global transcription is not shut down in mouse embryos at the time PGCs are born. However, microarray studies suggest

that transcription of only germline specific genes is active; expression of somatic genes is still repressed in mouse germ cell precursors. Although there are differences in the way different animals specify their germ cells during embryogenesis, repression of somatic gene transcription is evolutionarily conserved to protect germ cell identity from inappropriate somatic differentiation.

1. Global transcription is repressed around E8-9 of mouse PGCs

BMP signaling from extraembryonic ectoderm and endoderm activates *Blimp1* (B-lymphocyte-induced maturation protein 1, also known as Prdm1) expression in proximal epiblast cells to generate mouse PGCs and induce germ cell competence at around E6.5 stage of the mouse embryo (Raz, 2005). *Blimp1* is a transcriptional repressor with a PR domain and five zinc fingers (Kurimoto et al., 2008; Ohinata et al., 2005), previously shown to play a critical role in development of immunoglobulin-secreting B cells (Shaffer et al., 2002; Turner et al., 1994). *Blimp1* has been shown to interact with the Groucho co-repressors and with chromatin-modifying enzymes in the developing mouse somatic epidermal lineage (Kallies and Nutt, 2007). *Blimp1* is also expressed in mouse PGCs but not in surrounding cells of the embryo (Ohinata et al., 2005), and *Blimp1* null mutants have a dramatic reduction (~90%) in number of PGCs compared to wild type animals. *Blimp1* mutant PGCs form a tight cluster, cease to proliferate and have a defect in migration into somatic gonad (Ohinata et al., 2005; Vincent et al., 2005). Microarray analyses from wild type and *Blimp1* mutant primordial germ cells indicated that *Blimp1* downregulates expression of somatic genes and upregulates expression of germline-specific markers, suggesting that *Blimp1* does not globally repress mRNA transcription

(Hayashi et al., 2007; Kurimoto et al., 2008; Surani, 2007) but represses somatic transcription factors like Hoxb1, *Fgf8* and *Snail* to protect germ cells from inappropriate somatic differentiation (Ancelin et al., 2006; Hayashi et al., 2007). *Blimp1* activates transcription of germ cell specific genes as *stella, Sox2* and *fragilis* (Raz, 2005; Saitou et al., 2002), suggesting that *Blimp1* is critical for establishment of mouse PGCs (Hayashi et al., 2007). PGCs have transcriptional activity initially to produce germline specific mRNAs, and global transcription is shut down at a later stage, around E8-9, as detected by Bromo-UTP incorporation experiments and by markers specific to RNA Polymerase II (RNAPII) activity. Concurrent with the global transcriptional silencing, a stable repression marker H3 lysine 9 dimethylation (H3K9me2) is erased from mouse PGCs, but other markers associated with repressed chromatin like tri-methylation of H3K27 (H3K27me3) can be detected at high levels. Therefore, mouse PGCs use chromatin based transcriptional repression mechanisms to protect their pluripotency and do not rely on maternally inherited germline fate determinants (Extavour and Akam, 2003; Seki et al., 2007).

2. Global Transcription is repressed in *Drosophila* pole cells

Zygotic transcription begins in the somatic nuclei of the *Drosophila* embryo around stage 8, but transcription is kept repressed in the germ cells. [^{3}H] UTP incorporation assays did not detect incorporated [^{3}H] UTP in newly synthesized mRNAs, and also RNAPII CTD repeat serine 2 phosphorylation is absent in germ cell precursors by antibody staining (Seydoux and Dunn, 1997). Germ cells activate their own transcription

program right before their migration from midgut to somatic gonad (Zalokar, 1976), later than the onset of zygotic transcription.

Drosophila polar granule component (*pgc*) mutants have degenerate germ cells that fail to migrate during mid-embryogenesis and have ectopic transcriptional activity (Deshpande et al., 2004; Hanyu-Nakamura et al., 2008; Martinho et al., 2004; Nakamura et al., 1996). *pgc* was originally considered a noncoding RNA essential for transcriptional repression of embryonic germ cells (Martinho et al., 2004; Nakamura et al., 1996). More careful analyses comparing *pgc* loci from 12 different fly species enabled the identification of a 71 amino acid open reading frame. Pgc is required to block the establishment of an active chromatin state in germ cells of early *Drosophila* embryos, although the mechanism is not clear yet (Deshpande et al., 2004). In addition, Pgc represses transcription at the transcriptional elongation level by physically and genetically interacting with P-TEFb, the complex required for RNAPII CTD repeat serine 2 phosphorylation. Pgc inhibits recruitment of P-TEFb to transcription sites. Ectopic expression of Pgc in somatic nuclei is sufficient to repress transcription, suggesting that Pgc is the critical component of germ cells required for transcriptional repression during embryogenesis (Deshpande et al., 2004; Hanyu-Nakamura et al., 2008; Timinszky et al., 2008).

3. Global Transcription is repressed in *C. elegans* P lineage

Zygotic transcription starts at the 4-cell stage of *C. elegans* embryo, but only in somatic blastomeres (Seydoux and Dunn, 1997; Seydoux et al., 1996). In situ hybridization using known very early zygotic transcripts, and immunostaining with

antibody against phosphorylated Ser2 of RNAPII CTD repeats (marker for transcriptional elongation) detect signal in somatic blastomeres beginning at the 4-cells, but not in germline blastomeres: the P lineage (Figures 1.7) (Seydoux and Dunn, 1997; Seydoux and Fire, 1994; Seydoux et al., 1996). Germline precursors are kept transcriptionally repressed until around 100-cell stage, when PGCs are segregated from somatic blastomeres and move into the somatic gonad (Seydoux and Dunn, 1997; Seydoux and Strome, 1999).

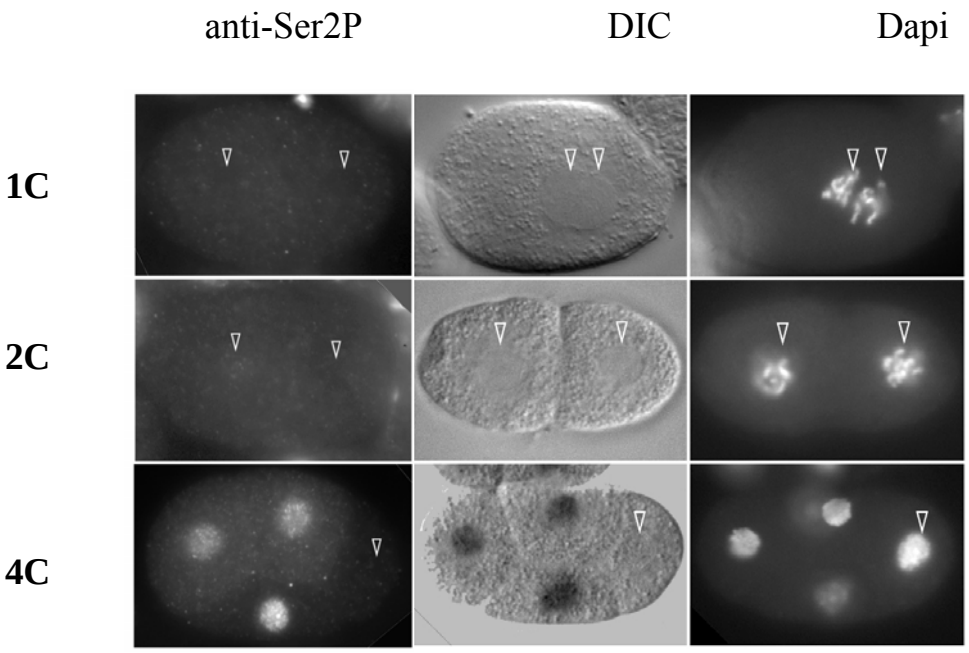


Figure 1.7. Transcription starts in somatic blastomeres of 4-cell stage embryo
Zygotic transcription of *C. elegans* is activated at 4-cell stage embryo. The first column on the left shows anti-Ser2P antibody staining for N2 (wild type) animals and the second column is in situ hybridization using *vet-5* (very early transcript) probe. Both markers detect transcription only in the somatic blastomeres, not in the P lineage. *vet-5* mRNA is nuclearly localized and is easier to detect by in situ. The last column is the corresponding DNA staining with Dapi.

PIE-1 is a tandem CCCH RNA-binding zinc finger protein that represses global transcription in the P lineage starting from the 4-cell stage. Expression of somatic zygotic transcripts occurs in germ blastomeres of *pie-1* mutant or RNAi embryos, which results in cell fate transformation with production of extra somatic cells in the expense of germline. Similar to P granule segregation to the P lineage with each asymmetric division, PIE-1 is asymmetrically localized to P blastomeres and all the residual PIE-1 protein is scavenged in the somatic daughter by proteasomal degradation (Mello et al., 1992; Mello et al., 1996; Seydoux et al., 1996). PIE-1 represses global transcription in germline precursors starting with P2 by inhibiting P-Tefb, the kinase complex required for RNAPII CTD serine 2 phosphorylation. Even though *Drosophila* Pgc and *C. elegans* PIE-1 do not show any protein sequence similarity, they inhibit the activity of the same kinase complex, P-Tefb, by targeting different components (Nakamura and Seydoux, 2008).

Markers associated with active chromation are present in the *C. elegans* P lineage, suggesting that transcriptional repression is not at the chromatin level (Schaner et al., 2003), enabling somatic sister cells generated after asymmetric P blastomere division to engage in transcription quickly, readily and reversibly. *C. elegans* P lineage blastomeres lack expression of anti-Ser2P transcriptional elongation marker, but when stained with anti-Ser5P transcriptional initiation marker, P blastomeres possess two distinct foci starting from P2. The presence of RNAPII transcriptional initiation marker in P blastomeres provides competence to transcribe somatic genes right after the somatic daughter is born (Seydoux and Dunn, 1997). Anti-Ser5P signal is not detected in P blastomeres before P2, in 1-cell and 2-cell embryos. In addition, transcription remains

repressed in 1-cell and 2-cell embryos of *pie-1* mutants, suggesting that PIE-1 is either redundant with some other factor(s) or not responsible before the onset of zygotic transcription, and the repression mechanism is likely to be at the level of transcriptional initiation.

IV. Post-transcriptional Control of mRNA during development

Early patterning of *C. elegans* embryo, before the onset of zygotic transcription relies heavily on maternally synthesized mRNAs and proteins. Maternal factors are expressed in or localized to specific blastomeres at specific time points during embryogenesis. Translational control of cell fate determinants is one mechanism to regulate embryonic development. Other mechanisms include directed movement of polarity factors, cell fate determinants and localized stabilization or degradation of key embryonic proteins (Evans and Hunter, 2005). Regulation of maternal mRNAs during oogenesis and embryonic development is a conserved mechanism in diverse species; specific RNA binding protein complexes control mRNA translation both spatially and temporally through binding to untranslated region (UTR) elements of the transcripts (de Moor et al., 2005; Evans and Hunter, 2005; Wilhelm and Smibert, 2005). mRNAs that are needed during oogenesis or embryogenesis are synthesized early in the mitotic zone or in early meiotic germ cells of *C. elegans* germline (Figure 1.4) and are kept untranslated until they are needed later during development. This allows accumulation of many transcripts without protein expression which may otherwise adversely interfere with germline development. When expression of genes in the *C. elegans* germline was compared using either a promoter or 3'UTR fusion reporter, 3'UTR sequences were sufficient for most genes to drive

expression similar to already known wild type protein expression pattern, suggesting that 3'UTRs and post-transcriptional regulation of mRNAs are the primary means of gene expression during *C. elegans* germline development with the exception of sperm specific genes (Merritt et al., 2008).

Post-transcriptional regulation can occur at different levels of mRNA metabolism such as splicing, polyadenylation, stabilization, localization and translation (de Moor et al., 2005; de Moor and Richter, 2001; Lee and Schedl, 2006). Translational control of gene expression is also observed in other developmental contexts such as cell proliferation, differentiation and interestingly also in adult brain, where translational regulation can mediate long lasting synaptic plasticity and memory locally at synapses (de Moor and Richter, 2001; Richter and Klann, 2009). FMRP (fragile X mental retardation protein) is an RNA binding protein and when dysfunctional leads to excess and altered mRNA translation at synapses that results in the loss of protein synthesis-dependent long term potentiation and memory, which is one cause of autism (Bassell and Warren, 2008; Costa-Mattioli et al., 2009).

Sequence elements of mRNA that mediate translational control can be found in both 5' or 3' untranslated regions (UTR), where RNA binding proteins can prevent translational initiation factors from associating with 5' cap or interfere with 3'UTR polyadenylation, deadenylation. Alternatively, micro RNAs can mediate post initiation repression (de Moor et al., 2005; de Moor and Richter, 2001). Once new RNA binding protein complexes are discovered and their sequence elements are uncovered, it would be interesting to find what stage of mRNA metabolism they function at. The study of RNA binding proteins, their RNA target elements and their mechanism of action is an

emerging field, and understanding their regulation will be invaluable to broaden our knowledge of post-transcriptional regulation of gene expression.

1. RNA binding proteins in *C. elegans*

The *C. elegans* genome encodes around 500 annotated RNA binding proteins with domains like RNA recognition motif (known as RRM, RBD or RNP), K homology (KH) domain, zinc finger (mainly CCCH), RGG box, DEAD/DEAH box, pumilio/FBF (PUF), double stranded RNA binding domain (ds-RBD), Piwi/Argonaute/Zwille (PAZ) and Sm domain, etc (Lee and Schedl, 2004), which are conserved in other species. RNA binding proteins have complex regulatory networks since their own expression in the germline or embryo also reling on other RNA binding proteins (Lee and Schedl, 2004). For example, the *C. elegans* KH domain protein GLD-1 is expressed in the pachytene (middle) region of adult germline (Figure 1.11), and its translation is repressed in the mitotic zone by PUF family members FBF-1/2 and reactivated by *nanos* homolog NOS-3 (Crittenden et al., 2002; Hansen et al., 2004). GLD-1 in turn represses many targets like another KH domain protein MEX-3 and the CCCH domain proteins OMA-1 and OMA-2 (Lee and Schedl, 2001; Lee and Schedl, 2004; Mootz et al., 2004). MEX-3 and OMA proteins in turn are likely to regulate many other mRNAs during oogenesis and embryogenesis as well.

Loss of function mutants of RNA binding proteins usually give pleiotropic phenotypes that cannot be rescued with the known mRNA targets, suggesting that they have many mRNA targets (Lee and Schedl, 2004). mRNA targets of RNA binding proteins can be identified by high throughput techniques like pull down of the protein of

interest followed by microarray to uncover associated mRNAs. Another method to find more mRNA targets is studying sequence specificity of RNA binding proteins towards their target mRNAs, to uncover similar sequence elements computationally in the genome to predict more mRNA targets (Lee and Schedl, 2004).

In this study, the focus will be on *C. elegans* tandem CCCH RNA binding zinc finger proteins and STAR/KH domain RNA binding proteins, which are known to have other essential functions in addition to mRNA regulation.

2. Tandem CCCH RNA binding zinc finger proteins in *C. elegans*

Maternally contributed proteins and mRNAs are crucial for the development of oocytes, for flawless oocyte to embryo transition, and for the establishment of embryonic polarity in *C. elegans.* Establishment of body axes and asymmetric localization of cell fate determinants occur before the onset of zygotic transcription by regulated translation of maternal mRNAs (Farley and Ryder, 2008).

The tandem CCCH RNA binding zinc finger protein class was first characterized in mammalian protein tristetraprolin (TTP), which regulates inflammation response by destabilizing TNF-α transcript (Carballo et al., 1998; Varnum et al., 1991). CCCH RNA binding zinc finger proteins play very important roles during *C. elegans* development ranging from oocyte maturation, germline fate specification and establishment of body axis to establishment of embryonic cell fates. Emerging evidence suggests that this class of RNA binding proteins regulates translation of maternal mRNAs by binding to their 3'UTR sequences (Jadhav et al., 2008).

In general, *C. elegans* CCCH zinc finger proteins like PIE-1, OMA-1, OMA-2, MEX-5, MEX-6, POS-1 and MEX-1 do not show sequence similarity outside of their CCCH zinc finger domains, suggesting that regulation of maternal mRNA may not be the only function of this group of proteins. For example, PIE-1 represses transcription in P blastomeres through its C terminus domain, downstream of zinc finger domains. Other zinc finger dependent or independent functions are likely to be assigned to this group of proteins in the future.

C. elegans tandem CCCH RNA binding proteins with known germline or embryonic phenotypes are summarized in Table 1.1.

2-1. PIE-1 is a bifunctional protein for germline cell fate specification

2-1a. PIE-1 represses transcription by inhibiting the P-TEFb kinase complex

PIE-1 represses global transcription in P blastomeres of *C. elegans* embryos to prevent germ lineage from differentiating into somatic fates (Seydoux et al., 1996). Nuclear localized PIE-1 is essential for transcriptional repression (Tenenhaus et al., 2001) by interacting with Cyclin T to inhibit CDK-9 kinase, two components of the P-TEFb complex (Hirose and Ohkuma, 2007). Cyclin T is the CTD binding subunit of the P-TEFb kinase complex; the complex which phosphorylates RNAPII CTD at serine 2 for transcriptional elongation (Peterlin and Price, 2006). PIE-1 C terminus contains YAPMAPT sequence repeats which are similar to an unphosphorylatable form of RNAPII CTD YSPTSPS hepta repeats. Cyclin T binds to the repeat domain of RNAPII CTD; therefore by competing with RNAPII hepta repeats using a similar

unphophorylatable sequence, PIE-1 displaces Cyclin T from the transcription complex (Figure 1.6) (Zhang et al., 2003). When the C terminal domain of PIE-1 was brought to promoters by fusing it to the DNA binding domain of yeast GAL4, it was able to repress transcription in a mammalian cell culture system (Batchelder et al., 1999), suggesting that transcriptional repression domain of PIE-1 is located at its C terminus, outside of the two CCCH RNA binding zinc finger domains (Batchelder et al., 1999; Tenenhaus et al., 2001; Zhang et al., 2003).

pie-1 mutant embryos possess transcriptional derepression in germline precursor cells starting from P2. Transcription remains repressed in diakinetic oocytes as well as in 1-cell and 2-cell stage embryos (Seydoux et al., 1996). When stained with RNAPII phospho antibodies, 1- and 2-cell stage embryos lack transcriptional initiation or elongation markers (Seydoux and Dunn, 1997). However, in later germline blastomeres starting from P2, transcriptional initiation markers can be detected in two distinc foci, while elongation markers are absent (Seydoux and Dunn, 1997), suggesting that regulation of transcriptional repression before the onset of zygotic transcription is likely to be different from later germline blastomeres and probably at the level of transcriptional initiation.

Recently, it has been suggested that PIE-1 may block transcription at initiation level besides elongation by a domain outside of the YAPMAPT repeats, but the mechanism is not known yet (Ghosh and Seydoux, 2008). PIE-1 may have a function in repression of transcriptional initiation in 1- and 2-cell embryos, but lack of ectopic transcription in these stages in *pie-1* null embryos suggest that it is either not required during these stages or redundant with other factors.

2-1b. PIE-1 can regulate stability and translation of nos-2 mRNA

PIE-1 is localized primarily to nuclei, consistent with a function in transcriptional repression, and interestingly some portion of PIE-1 is cytoplasmic, most notably associated with P granules, RNA-rich organelles specific to the germline; suggesting a role in regulation of mRNA metabolism (Mello et al., 1996; Pitt et al., 2000; Seydoux and Fire, 1994; Strome and Wood, 1982). The second zinc finger of PIE-1 is sufficient to target its localization to P granules (Reese et al., 2000). PIE-1 promotes maternal *nos-2* mRNA maintenance and expression through its CCCH RNA binding zinc finger domains, which are independent of its C terminal transcriptional repression domain (Tenenhaus et al., 2001). NOS-2 is homologous to Nanos from other animals, and it is essential for PGC development and efficient incorporation into somatic gonad (Subramaniam and Seydoux, 1999). In addition to a function in transcriptional repression, a role in maintenance and expression of a PGC specific gene like *nos-2* suggests that PIE-1 contributes to specification of *C. elegans* germline fate by two different mechanisms.

2-1c. Asymmetric localization of PIE-1 to P lineage

P lineage blastomeres divide asymmetrically to generate one somatic and one germline daughter during specification of germline precursors of early *C. elegans* embryos. PIE-1 is a maternal protein expressed in the oocytes and the P lineage of early embryos with a primarily nuclear but also some cytoplasmic localization. *C. elegans* embryos are polarized after fertilization, and establishment of polarity results in PIE-1's inheritance primarily by the P lineage and exclusion from the somatic blastomeres after each of four asymmetric divisions (Figure 1.8).

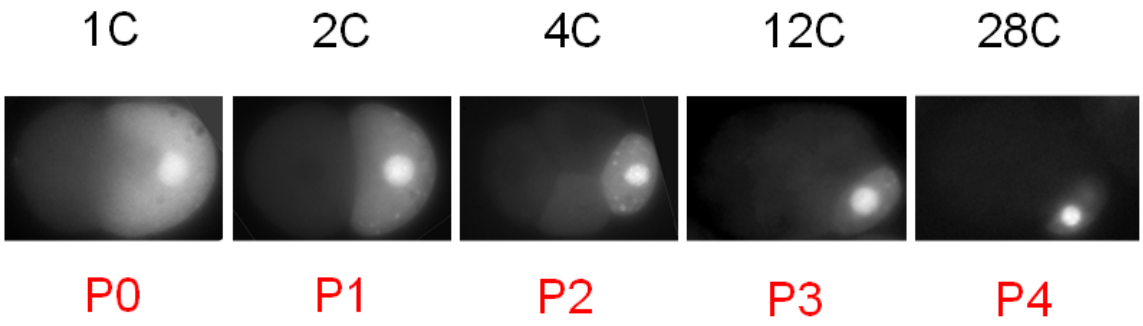


Figure 1.8. PIE-1 protein localizes asymmetrically to P blastomeres
PIE-1 is a maternally expressed protein detected as early as proximal oocytes, and the level of protein increases in the embyos. PIE-1 predominantly localizes to nuclei, consistent with a direct repression of P-TEFb complex. Cytoplasmic PIE-1 is enriched in P granules, and the second zinc finger is required for this localization pattern. Sperm-derived polarity results in asymmetric localization of PIE-1 towards the posterior of the embryo starting from the 1-cell stage. Asymmetric localization of PIE-1 to P lineage is reiterated after each asymmetric division of P blastomeres and requires two mechanisms: directed enrichment in the posterior before division and somatic degradation after each division.

Two distinct domains of PIE-1 that are regulated by two different mechanisms are required for its asymmetric localization (DeRenzo et al., 2003; Reese et al., 2000). Firstly, PIE-1 is directed towards the posterior cytoplasm right before each asymmetric cell division. Secondly, residual amounts of PIE-1 protein left in the somatic daughter cell right after the cell division are degraded by proteosome, along with some other maternal tandem CCCH RNA binding zinc finger proteins as POS-1, MEX-1, MEX-5 and MEX-6. Asymmetric localization of PIE-1 is reiterated through the four asymmetric P lineage divisions (Figure 1.3), and finally when P4 divides symmetrically to generate Z2 and Z3, PIE-1 is no longer detected (DeRenzo et al., 2003; Mello et al., 1996; Reese et al., 2000).

-Directed Localization of PIE-1 to P lineage before cleavage

The sperm-derived centrosome initiates the formation of polarity in the newly fertilized zygote of *C. elegans* (Cowan and Hyman, 2004; Goldstein and Hird, 1996) by inducing contractions of the cortical actinomyosin network that lead to anterior localization of cortical PAR-3/PAR-6/aPKC complex (Lyczak et al., 2002; Munro et al., 2004). Anterior Par proteins in turn restrict cortical PAR-1 and PAR-2 to the posterior of the embryo. Posterior PAR-2 counteracts PAR-3/PAR-6/aPKC (anterior Par) complex, reinforcing its anterior localization and the establishment of embryonic polarity (Figure 1.9) (Cuenca et al., 2003; Hao et al., 2006).

Established AP polarity in turn causes unequal segregation of cell fate determinants in the 1-cell embryo. MEX-5 and MEX-6 tandem CCCH zinc finger proteins are localized anteriorly by counteraction of PAR-1 but also themselves further reinforce polarity and unequal cell division (Nishi et al., 2008; Schubert et al., 2000). MEX-5 and MEX-6 exclude posteriorly required maternal proteins from the anterior of the embryo, like PIE-1 and P granules (Figure 1.9).

Directed localization of PIE-1 to the posterior of the embryo along the AP axis involves a cascade of events that establish AP polarity and PAR protein domains (Cuenca et al., 2003). After the first mitotic division, the majority of PIE-1 is inherited posteriorly by the P daughter cell. Similar to the first mitotic division of the zygote, during other asymmetric divisions of P blastomeres, PAR proteins and MEX-5 reiterate their asymmetric localization to promote asymmetric segregation of PIE-1 and P granules to P daughter blastomeres. How MEX-5 contributes to asymmetric localization of PIE-1 is not clear yet.

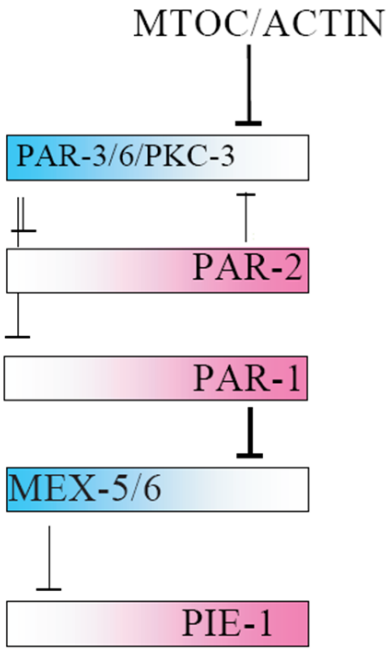


Figure 1.9: Establishment of embryonic polarity in *C. elegans* (Reproduced from Cuenca et al, 2003)
Microtubules derived from the asters of the sperm pronucleus initiate polarity formation in newly fertilized *C. elegans* embryos by initiating cortical movement of the PAR-3/6/PKC-3 complex anteriorly. Anteriorly localized PAR complex can counteract PAR-1 and PAR-2 to force their posterior enrichment. PAR-2 can in turn inhibit posterior localization of PAR-3/6/PKC-3 complex. Initiated PAR polarity results in MEX-5 to localize to the anterior of the embryo. MEX-5 displaces PIE-1 and P granules posteriorly to convert initiated PAR polarity to polarization of cell fate determinants. Anterior proteins are shown with blue, posterior proteins with pink bars.

-PIE-1 is excluded from soma by ZIF-1 dependent degradation

After division of P blastomeres, the majority of PIE-1 is localized to the P daughter, but traces of protein can be detected in the somatic daughter (Reese et al., 2000). The second mechanism to remove all residual PIE-1 from soma is ubiquitin mediated degradation by CUL-2 based cullin-RING ubiquitin ligase degradation complex. The first

zinc finger of PIE-1 is required for its somatic degradation (Reese et al., 2000). A yeast two hybrid screen to identify interaction partners of this domain uncovered ZIF-1 (zinc finger interacting factor), the substrate specific binding partner that targets PIE-1 for degradation specifically in somatic blastomeres (DeRenzo et al., 2003; Reese et al., 2000). ZIF-1 is a SOCS box protein and can interact with both its substrate and a component of the CUL-2 complex: Elongin C (Figure 1.10).

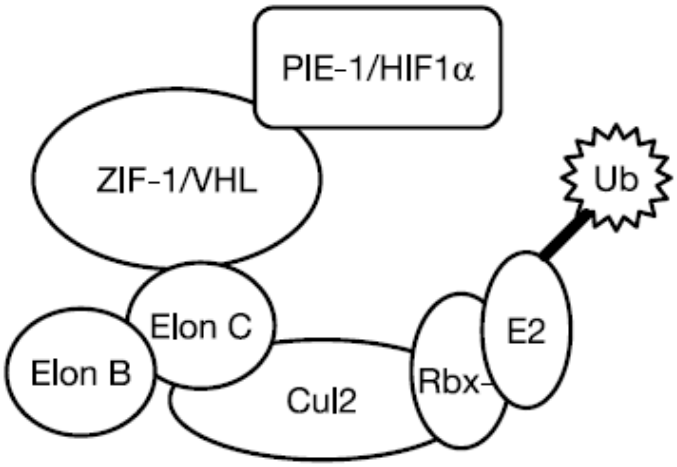


Figure 1.10. PIE-1 degradation complex
(Adapted from DeRenzo *et al,* 2003)
PIE-1 is degraded by CUL-2 based cullin-RING ubiquitin ligase degradation complex. ZIF-1 (Zinc finger interacting factor**)** is the substrate specific binding partner that interacts with the first zinc finger of PIE-1 and also with Elongin C.

ZIF-1 targets other CCCH zinc finger proteins POS-1, MEX-1, MEX-5 and MEX-6 for degradation as well, through binding to one of their two CCCH zinc finger domains

(DeRenzo et al., 2003). All five known targets of ZIF-1 are required for proper embryogenesis; thus ZIF-1 activity must be controlled tightly both spatially and temporally to activate ZIF-1 at the right time, only in somatic blastomeres.

2-2. MEX-5/6, POS-1 and MEX-1 are other known members of CCCH zinc finger proteins in *C. elegans*

There are other CCCH zinc finger proteins essential for viability during *C. elegans* embryogenesis, which will be described briefly here. Interestingly, except for OMA-1/2, all characterized embryonic tandem CCCH RNA binding zinc finger proteins require ZIF-1 for their degradation. ZIF-1 binds to one of their two zinc finger domains in order to target them for destruction, similar to PIE-1 protein degradation (DeRenzo et al., 2003).

2-2a. MEX-5/6 link polarity to cell fate determination

Initial anterior posterior (AP) polarity of *C. elegans* embryo is established by cortical contractions that the sperm centrosome causes at fertilization (Goldstein and Hird, 1996). Cortical contractions move anteriorly, resulting in anterior localization of PAR-3/PAR-6/aPKC complex, while PAR-1 accumulates posteriorly (Figure 1.9) (Goldstein and Macara, 2007; Munro et al., 2004; Munro, 2006). MEX-5 and MEX-6 are two CCCH RNA binding zinc finger proteins like PIE-1, but they are the only two embryonic *C. elegans* proteins in this class with an anterior localization (Schubert et al., 2000). Anterior localization of MEX-5/6 requires PAR-1 and PAR-4 phosphorylation (Tenlen et al., 2008). MEX-5 and MEX-6 convert established PAR asymmetry to

asymmetric localization of maternal mRNAs and proteins (Schubert et al., 2000) by (1) activating ZIF-1 dependent germline protein degradation anteriorly (DeRenzo et al., 2003), (2) displacing PIE-1 and P granules to the posterior of the embryo (Cuenca et al., 2003), and by (3) maintaining established embryonic polarity via a feedback loop with posterior PAR-1. Recently, it has been suggested that MEX-5/6 can interact with polo kinases PLK-1 and PLK-2, which are localized to the anterior of the embryo similar to MEX-5/6, and this interaction is suggested to be important for MEX-5/6 function (Nishi et al., 2008).

MEX-5/6 have two CCCH RNA binding zinc finger domains, and it has been suggested that their interaction with unidentified target mRNA 3'UTRs requires a tract of six or more uridines within a 9–13 nucleotide window, which is fairly abundant in *C. elegans* transcripts. Therefore, MEX-5/6 are likely to provide specificity by interacting with other RNA binding proteins (Pagano et al., 2007). So far no direct mRNA target of MEX-5/6 has been described; it will be interesting to define some targets that may shed light on function(s) of these highly redundant embryonic proteins in establishment of anterior cell fate.

2-2b. POS-1 and MEX-1 are determinants of posterior cell fate

Mutations in *pos-1 (posterior lineage defective)* or *mex-1 (muscle excess)* cause P blastomeres to adopt a somatic cell fate (Mello et al., 1992; Tabara et al., 1999). Similar to PIE-1, POS-1 and MEX-1 are localized predominantly to P lineage, but the somatic cell fate switches observed in germline blastomeres in mutants of all three genes have

different reasons and outcomes. POS-1 and MEX-1 are both localized to cytoplasmic P granules, and they are from CCCH zinc finger family.

In *pos-1* mutant embryos, P4 adopts the fate of its sister D, producing extra muscle tissue at the expense of germline (Tabara et al., 1999). Ectopic transcription is detected in P4 (refer to figure 1.3 for cell lineage) of *pos-1(-)* embryos, but inappropriate transcription in P4 alone cannot explain the pleiotropic defects in *pos-1* mutant embryos. In addition to a poorly characterized transcriptional repression role, POS-1 can regulate translation of maternal transcripts of *apx-1* (Notch ligand), *glp-1* (Notch receptor) and *nos-2* (nanos), and more targets are likely to emerge (D'Agostino et al., 2006; Evans et al., 1994; Evans and Hunter, 2005; Jadhav et al., 2008; Ogura et al., 2003; Tabara et al., 1999).

mex-1 mutants express PIE-1 ectopically in somatic blastomeres and have reduced POS-1 expression (Guedes and Priess, 1997; Tabara et al., 1999). Although the molecular mechanism is not clear yet, *mex-1* mutants have a P granule segregation defect that causes loss of the germline lineage. MEX-1 protein is localized predominantly to the P lineage and has a high P granule signal, suggesting that it may be regulating translation of maternal transcipts with its two CCCH RNA binding zinc finger domains.

Refer to Table 1.1 for a summary of known germline and embryonic CCCH RNA binding zinc finger proteins of *C. elegans*. OMA-1 and OMA-2 are two other members of the CCCH RNA binding zinc finger protein family and they are the focus of my thesis, so they will be described in more detail in the following pages.

	Subcellular Localization	Expression pattern	Known Function	Degradation by ZIF-1
MEX-1	Cytoplasmic P granule	Oocytes 1-cell P lineage Weak anterior sister of P	Germline vs soma decision(not clear)	Yes
MEX-5/6	Cytoplasmic some P granule (weak)	Oocytes 1-cell embryo *Anterior sister of P *Weak P lineage	Germline vs soma decision Establishment and maintenance of embryonic polarity Possible translational control	Yes
OMA-1/2	Cytoplasmic	Oocytes 1-cell embryo *Very little expression in 2-cell	*Oocyte maturation Embryonic development (was not clear how)	*No
PIE-1	*Nuclear enriched Some cytoplasmic P granule	Oocytes P lineage Weak anterior sister of P	Germline vs soma decision Transcriptional repression starting from P2 Stability of *nos-2* RNA (not clear)	Yes
POS-1	Cytoplasmic P granule	Oocytes (weak) 1-cell (weak) P lineage Weak anterior sister of P	Germline vs soma decision Transcriptional repression in P4 (not clear) Translational control	Yes

Table 1.1 *C. elegans* embryonic tandem CCCH RNA binding zinc finger proteins
CCCH zinc finger proteins are summarized in alphabetical order. Their subcellular localizations, expression patterns, known functions and whether ZIF-1 is involved in their degradation are shown.
* **Asterisks:** Proteins with unique feature among the other group members.

3. KH domain RNA binding proteins

The hnRNP K homology (KH) domain is a nucleic acid recognition motif that can bind to RNA or single stranded DNA molecules. KH domain proteins may have roles in different cellular functions ranging from haematopoietic cell differentiation (Ostareck-Lederer and Ostareck, 2004), glial cell fate differentiation and myelination (Larocque and Richard, 2005) to development of germ cells (Draper et al., 1996; Francis et al., 1995a; Tanaka et al., 2006). When dysfunctional this class of proteins may cause diseases, like cancer (Lukong and Richard, 2007), fragile X syndrome (Siomi et al., 1994; Siomi et al., 1993) or polycystic kidney disease (Bouvrette et al., 2008).

C. elegans KH domain proteins GLD-1 and MEX-3 are expressed maternally in the germline and early embryos with a complementary expression pattern (Figure 1.11) (Draper et al., 1996; Jones et al., 1996). Although their expression is not overlapping, they are redundantly required to maintain totipotency of adult germ cells (Ciosk et al., 2006). *gld-1; mex-3* double loss of function results in differentiation of germ cells into somatic cell types like muscle, gut or neurons causing worm teratomas (Ciosk et al., 2006). Contrary to embryonic germ lineage, transcription is active in adult germ cells to provide the mother and her future progeny with maternal mRNAs. Therefore, transcriptional repression cannot be the primary mechanism to maintain germline identity during adult germline development. Although neither how GLD-1 and MEX-3 maintain germline totipotency nor their in vivo common targets are yet clear, translational control of mRNAs seems to be the primary mechanism to protect adult germline against somatic differentiation (Ciosk et al., 2006).

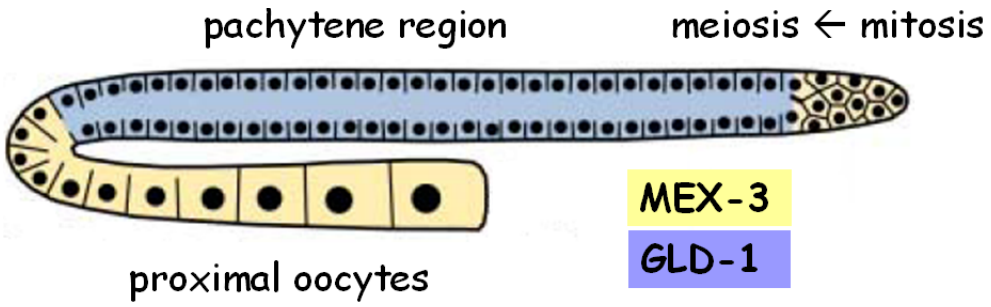


Figure 1.11: Non overlapping expression patterns of GLD-1 and MEX-3 in adult germline (Reproduced from Ciosk *et al,* 2006)
Germline stem cell nuclei are located at the distal end of the gonad loop; these cells express MEX-3 but have very minor GLD-1 expression. When nuclei enter meiosis, MEX-3 levels are down but GLD-1 levels go up. As germ cells start cellularizing and forming maturing oocytes GLD-1 levels drop dramatically and MEX-3 protein starts to accumulate. MEX-3 and GLD-1 have non-overlapping expression patterns but they are still redundantly required to protect germline totipotency.

3-1. Loss of GLD-1 causes tumorous germline

Adult germline is generated from the primordial germ cells (PGCs) of the embryo by many mitotic divisions resulting in over a thousand germ nuclei for each hermaphrodite gonad arm of *C. elegans*. Germline stem cells of *C. elegans* are located at the distal region of the gonad, and their continued mitotic division requires Notch signaling from distal tip cell and PUF family RNA binding proteins FBF-1 and FBF-2 (Kimble and Crittenden, 2005). Germ cells move along the gonad arms, and when they leave the mitotic region they enter into early stages of meiosis in the transition zone. FBF-1/2 repress translation of GLD-1 (for germline development) in the mitotic zone, and GLD-1 can be expressed in the pachytene region when FBF-1/2 expression is lost (Crittenden et al., 2002; Hansen et al., 2004; Kimble and Crittenden, 2005). GLD-1 is a sequence

specific KH domain RNA binding protein and functions as translational repressor in the center region of the *C.elegans* germline to control proliferation and the mitotic entry decision (Jan et al., 1999; Jones et al., 1996; Lee and Schedl, 2001; Ryder et al., 2004). The GLD-1 pathway is redundant with the GLD-2 pathway; therefore in *gld-1* single mutant animals, germline proliferation and entry into meiosis are essentially normal. (Eckmann et al., 2004; Francis et al., 1995b; Hansen et al., 2004; Kadyk and Kimble, 1998). However, single *gld-1* mutants lack oogenesis and have a tumorous germline phenotype, which is a result of pachytene germ cells returning to the mitotic cell cycle. These germ cells ectopically proliferate and form germline tumors. GLD-1 is expressed in the pachytene region of the germline and is missing in the proximal oocytes. Protein instability and repression of new translation result in the absence of GLD-1 in the growing oocytes (Lee and Schedl, 2001). GLD-1 represses translation of maternal genes from early oogenesis until late oogenesis (Jones et al., 1996), and some mRNA targets of GLD-1 have been identified (Lee and Schedl, 2001; Lee and Schedl, 2004; Ryder et al., 2004). However, it is not clear which mRNA target(s) result in the worm teratoma phenotype when functional GLD-1 is removed together with MEX-3.

3-2. MEX-3 is required for anterior fate specification of the embryo

Isolated blastomeres of the *C. elegans* early embryo can be cultured to study the cell fates of those blastomeres. When the two blastomeres AB and P1 of 2-cell embryo are separated and cultured, AB does not produce any muscle but P1 does, indicating that cell fate commitment begins as early as the 2-cell stage of the *C. elegans* embryo (Priess and Thomson, 1987). *mex-3 (muscle excess)* mutant AB blastomeres produce muscle ectopically (Draper et al., 1996). MEX-3 inhibits translation of *pal-1,* a Caudal like homeodomain protein required for specification of posterior fates, to prevent body wall muscle formation in AB lineage (Hunter and Kenyon, 1996). MEX-3 is expressed in the distal germline and in the proximal oocytes but it is downregulated by GLD-1 in the center region of the gonad (Figure 1.11). GLD-1 represses *pal-1* translation in the region of the gonad with no MEX-3 expression (Mootz et al., 2004).

In addition to *pal-1,* another mRNA target of MEX-3 has been recently identified; MEX-3 can also repress nanos homolog *nos-2* in anterior blastomeres to restrict NOS-2 expression to P blastomeres (Jadhav et al., 2008). Although a few mRNA targets of MEX-3 during embryonic development have been identified, the function of MEX-3 in the gonad of adult animals is poorly understood. The latest study suggests that MEX-3 protein in the mitotic zone of the adult gonad is redundantly required with PUF-8 to promote germline stem cell mitosis (Ariz et al., 2009). *mex-3* mutantion causes 100% embryonic lethality with overall normal looking gonads, suggesting that possible function(s) of MEX-3 in the gonad might be redundant with other factors.

IV. OMA-1 and OMA-2 are required for oocyte maturation

OMA-1 and OMA-2 (OMA-1/2) are CCCH RNA binding zinc finger proteins redundantly required for completion of oocyte maturation (Detwiler et al., 2001). *zu405,* an embryonic lethal allele of *oma-1,* results in delayed degradation of OMA-1 protein in embryos (Lin, 2003). Although *zu405* causes temperature sensitive embryonic lethality, single loss of function mutants of *oma-1(te33)* and *oma-2(te51)* do not show any abnormal phenotype. Removal of *oma-1* and *oma-2* simultaneously causes an oocyte maturation defect and fully penetrant sterility, but the reason for the sterility phenotype is still not clear. The sterility observed in *oma-1/2(-)* double mutant is female specific, because sperm of double mutants are functional (Detwiler et al., 2001).

Similar to PIE-1, OMA-1/2 proteins have two CCCH zinc finger domains. OMA-1 and OMA-2 share overall 64% sequence identity; they are even more similar in their CCCH domains. The two CCCH RNA binding domains OMA proteins contain make them very strong candidates for post-transcriptional regulators of maternal mRNAs. So far, *nos-2* is the only direct mRNA target identified for translational control by OMA-1/2 (Jadhav et al., 2008). Discovering molecular functions for OMA proteins during oogenesis or embryogenesis and uncovering mRNA targets will give more insight into OMA protein function(s) and the oocyte to embryo transition of *C. elegans.*

1. Regulated expression pattern of OMA-1/2

OMA-1 and -2 are two very similar maternal proteins with cytoplasmic expression in oocytes and in newly fertilized embryos of *C. elegans*. Expression peaks in the most matured oocyte and in the 1-cell embryo (Figure 1.12) (Lin, 2003). OMA-1/2 proteins are degraded right after the first mitosis of the zygote, and ectopic OMA-1 beyond the 1-cell stage causes embryonic lethality (Lin, 2003; Nishi and Lin, 2005).

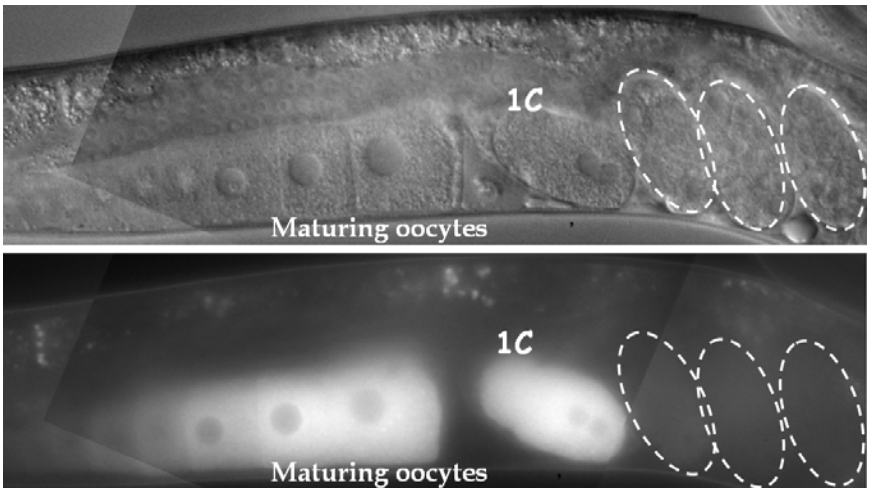


Figure 1.12. OMA-1::GFP live animal
An OMA-1::GFP translational fusion shows expression of OMA proteins in maturing oocytes and in a 1-cell embryo. Intensity of the signal is highest in the largest oocyte and in the 1-cell embryo. OMA proteins are developmentally degraded right after the first division of the zygote, and only a trace amount of protein is detected in the 2-cell embryo, and almost no protein is detected in later stages. Top image is DIC (differential interference contrast) and bottom is the fluorescent image of OMA-1::GFP animal. The gonad of the animal is located on the left and the uterus on the right. Maturing oocytes are labeled below. 1C: 1-cell embryo (at sperm and oocyte pronuclei meeting stage); dashed lines outline later stage embryos with no OMA-1::GFP signal.

The restricted oocyte and 1-cell stage expression pattern of OMA-1/2 proteins requires release of translational repression of their mRNAs by GLD-1 in the pachytene zone of the germline (Lee and Schedl, 2004). *oma-1/2* mRNA is present both in the pachytene zone and oocytes; STAR/KH domain RNA binding protein GLD-1 represses translation of *oma-1/2* transcripts in the pachytene region by direct association with their 3'UTRs (Lee and Schedl, 2004). GLD-1 expression disappears in the oocytes, enabling activation of OMA-1/2 translation in the proximal gonad (Detwiler et al., 2001; Lin, 2003). OMA proteins disappear after first mitosis through proteosome mediated degradation during embryogenesis (Lin, 2003; Nishi and Lin, 2005; Shirayama et al., 2006).

In *C. elegans,* there is no obvious delay between oocyte maturation and fertilization; thus the oocyte to embryo transition is very quick and must be tightly controlled. OMA protein expression overlaps with completion of oocyte maturation and the initial stages of embryogenesis; therefore understanding the functions of OMA proteins will shed light on developmental control of the oocyte to embryo transition, the most dramatic switch in an organism's life.

1-1. MBK-2 marks oocyte proteins for degradation

OMA-1/2 proteins are rapidly degraded after the first mitosis of the embryo, and ectopic OMA-1 beyond the 1-cell stage causes embryonic lethality as observed with the *zu405* allele (Detwiler et al., 2001; Lin, 2003). Similar to OMA-1/2, some of the maternal proteins required for oocyte development or meiosis must be scavenged for embryogenesis to proceed normally. For example, two subunits of meiotic spindle

severing complex, homologous to sea urchin katanins, MEI-1 and MEI-2, are degraded right after completion of meiosis for proper progression of mitosis (McNally and Vale, 1993). If MEI-1/2 persist through mitosis, short, barrel shaped meiotic spindles interfere with large mitotic spindle dynamics and cause embryonic lethality with cleavage defects (Dow and Mains, 1998; Kurz et al., 2002; Mains et al., 1990; Srayko et al., 2000). MBK-2 is a serine/threonine directed kinase homologous to DYRK2 (dual-specificity Yak1-related kinase), and it is required for the oocyte to embryo transition for tagging some maternal proteins for destruction by the proteasome. Additionally, MBK-2 phosphorylation may trigger change of maternal protein functions to better fit into the embryonic context (Nishi et al., 2008; Pellettieri et al., 2003; Stitzel et al., 2006). MBK-2 directly phosphorylates MEI-1 to mark it for degradation during the oocyte to embryo transition; in *mbk-2* mutants there is a MEI-1 degradation delay and ectopic MEI-1 localizes to mitotic spindles, causing embryonic cell cleavage defects (Pellettieri et al., 2003; Quintin et al., 2003).

The rapid developmental degradation of OMA proteins after the first mitosis of the embryo is regulated by MBK-2 phosphorylation as well. MBK-2 directly phosphorylates OMA-1 at threonine 239, and mutating that residue to an alanine delays OMA-1 degradation in vivo (Nishi and Lin, 2005; Stitzel et al., 2006). Interestingly, the *oma-1(zu405)* allele has a proline to leucine mutation at amino acid 240 (Lin, 2003), just one residue downstream of the MBK-2 phosphorylation site. Therefore, the *zu405* allele of *oma-1* interferes with MBK-2 phosphorylation, and ectopic OMA-1 beyond the 1-cell stage is due to impaired MBK-2 phosphorylation (Nishi and Lin, 2005). MBK-2 is localized to the cell cortex in the oocyte. Upon fertilization, right before meiosis II,

around the anaphase/prophase stage of meiosis I, MBK-2 protein localization undergoes a dramatic rearrangement, changing from uniform to punctate pattern at the cortex. This change in protein localization pattern has been proposed to correlate with MBK-2 kinase activation (Figure 1.13) (Pellettieri et al., 2003). During meiosis II and meiotic exit, MBK-2 progressively becomes cytoplasmic. Progression through meiotic division and meiotic exit are important steps for the change in subcellular localization of GFP::MBK-2, independent of fertilization (Stitzel et al., 2006).

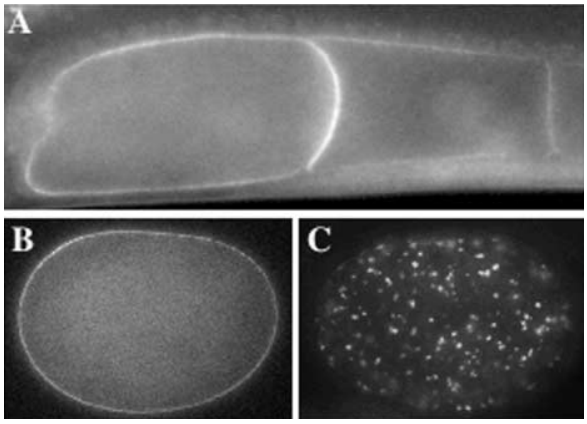


Figure 1.13. Dynamic localization change of MBK-2 at the oocyte to embryo transition (Reproduced from Pellettieri *et al,* 2003)
GFP::MBK-2 fluorescence images from A. ooctyes, B. newly fertilized embryo, right after sperm entry C. zygote in meiosis II.

While MEI-1 degradation starts around the proposed MBK-2 activation time, OMA-1 is degraded slightly later, after the first mitosis of the embryo (Lin, 2003; Pintard et al., 2003), suggesting that OMA-1/2 degradation requires additional regulation or/and that

MBK-2 phosphorylated OMA-1/2 may need to fulfill an embryonic function right before their destruction.

1-2. MBK-2 primes OMA-1 for GSK-3 phosphorylation

GSK-3, another serine/threonine directed kinase, is required for OMA-1 degradation in vivo and in vitro (Nishi and Lin, 2005; Shirayama et al., 2006). GSK-3 phosphorylation usually requires a priming phosphorylation in the substrate (Biondi and Nebreda, 2003). OMA-1 phosphorylation by MBK-2 serves as a priming event for GSK-3 phosphorylation in vitro (Nishi and Lin, 2005; Shirayama et al., 2006). A T239A mutation in the MBK-2 site greatly reduces phosphorylation by GSK-3 at position T339, using in vitro kinase assays (Nishi and Lin, 2005). Mutation of the GSK-3 phosphorylation site of OMA-1 threonine 339 to alanine in vivo results in an OMA-1 degradation defect similar to the MBK-2 site mutation (Nishi and Lin, 2005). It is not known at what stage of the 1-cell embryo GSK-3 phosphorylates OMA-1, but it is clear that OMA-1 is not degraded immediately after MBK-2 phosphorylation. Therefore, it was not known if OMA-1 phosphorylated at the MBK-2 site needed to execute an embryonic function between the time points of MBK-2 activation at meiosis II and OMA-1 destruction after first mitosis.

2. OMA-1/2 have redundant roles in 1-cell embryo

OMA-1 and -2 are redundantly required for oocyte maturation, but three pieces of evidence suggest that they may have redundant roles during embryogenesis as well. Firstly, unlike MEI-1, which is a target of MBK-2 phosphorylation and is degraded after

meiosis (Pellettieri et al., 2003; Quintin et al., 2003), OMA-1 is degraded after anaphase of the first mitosis, slightly later than MEI-1. Therefore, OMA-1 may have an embryonic role during the time it persists in the 1-cell embryo. Secondly, *oma-1; oma-2* double loss of function causes 100% penetrant sterility (Detwiler et al., 2001); however, milder double RNAi or double mutant mothers for reduction of function alleles can produce some dead embryos with pleiotropic defects (Nishi and Lin, 2005). Thirdly, when grown at permissive temperature (16ºC), *oma-1(zu405)* embryos display around 50% embryonic lethality; however when functional *oma-2* is depleted by RNAi *zu405* itself cannot support embyogenesis and results in 100% embryonic lethality even at permissive temperature. Interestingly, *oma-1(zu405); oma-2(RNAi)* animals do not display any sterility even at restrictive temperature (25ºC), suggesting that impaired function of the *zu405* allele is specific to embryogenesis. It is highly likely that that embryonic function(s) of OMA proteins require phosphorylation by MBK-2, since *zu405* P240L mutation interferes with proper MBK-2 phosphorylation.

CHAPTER TWO

OMA-1/2 repress global transcription in germline precursors of *C. elegans* by sequestering TAF-4 in the cytoplasm

SUMMARY

Germ cells are tightly regulated during animal development to achieve correct passage of genetic material to subsequent generations. Embryonic germ cell precursors are transcriptionally repressed in a wide range of animals to protect them from premature expression of somatic genes. Global transcription is repressed in maturing oocytes of adult *C. elegans* germline and maintained in repressed state right after fertilization. Zygotic transcription is activated in somatic blastomeres at the 4-cell stage of the *C. elegans* embryo. PIE-1 is a tandem CCCH RNA binding zinc finger protein. It represses transcription in the germline blastomeres, the P lineage, beginning with the 4-cell stage embryo. However, PIE-1 is not required for transcriptional repression in earlier embryonic stages, in 1-cell and 2-cell embryos before the onset of zygotic transcription. In this chapter, I present my data showing that two tandem CCCH RNA binding zinc finger proteins OMA-1 and OMA-2 that have been shown to be required for oocyte maturation redundantly repress global transcription in newly fertilized embryos by sequestering TAF-4, a key component for the RNA Polymerase II transcription machinery, in the cytoplasm. OMA-1 competes with TAF-12 to interact with TAF-4. TAF-12 is a histone fold binding partner of TAF-4. OMA-1/TAF-4 interaction is regulated by direct phophorylation of OMA-1 by MBK-2, a *C. elegans* DYRK kinase required for a proper oocyte to embryonic transition. MBK-2 phosphorylation enables

OMA/TAF-4 interaction to occur only during embryogenesis, not during oogenesis. Therefore, we believe this phosphorylation event converts OMA-1/2 from oocyte to embryonic regulators. Finally, my data demonstrate that properly phosphorylated ectopic OMA-1/2 are sufficient to repress transcription in all blastomeres and substitute for PIE-1 in the germ lineage during later stages of embryogenesis.

INTRODUCTION

Transcription is active during early stages of adult germline development to deposit maternal mRNAs and proteins required for proper gametogenesis and early embryogenesis (Nakamura and Seydoux, 2008). However, the transcription machinery is shut down in maturing late oocytes during diakinesis of meiotic division. Zygotic transcription resumes at the 4-cell stage of *C. elegans* embryo, but only in somatic blastomeres. Transcription is kept repressed in germline precursor cells, the P lineage, until they generate the primordial germ cells (PGCs) Z2 and Z3 (Blackwell and Walker, 2006; Seydoux and Dunn, 1997). Embryonic germ cell precursors are kept transcriptionally repressed to protect germ cell identity and to prevent their premature differentiation into somatic fates during embryogenesis. The *C. elegans* germ lineage is generated through four asymmetric divisions and one equal division starting from the zygote, the first germline blastomere, also called P0, to generate the PGCs of *C. elegans,* Z2 and Z3 (Figure 1.3). P blastomere asymmetric divisions are similar in a way to stem cell divisions, because each germ cell precursor generates another germ cell (P1-P4), which is transcriptionally repressed to block differentiation, and a somatic sister cell that

activates transcription of somatic genes soon after its birth to adopt a restrictive developmental program (Seydoux and Dunn, 1997; Seydoux and Fire, 1994). To activate its own differentiation program quickly after its birth, transcriptional repression in somatic sister cells of *C. elegans* germline precursors must involve readily reversible and transient mechanism(s). Consistent with this, transcriptional repression in the *C. elegans* P lineage does not involve stable chromatin-based transcriptional regulation. P blastomeres express epigenetic markers associated with active chromatin and lack markers associated with silent chromatin, which enables their somatic daughters to engage in transcription dynamically and quickly (Schaner et al., 2003).

Tandem CCCH RNA binding zinc finger protein PIE-1 represses transcription in the P lineage of 4-cell and later stages of *C. elegans* embryo, from P2 to P4 blastomeres (Seydoux et al., 1996). PIE-1 localizes preferentially to the P lineage, and nuclear localization of PIE-1 is essential to repress transcriptional elongation by binding to Cyclin T, a component of the P-TEFb kinase complex which phosphorylates RNAPII CTD serine 2 for transcription elongation (Batchelder et al., 1999; Mello et al., 1996; Tenenhaus et al., 2001; Zhang et al., 2003). Interestingly, similar to the mechanism of PIE-1 in *C. elegans* P lineage, *Drosophila* protein Pgc also blocks transcription in germline precursor pole cells by interfering with the P-TEFb kinase complex (Hanyu-Nakamura et al., 2008; Timinszky et al., 2008). Although *C. elegans* PIE-1 and *Drosophila* Pgc do not show any domain or sequence similarity, P-TEFb inhibition seems to be conserved between the two animals' germ cell precursors.

PIE-1 is a maternal protein expressed in the oocytes and in the P blastomeres of the embryo, yet it is required for transcriptional repression only in P2 and P3 and partially in

P4 blastomeres (Seydoux and Dunn, 1997; Seydoux et al., 1996). Transcription remains repressed in *pie-1* mutants in maturing oocytes as well as in 1-cell and 2-cell stage embryos, suggesting that PIE-1 is either not responsible for transcriptional repression at earlier stages or is redundant with some other factors. In addition, transcriptional repression in P0 and P1 occurs prior to transcription initiation, not elongation, because phosphorylation of RNAPII CTD repeats at serine 5, a transcriptional initiation marker, is not detected in P0 and P1 but can be detected at low levels in P2-P4 stages (Seydoux and Dunn, 1997). This suggests that the mechanism of transcriptional inhibition is likely to be different before and after the onset of zygotic transcription, during different stages of P lineage development.

OMA-1 and OMA-2 are maternal tandem CCCH RNA binding zinc finger proteins that are redundantly required for oocyte maturation (Detwiler et al., 2001). Loss of both *oma-1* and *oma-2* function results in 100% penetrant sterility, with very large but still not mature enough oocytes (Detwiler et al., 2001). OMA-1 and -2 are expressed in the proximal oocytes and in the 1-cell embryo, and they are developmentally degraded right after the first mitosis (Detwiler et al., 2001; Lin, 2003; Shimada et al., 2006). Rapid proteasomal degradation of OMA-1/2 is tightly regulated, requiring tandem phosphorylations by DYRK2 kinase MBK-2 and GSK-3 kinases (Nishi and Lin, 2005). *oma-1(zu405)* is a mutation interfering with MBK-2 phosphorylation that results in ectopic OMA-1 beyond the 1-cell stage, causing embryonic lethality (Nishi and Lin, 2005; Shirayama et al., 2006). Although double loss of function mutants of *oma-1* and *-2* are sterile, OMA proteins are implicated to have an embryonic function before their

proteasomal degradation, during the short time window in which they are expressed in the 1-cell embryo (Nishi and Lin, 2005).

In this chapter, I present my work demonstrating one embryonic function of OMA-1/2 in the newly fertilized zygote of *C. elegans*. OMA-1/2 repress global transcription in 1-cell and 2-cell embryos by a novel mechanism. OMA-1/2 are tandem CCCH RNA binding proteins similar to PIE-1, but they do not have similarity to PIE-1 outside of the zinc fingers. I demonstrate that OMA-1/2 interact physically and genetically with a keystone subunit of the basal transcription machinery, TAF-4 (TATA binding protein associated factor 4) and sequester TAF-4 in the cytoplasm to repress global transcription in 1-cell and 2-cell embryos of *C. elegans*. TAF-4 is required for the nucleation and stability of the TFIID (Transcription factor IID) complex (Wright et al., 2006) and RNAi depletion of *taf-4* in *C. elegans* embryos cause phenotypes similar to depletion of the RNAPII large subunit, AMA-1, suggesting that TAF-4 is essential for zygotic transcription of *C. elegans* embryos (Walker and Blackwell, 2003). OMA-1/2 block transcription globally at the "transcriptional initiation" level by sequestering a key component of the transcription machinery, TAF-4, consistent with the absence of serine 5 phophorylation of RNAPII CTD in 1-cell and 2-cell stage embryos.

I show that interaction of TAF-4 and its histone fold domain (HFD) binding partner TAF-12 is conserved in *C. elegans* and this interaction is required for nuclear localization of TAF-4 both in vivo and in HEK293T cells. The OMA-1 N terminal domain binds to the conserved HFD of TAF-4 via a domain predicted to have secondary structure similarity to HFD of TAF-12. Interestingly, I further show that OMA-1 and TAF-4 interaction is a regulated event requiring OMA-1 phosphorylation by MBK-2 at the

oocyte to embryo transition. When ectopically expressed, properly phosphorylated OMA-1 is sufficient to repress transcription and sequester TAF-4 in later stage embryos and substitute for PIE-1 for transcriptional repression in the P lineage. Finally, I show that the TAF-4 interaction domain of OMA-1 is essential for embryonic viability because its deletion causes 100% penetrant embryonic lethality, with no sign of an oocyte maturation phenotype, demonstrating for the first time that the oocyte and embryonic functions of OMA-1 can be uncoupled.

EXPERIMENTAL PROCEDURES

Strains

N2 was used as the wild-type strain (Brenner, 1974). Genetic markers used are: LGIII, *unc-119(ed3)*; LGIV, *oma-1(te21), oma-1(te33), oma-1(zu405)*; and LGV, *oma-2(te50), oma-2(te51)*. Transgenic strains were generated by microparticle bombardment (Praitis et al., 2001), and consistency of expression patterns was confirmed in at least two transgenic lines.

Plasmids used, strain names and integrations are as follows: TX903 (*teIs90 [P_{pie-1}gfp::taf-4]*), TX909 (*teIs96 [P_{pie-1}gfp::taf-4Δ333-382]*), TX1014 (*teIs102 [P_{pie-1}gfp::taf-12]*), TX864 (*te33; teIs76[P_{oma-1}oma-1::gfp]*), TX1155 (*te33; teEx559[P_{oma-1}oma-1Δ46-80::gfp]*) and TX1162 (*te33; teIs108[P_{oma-1}oma-1Δ46-80::gfp]*). TX189, ET113, and AZ212 contain *P_{oma-1}oma-1::gfp*, *P_{pie-1}gfp::cyb-1* and *P_{pie-1}gfp::h2b* transgenes, respectively, as described before (Lin, 2003; Liu et al., 2004; Praitis et al., 2001).

Plasmid Construction

Partial cDNA for *taf-1* and full-length cDNAs for *taf-4*, *taf-5*, *taf-6.1*, and *taf-10* were amplified from *yk14c2*, *yk326f12*, *yk1669h05*, *yk850e10*, and *yk1035g02* clones, respectively. Full-length cDNAs for *taf-8*, *taf-9*, and *taf-12* were amplified from embryos by RT-PCR. Most plasmids were constructed with the Gateway cloning technology. Germline expression constructs were derived from pID3.01B a *pie-1* promoter Gateway destination vector (Reese et al., 2000) or from pRL475, an *oma-1* promoter driving expression of OMA-1::GFP fusion protein (Lin, 2003). All the deletions were generated using the Quick Change site directed mutagenesis kit (Stratagene). For yeast two hybrid interaction assays, pGBKT7 or pASII-derived Gateway destinations were used as bait vectors, and a pACTII-derived Gateway plasmid was used as prey vector.

RNA interference (RNAi)

Feeding RNAi was performed as described (Timmons and Fire, 1998) using HT115 bacteria seeded on NGM plates containing 1mM IPTG. L1 larvae were fed for 2 days at 25ºC or 3 days at 20ºC to score either gonad phenotypes or embryonic phenotypes of the progeny. RNAi of various TAFs was done by double stranded RNA (dsRNA) injections into TX903 ($P_{pie\text{-}1}$*gfp::taf-4*), and GFP::TAF-4 localization was analyzed 1 day or 2 days later. RNA was synthesized in vitro (Ambion T7 transcription kit) and annealed to obtain dsRNA. A reduction in nuclearly localized GFP::TAF-4 was observed 1 day after injection of *taf-12* dsRNA; for the other TAFs: *taf-1, taf-5, taf-6.1, taf-7.1, taf-8, taf-9* and *taf-10*, nuclear GFP::TAF-4 was somewhat normal 1 day later, but dramatically

reduced 2 days post injection, suggesting that any of the *taf* genes affect GFP::TAF-4 localization eventually, probably by disrupting the stability of the TAF complex.

HEK293T Cell Transfection Assay

HEK293T cells were cultured on 1.5 mm coverslips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and transfected 16 hr later with the Fugene 6 transfection reagent (Roche) using 1 μg of total DNA for each coverslip. Transfected cells were fixed with 4% paraformaldehyde 24 hours to 48 hours later. GFP::TAF-4, HA::TAF-12, and FLAG::OMA-1 were all driven by the cytomegalovirus (CMV) promoter and were tagged N-terminally. The myristylation sequence MGSNKSKPKDASQ from human Src N terminus was inserted by PCR to target FLAG::OMA-1 full length and FLAG::OMA N terminus to the cell cortex. MBK-2 and kinase-dead MBK-2 (Y237A) were not tagged (Nishi and Lin, 2005; Shirayama et al., 2006).

Immunofluorescence

Immunofluorescence for *C. elegans* embryos are carried out as follows: for anti-Ser2P (Covance, MMS-129R) 1/300 dilution, α -PIE-1 (Mello et al., 1996) 1/50 dilution, α -OMA-1a (Shimada et al., 2006), 1/100 dilution, α –TAF-4 (Walker et al., 2001) 1/100 dilution, α -GFP (Invitrogen both mouse and rabbit antibodies were used) 1/250 dilution were used and the protocols were as desribed (Mello et al., 1996; Seydoux and Dunn, 1997; Shimada et al., 2006; Walker et al., 2001). Immunofluorescence staining of HEK293T cells was as follows: HEK293T cells were fixed with 4% paraformaldehyde

for 15 minutes, permeabilized with 0.3% Triton-X100 and blocked with 5% BSA. Antibodies used were rabbit anti-GFP (1/250 Invitrogen), mouse anti-FLAG (1/250, Sigma, F3165), rat anti-HA (1/50, Roche, 3F10). Secondary antibodies for all immunofluorescent analyses were from Invitrogen, goat anti-rabbit Alexa488, goat anti-mouse Alexa568, and goat anti-rat Alexa647.

In situ Hybridization

In situ hybridization for *vet-5* (very early transcript 5) (Schauer and Wood, 1990) was as described (Seydoux and Fire, 1995) with the exception that to obtain one-cell images, *oma-1(RNAi); oma-2(RNAi)* mothers were dissected in PBS on polylysine-treated teflon slides rather than by hypochlorite treatment. *oma-1(RNAi); oma-2(RNAi)* embryos have weak egg shell, so they were not compatible with hypochlorite treatment. *vet-5* DNA probes were synthesized by PCR from linearized pC101 plasmid with the DIG probe synthesis kit (Roche). To detect the probe, anti-DIG-AP antibody (Roche, 1/2000 dilution) was incubated 2 hours at room temperature, followed by NBT/BCIP (Roche) color reaction.

Analysis of embryos, imaging and quantification of cell cycle stage

Imaging of immunofluorescence, in situ, and live embryos was performed with an Axioplan microscope (Zeiss) equipped with a MicroMax-512EBFT CCD camera (Princeton Instruments) controlled by the Metamorph acquisition software (Molecular Devices). Imaging of HEK293T cells was performed with a LSM 510 Meta confocal microscope (Zeiss).

The percentage of early embryos at different cell cycle stages as meiosis I, meiosis II, 1-cell, 2-cell, and 4-cell stages were determined by fixation of embryos followed by DAPI staining and shown to be comparable between *oma-1(te33);oma-2(RNAi); teIs108 (OMA-1Δ46-80::GFP)* and *oma-1(te33); teIs76 (OMA-1::GFP)* strains.

Yeast Two Hybrid Assay

Yeast two hybrid analysis was done using GAL4 based transcription system in Mav203 strain grown on 50 mM 3AT (3-amino-1,2,4-triazole) Trp- Leu- His- plates, a stringent condition to test interactions.

oma-1; oma-2 Rescue Assay with transgenic OMA-1::GFP lines

Two OMA-1Δ46-80::GFP-expressing lines were generated by microparticle bombardment: TX1162 (*oma-1(te33); teIs108[P_{oma-1} oma-1Δ46-80::gfp]*), in which the transgene is integrated, and TX1155 (*oma-1(te33); teEx559[P_{oma-1} oma-1Δ46 80::gfp]*), a non-integrated line. The same results were obtained in both cases. Approximately 200 L1 larvae were placed on each *oma-2(RNAi)* plate and were grown at 20ºC for 3 days and animals were scored for the Oma (oocyte maturation defective) phenotype. No animal with Oma phenotype was observed with TX864 (*oma-1(te33); teIs76[P_{oma-1} oma-1::gfp]*), TX1155 (*oma-1(te33); teEx559[P_{oma-1} oma-1Δ46 80::gfp]*), a non-integrated line, or TX1162 (*oma-1(te33); teIs108[P_{oma-1} oma-1Δ46-80::gfp]*), an integrated line; whereas 100% of *oma-1(te33);oma-2(RNAi)* animals were Oma. To score for the rescue of embryonic lethality, only two L1 larvae were placed on each *oma-2* feeding RNAi

plate. The number of larvae and dead embryos on a total of three plates were counted and scored 5 days later, when all the embryos were laid.

RESULTS

At the time I joined the lab, three papers were published on OMA-1 by our lab showing (1) requirement for OMA-1/2 for oocyte maturation (Detwiler et al., 2001), (2) characterization of embryonic lethality caused by *oma-1 zu405* (P240L) allele by demonstrating mislocalization of some embryonic cell fate determinants as PIE-1, POS-1, SKN-1 etc (Lin, 2003) and (3) timely degradation of OMA proteins by tandem phosphorylation events by MBK-2 and GSK-3 kinases and it was suggested that OMA proteins likely to have an embryonic function (Nishi and Lin, 2005). However, neither gonad, nor embryonic functions of OMA proteins were clear. OMA-1/2 have tandem CCCH RNA binding zinc fingers but it was not known if these domains or others are important for OMA protein function. To get a clue on the function of OMA-1, a yeast two hybrid screen was carried out by a summer student Angela Collins. One of the interesting binding partners was TAF-4, an essential component of transcription machinery. Yuichi Nishi confirmed the interaction in yeast and this interaction seemed to be quite strong as it occurred even on 50mM 3AT plates, a stringent condition for yeast two hybrid interaction testing. While OMA-1/2 are known to be exclusively cytoplasmic both in the oocytes and in the 1-cell embryo, TAF-4 is a transcription factor expected to be nuclear. Therefore, it was not clear how this interaction might occur in vivo.

I. OMA-1/2 Interact with TAF-4, a key component of the TFIID Complex

To get some clue on the function(s) of OMA-1, yeast two hybrid screen was carried out using both full length and N terminus of OMA-1. N terminal 117 amino acid domain interacts with both full length and C terminal portions of TAF-4. Similarly, OMA-2 N terminus can also interact with TAF-4. I collected cDNAs of some other TAFs and tested if they could interact with OMA-1 in yeast as well. None of the TAFs I have tested interacted with OMA-1 (Figure 2.1), suggesting that OMA-1/TAF-4 interaction is specific to TAF-4 component of the TAF complex.

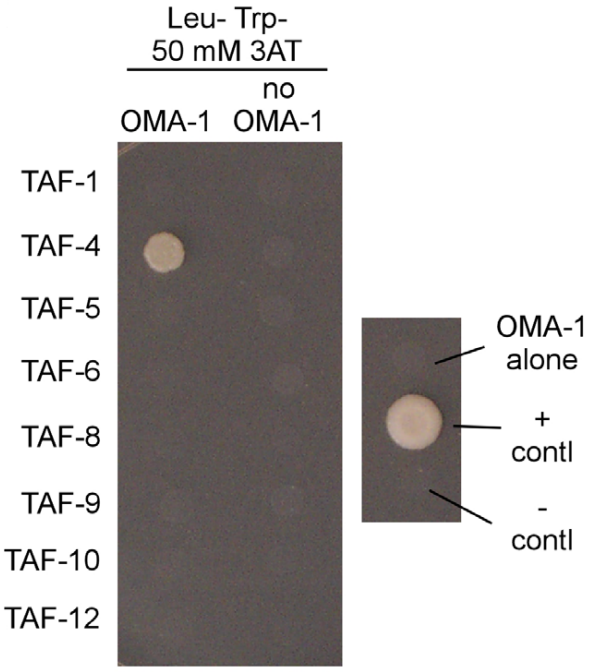


Figure 2.1: OMA-1 specifically interacts with TAF-4
Both full length OMA-1 cDNA and N terminal 117 amino acids interact with full length TAF-4 in Mav203 strain on stringent growth condition: leu-, trp- and supplemented with 50mM 3AT. Growth of a spotted colony indicates interaction. Other TAFs with an available cDNA tested for OMA-1 interaction and none of them interacted with N

terminus of OMA-1 in yeast. Left most column of spotted yeast are interaction test and right column demonstrates individual TAFs alone with and without transformed OMA-1. Third right separate column shows positive, negative controls for growth and OMA-1 single control.

Scott Robertson confirmed that OMA-1 and TAF-4 interaction using in vitro pull-down assays. MBP (maltose binding protein) tagged full length and N terminal OMA-1 was mixed with TAF-4 synthesized in rabbit reticulocyte lysate system and OMA-1 can pull down ^{35}S-labeled TAF-4. This interaction can stand even 750 mM NaCl high salt concentration, demonstration that this is a quite strong interaction (data not shown).

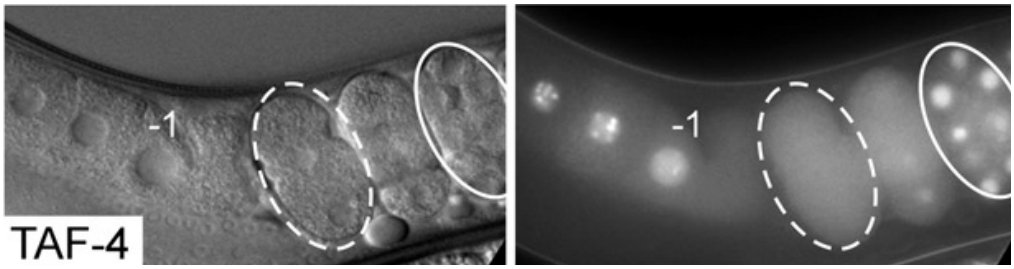
C. elegans adult germ cells shut down their transcription machinery in diakinesis stage of meiosis in maturing oocytes as indicated by UTP incorporation experiments and immunostaining with active transcription markers (Kelly et al., 2002; Schisa et al., 2001). Transcription can resume in somatic blastomeres starting from 4-cell stage embryo (Seydoux and Dunn, 1997). P blastomere transcriptional repressor, CCCH RNA binding zinc finger protein PIE-1 is not required for transcriptional repression in the late oocytes, nor in 1- and 2-cell stage embryos (Seydoux and Dunn, 1997; Seydoux and Fire, 1994; Seydoux et al., 1996). Very high levels of other CCCH RNA binding zinc finger proteins OMA-1/2 in the oocytes and in the zygote, in addition to their ability to interact with TAF-4 in vitro, prompted us to test a possible involvement of OMA proteins in regulating transcriptional repression either in the oocytes or in the newly fertilized embyos of *C. elegans*.

II. TAF-4 is nuclearly enriched except 1-cell and early 2-cell stages

Previous report using TAF-4 antibody showed that TAF-4 is nuclear in the oocytes and in the early embryos but nuclear signal was not very obvious in 1- and 2-cell stage embryos (Walker et al., 2001). Yuichi Nishi generated a GFP::TAF-4 germline expression transgenic animals by complex array injection, which was similar to reported TAF-4 localization with overall nuclearly enriched GFP::TAF-4 in the oocytes and embryos except in 1- and early 2-cell stages (Figure 2.2). I started the project by generating integrant lines for the same transgene because injection lines were both weak and extra chromosomal. My brighter GFP::TAF-4 integrant lines along with my immunofluorescence analyses using TAF-4 antibody reproduced the same expression pattern, with reduced nuclear signal of GFP::TAF-4 in 1-cell and early 2-cell embryos (Figure 2.2).

When I compared 1-cell localization of GFP::TAF-4 to GFP::H2B (histone 2B) and GFP::TAF-12, two other nuclear proteins expressed from the same vector, nuclear decrease of GFP::TAF-4 was even more obvious (Figure 2.3A). GFP::H2B and GFP::TAF-12 were clearly nuclearly enriched compared to cytoplasmic signal throughout all embryonic stages; including 1-cell embryo.

(A)



(B)

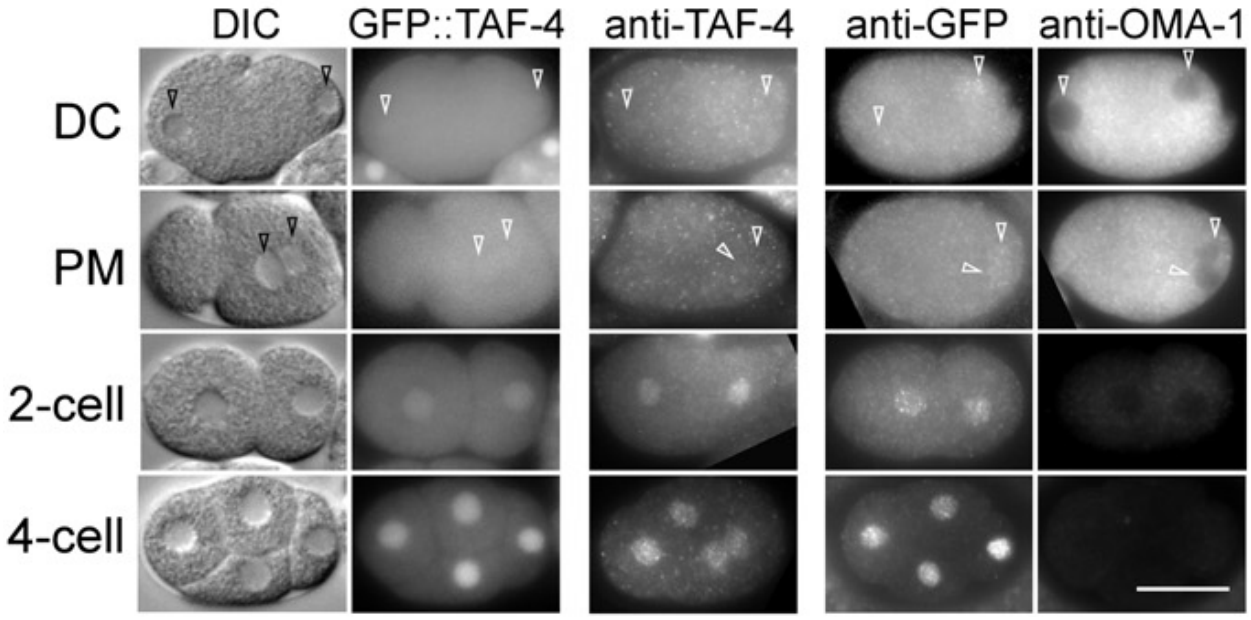


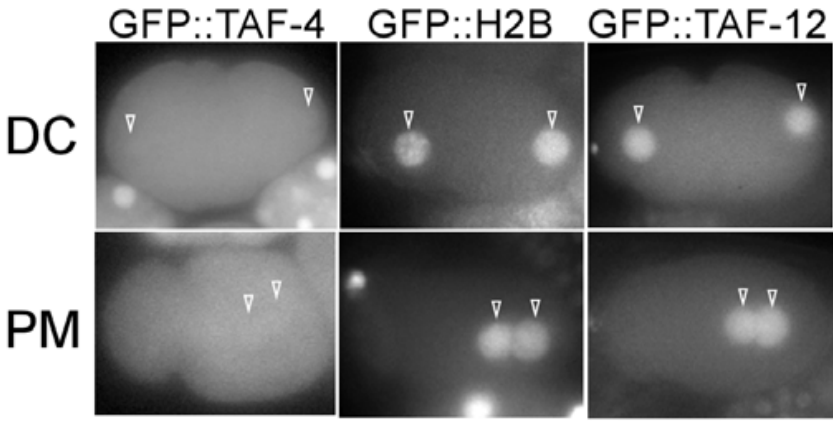
Figure 2.2 TAF-4 is nuclearly enriched in oocytes and embryos except 1-cell and early 2-cell embryos

A. DIC (left) and GFP fluorescent (right) images of GFP::TAF-4 strain. Shown is an adult animal, -1 is the most matured oocyte and located on the right of the oocytes is the uterus of the animal filled with embryos, arranged in developmental progression from youngest (left) to oldest (right). 1- cell embryo outlined in dashed line and 12-cell embryo in solid white line.

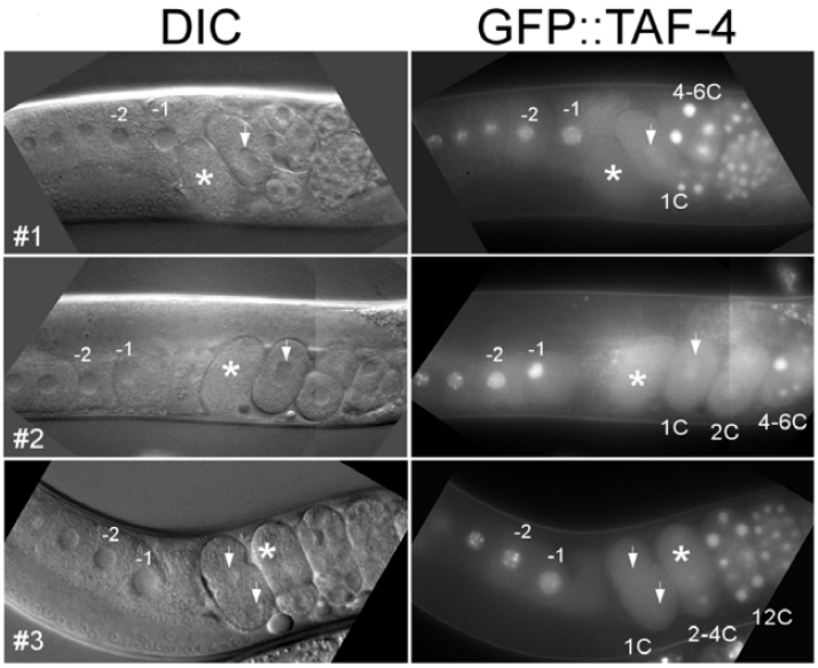
B. First two columns show DIC and GFP fluorescence images of live GFP::TAF-4 embryos, anti-TAF-4 immunostaining of wild type embryos shown in third column. anti-GFP and anti-OMA-1 staining of GFP::TAF-4 embryos are shown on the last two columns.

DC: pronuclei decondensation, PM: pronuclei meeting. DC and PM are different stages of 1-cell embryo. Arrowheads point to pronuclei in 1-cell embryos

(A)



(B)



(C)

	#1	#2	#3
-2 oocyte	1.08	0.89	**1**.23
-1 oocyte	1	1	**1**
1C embryo	**0.53**	**0.39**	**0.48**
2C embryo		0.52	
>4C embryo	1.01	0.67	**1**.67

Figure 2.3: TAF-4 has lower nuclear signal in 1-cell embryos
A. 1-cell GFP fluorescencent images from three transgenic animals expressed under the same vector (*pie-1* promoter, *pie-1* 3'UTR)**.** GFP signal is clearly nuclearly enriched in GFP:: H2B (histone 2B) and GFP::TAF-12 embryos, but not in GFP::TAF-4. DC: pronuclei decondensation, PM: pronuclei meeting. DC and PM are different stages of 1-cell embryo. Arrowheads point to pronuclei in 1-cell embryos.
B. Quantification of nuclear to cytoplasmic GFP intensity in GFP::TAF-4 animals at different stages of development from three independent animals. -2, -1: oocyte positions relative to uterus, arrows point to 1-cell pronuclei. Asteriks denotes cells undergoing division that were not quantified. DIC images are on the left column and corresponding GFP fluorescent micrographs are on the right.
C. Average nuclear to cytoplasmic GFP intensity of -1 oocyte is given a constant value of 1; fold difference of other stages of oocytes and embryos from three animals shown in B are calculated. 1-cell embryos had consistently lowered nuclear to cytoplasmic ratio relative to other stages.

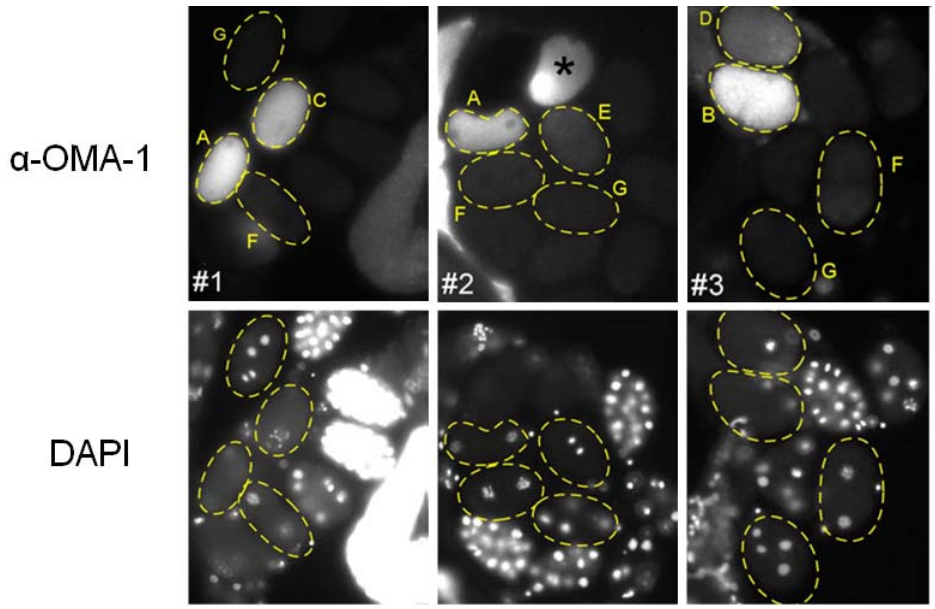
Nuclear to cytoplasmic ratio of GFP::TAF-4 signal in three independent animals were quantified by measuring average pixel intensity of GFP signal in -2, -1 oocytes and in 1-cell, 2-cell, 4-cell and older stage embryos. We assigned -1 oocyte an average intensity value of 1 for each animal, and quantified the average fold difference relative to -1 oocyte signal measurement in other oocytes and embryos within the same animal. We detected a consistently lowered nuclear to cytoplasmic GFP::TAF-4 in 1-cell embryos compared to oocyte and later embryonic signals, confirming that GFP::TAF-4 is not nuclearly enriched during at least one cell cycle, until completion of first mitosis (Figure 2.3B, C).

III. OMA-1/2 Sequester TAF-4 in the Cytoplasm

Transient decrease in nuclear enrichment of TAF-4 during 1-cell and early 2-cell stage of embryogenesis coincides with the brief time point OMA proteins can be detected in the embryo with very high levels in 1-cell and residual amount in 2-cell embryos (Figure 2.4) (Lin, 2003; Shimada et al., 2006). To analyze the degradation timing of OMA-1 protein more carefully, we stained wild type embryos with anti-OMA-1 antibody (Figure 2.4A) and quantified the average pixel intensity from early embryos. 1-cell embryos have the highest level of OMA-1, because the amount of protein reaches maximum levels right before initiation of its degradation. We assigned 1-cell embryo signal intensity, a constant value of 100 and calculated the relative values at different stages of early embryos ranging from 1-cell decondensation to 4-cell stage (Figure 2.4B, C). OMA-1 levels decrease sharply after 1-cell mitotic division stages; less than 10% of the protein remains around 2-cell stage embryos and by 4-cell stage the value of GFP intensity falls to zero relative to 1-cell embryo (Figure 2.4B, C).

1-cell and early 2-cell stage is the only time point OMA proteins exist during embryogenesis and this is the only time TAF-4 has lowered nuclear levels. Therefore, we believed that OMA-1/2 and TAF-4 could coexist in the same subcellular compartment only during 1-cell and early 2-cell stages to be able to interact. We believe this interaction occurs only in the newly fertilized embryos, in order to sequester TAF-4 in the cytoplasm and repress transcription by displacing a key component of transcription machinery away from DNA and mRNA transcription machinery.

(A)



(B)

Embryo symbol	Embryonic Stages	#1	#2	#3
A	1-cell, decondensation	100	100	-
B	1-cell, pronuclear migration	-	-	100
C	1-cell, pronuclei meet	95	-	-
D	1-cell, mitotic metaphase	-	-	34
E	1-cell, mitotic anaphase	-	21	-
F	2-cell	9	9	7
G	4-cell	0	0	0

(C)

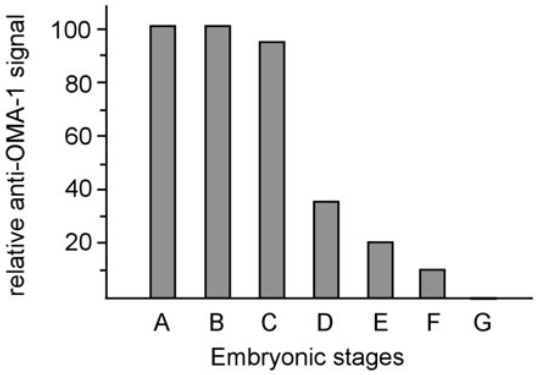
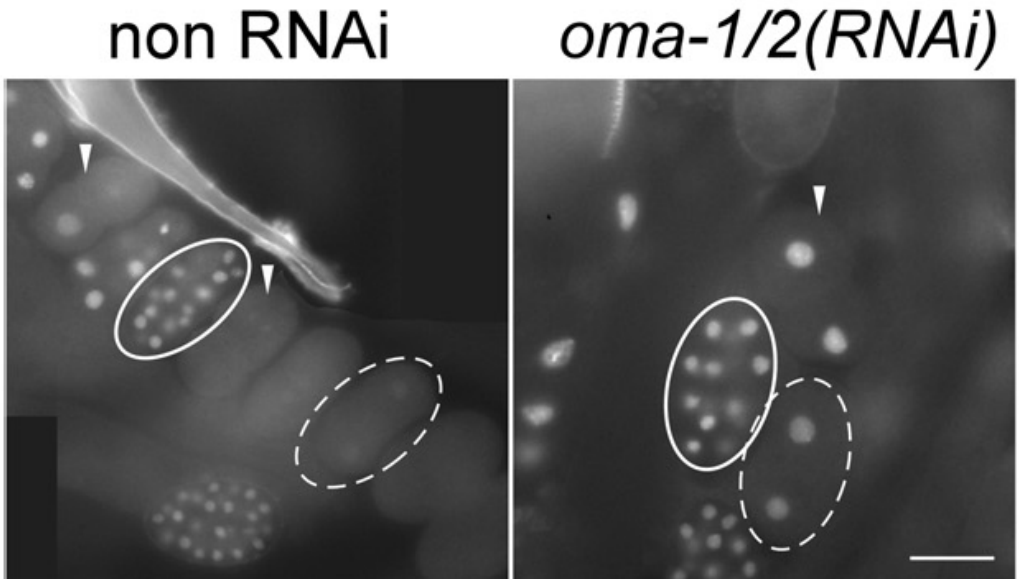


Figure 2.4: Quantification of OMA-1 levels in early embryos
A. anti-OMA-1 and Dapi staining of wild type embryos, three independent slide sections are shown. Embryos are given a symbol depending on the stage they are at. Stages corresponding to the symbols are shown in B.
B. Signal intensity from anti-OMA-1 stained wild type embryos are quantified by measuring average pixel intensity from different stages of early embryos. 1-cell stage embryos during pronuclei decondensation and pronuclear migration are assigned a value of 100 and other embryos were normalized relative to that value. Embryos in the above figure are given symbols depending on their stage.
C. Bar graph representing relative anti-OMA-1 intensity values vs embryonic stages using data from the above figure and table.

To test a possible role for OMA-1/2 in lowered nuclear TAF-4 levels in 1-cell and early 2-cell embryos, I depleted *oma-1/2* by RNAi in GFP::TAF-4 worms. RNAi was done with a series of dilutions using untransformed HT115 RNAi bacteria to get a milder RNAi phenotype, to bypass the requirement for OMA-1/2 during oocyte development and to get morphologically somewhat normal looking embryos in order to analyze GFP::TAF-4 localization during early stages of embryogenesis. Depletion of *oma-1/2(-)* resulted in a higher nuclear GFP::TAF-4 compared to non RNAi treated control strain (Figure 2.5A). We quantified nuclear to cytoplasmic ratio of average signal intensity in different early embryonic stages and also calculated the total level of GFP::TAF-4 in non RNAi and *oma-1/2* RNAi depleted embryos (Figure 2.5B). *oma-1/2* RNAi depletion resulted in nuclear retention of GFP::TAF-4 and nuclear to cytoplasmic ratio of GFP signal intensity increased about three fold upon reduction of *oma-1/2(-)* without affecting overall GFP::TAF-4 protein levels because the total signal intensity was not very different between 1-cell stage embryos from non RNAi and *oma-1/2* RNAi embryos (Figure 2.5B).

(A)



(B)

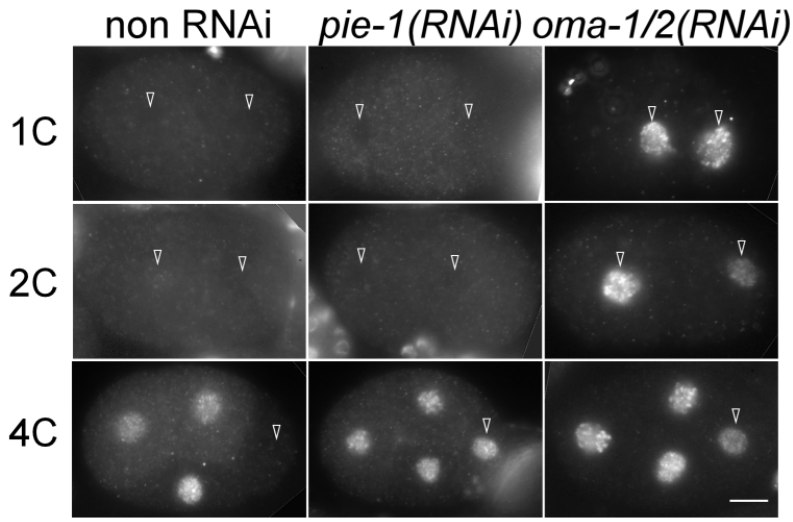
	Nuclear to Cytoplasmic Ratio		Total Embryonic GFP
	non RNAi	*oma-1/2(RNAi)*	RNAi/non RNAi
1-cell, decondensation	1.2	3.4	0.97
2-cell #1	ND	4.5	1.16
2-cell #2	ND	-	1.01
≥ 4C	3.2	4.9	1.09

Figure 2.5: *oma-1; oma-2* double RNAi in GFP::TAF-4 embryos
A. GFP fluorescence micrographs of fixed GFP::TAF-4 embryos with (right image) or without (left image) *oma-1/2* RNAi depletion. Outlined in dashed circles are 1-cell embryos, white solid line later stage embryos for comparison. Arrowheads point to 2-cell stage embryos. Scale bar represents 25μm.
B. Quantification of embryos in above images. Average GFP intensity of nuclear to cytoplasmic ratio was determined for each individual fixed embryo and shown in the second and third columns. Ratio of total GFP intensity of *oma-1/2* RNAi and non RNAi embryos from comparable stages is shown on the last column. ND: not determined because embryos are undergoing division.

IV. OMA1/2 are required for transcriptional repression in the newly fertilized embryos of *C. elegans*

oma-1/2 RNAi depletion results in a higher nuclear GFP::TAF-4 in 1-cell and 2-cell embryos, stages in which transcription is normally repressed in wild type animals. Therefore, we asked whether ectopic higher nuclear TAF-4 as a result of removal of *oma* genes caused ectopic transcriptional derepression. To test transcriptional status of the embryos, we used two markers: immunostaining using anti-Ser2P antibody, which detects phosphorylated serine 2 residues of RNAPII CTD repeats, a marker for transcriptional elongation (Kim et al., 1997; Komarnitsky et al., 2000; Seydoux and Dunn, 1997), and in situ hybridization to detect one of the known early zygotic transcripts (Seydoux and Fire, 1994). We chose to use *vet-5* (very early transcript 5), a gene with unknown function (Schauer and Wood, 1990; Tenenhaus et al., 1998) for in situ analysis because *vet-5* RNA accumulates in the nuclei providing easier detection. Using both markers, transcription is detected only in somatic blastomeres of 4-cell and later stage embryos in wild type animals (Figure 2.6). Germline blastomeres are transcriptionally repressed by C terminal portion of CCCH RNA binding zinc finger protein PIE-1(Seydoux et al., 1996; Zhang et al., 2003). However, transcription remains repressed in 1-cell and 2-cell stages of *pie-1* mutant embryos. I could reproduce the published results using the two transcription markers, anti-Ser2P immunostaining and *vet-5* in situ hybridization in *pie-1* RNAi animals; and I detected transcription both in somatic and germline precursors of embryos starting from 4-cell stage and transcription was still repressed in 1- and 2-cell stage of *pie-1* embryos (Figure 2.6) (Seydoux et al., 1996).

(A)



(B)

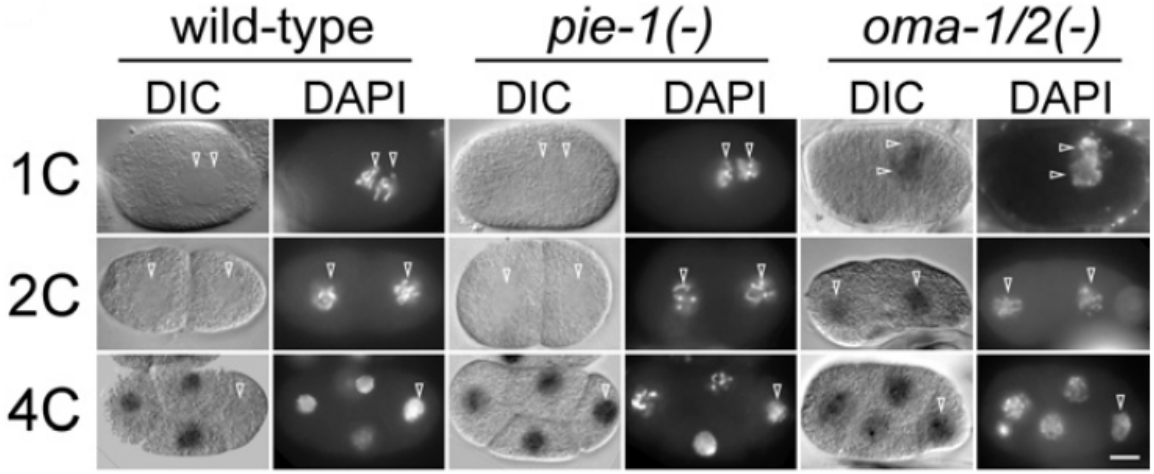


Figure 2.6 OMA-1 and OMA-2 are required for transcriptional repression
A. Anti-Ser2P immunostaining of wild type, *pie-1* and *oma-1/2* double RNAi embryos. Arrowheads point to pronuclei in 1-cell (1C), nuclei in 2-cell (2C) and germline blastomere P2 of 4-cell (4C) stage embryos. 1-cell and 2-cell embryos with no nuclear signal are enhanced to show the outline of the embryos.
B.In situ hybridization of *vet-5* mRNA. DIC and Dapi images are shown for wild type, *pie-1* and *oma-1/2* RNAi embryos. Arrowheads point to pronuclei in 1-cell (1C), nuclei in 2-cell (2C) and germline blastomere P2 in 4-cell (4C) embryos.
Scale bars represent 10μm.

To test a possible role for OMA-1/2 in transcriptional repression of 1-cell and 2-cell embryos, I used serial dilutions of *oma-1/2* feeding RNAi by using non transformed HT115 RNAi bacteria, because double loss of *oma-1/2* function results in sterility with no embryos. I could get a lot of dead embryos bypassing the sterility phenotype with milder RNAi. Phenotypes of dead embryos were variable depending on the strength of the RNAi condition; using milder conditions, I could get morphologically normal looking embryos, with slightly stronger RNAi, embryos had severe cleavage defects. Nonetheless, I analyzed a lot of wild type looking embryos based on DAPI staining to detect DNA morphology and I used transcriptional markers described above to see transcriptional activity of *oma-1/2(-)* embryos. Interestingly, *oma-1/2(-)* embryos had transcriptional derepression as early as 1-cell and 2-cell stage embryos (Figure 2.6). Detected premature activation of transcription in 1-cell and 2-cell stage embryos was not result of ectopic transcription in the late oocytes, which were still transcriptionally repressed even in *oma-1/2(-)* double null mutant animals. *oma-1/2(-)* oocytes showed transcriptional repression pattern similar to wild type of the diakinetic oocytes (Figure 2.7) (Walker et al., 2007). My results indicate that OMA-1/2 are required for transcriptional repression only in the early embryos, not in the late oocytes by sequestering an essential component of zygotic transcription machinery, TAF-4 in the cytoplasm. TAF-4 protein is nuclearly enriched in the oocytes suggesting that the mechanism of transcriptional repression in the oocytes is likely to be different than the one in the newly fertilized zygote.

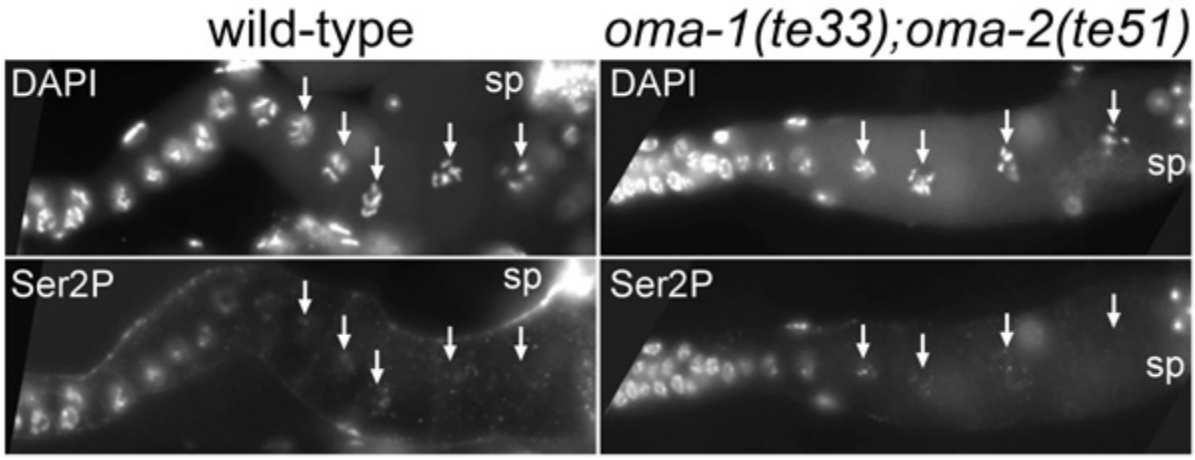


Figure 2.7: OMA-1/2 are not required for transcriptional repression in late oocytes
Immunofluorescence images from anti-Ser2P staining of wild type and TX183 (*oma-1/2(-)* double loss of function mutant) oocytes. Top images show co-staining with Dapi. Arrows point to oocyte nuclei. **sp:** spermatheca

Interestingly, transcription was not derepressed only in 1- and 2-cell stage embryos of *oma-1/2(-)* animals. I also detected derepression in germline blastomeres of later stage embryos, like P2 and P3 germline blastomeres (Figure 2.6, embryos beyond 4-cell are not shown). OMA proteins are normally degraded in later stage embryos, suggesting that the transcriptional derepression defect we observed in later germline blastomeres is probably not directly regulated by OMA-1/2, likely to be an indirect effect. It is already known that PIE-1 is responsible for transcriptional repression in germline blastomeres beginning from P2, therefore I checked PIE-1 protein levels in *oma-1/2* RNAi embryos using GFP::PIE-1 animals (Figure 2.8). As we predicted, *oma-1/2* RNAi embryos had reduced PIE-1 protein levels, which was inversely correlated with transcriptional derepression detected by anti-Ser2P staining. Embryos with almost no or very low PIE-1 had a

stronger derepression signal (Figure 2.8). 4-cell and older embryos I obtained from *oma-1/2* RNAi depletion were mildly treated compared to 1-cell embryos, in order to get morphologically somewhat normal looking embryos. Therefore, I detected almost no PIE-1 in 1-cell embryos with ectopic transcription but slightly more in later stage P blastomeres. Strength of *oma-1/2* depletion was correlated with the level of PIE-1 protein. Undiluted, strongest RNAi results in sterile, Oma (oocyte maturation defect) phenotype, which results in loss of PIE-1 expression completely in the gonads. In conclusion, OMA-1/2 indirectly repress transcription in later P blastomeres by regulating PIE-1 protein levels. This result suggests that a set of redundant proteins OMA-1/2 that are expressed maternally in the oocytes and in 1-cell embryos are crucial for the establishment of future germ lineage of the *C. elegans*, details of this regulation will be the topic of Chapter 3.

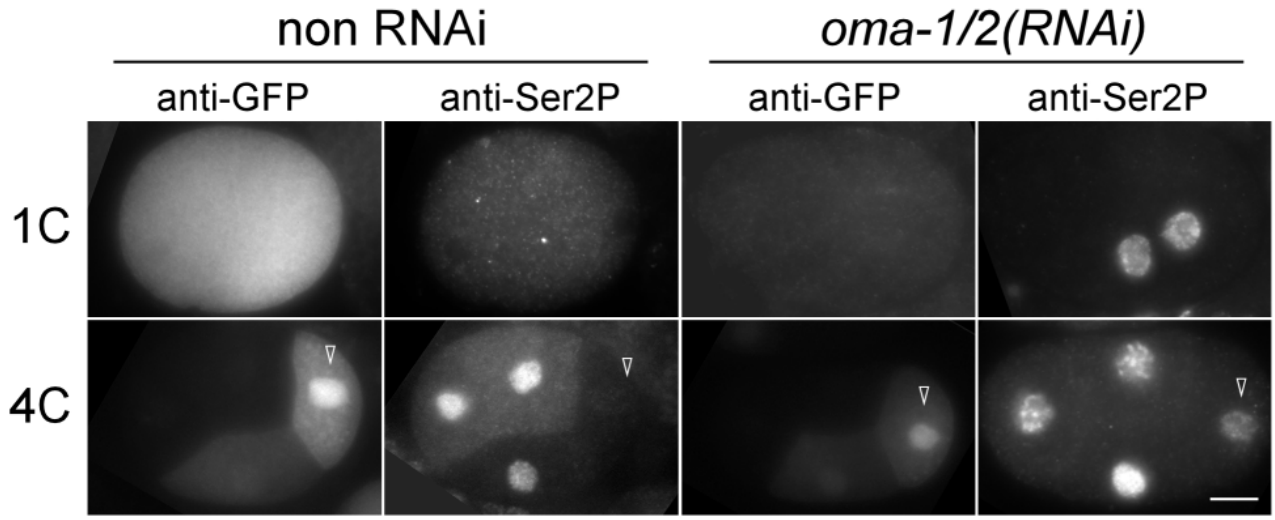


Figure 2.8: OMA-1/2 repress transcription in germline precursors beyond 4-cell stage indirectly by regulating PIE-1 levels
Immunofluorescence images of anti-GFP and anti-Ser2P double staining in GFP::PIE-1 animals with or without *oma-1/2* RNAi depletion. 1-cell and 4-cell embryos (labeled on the left) from non RNAi (left two columns) and *oma-1/2* RNAi (right two columns) embryos are shown. Arrowheads point to P2 blastomere of 4-cell stage embryo.
Scale bar represents 10μm.

V. OMA-1/2 and TAF-4 interact genetically

When I depleted *oma-1/2* in N2 and GFP::TAF-4 strains, I noticed that GFP::TAF-4 was more sensitive to reduction in *oma-1/2(-)* levels. RNAi dilution that results in no sterility with few dead embryos in wild type strain causes around 50% oocyte maturation defect in GFP::TAF-4 overexpressing strain, and 50% of the laid embryos were not viable (Table 2.1).

Increased sensitivity of TAF-4 overexpressing strain to reduction in *oma-1/2* function is in agreement with our model. When there is more TAF-4 expressed using a transgene, there will be an increased need for extra OMA-1/2 proteins to displace TAF-4 away from transcription machinery. Consistently, the severity of embryonic lethality in GFP::TAF-4 was correlated with more enriched nuclear GFP::TAF-4 signal. This increased sensitivity is not result of the overexpression of a transgene, because we used GFP::CYCLIN expressed from the same vector as control and there was no change in sensitivity to *oma-1/2* RNAi compared to GFP::TAF-4 animals. GFP::CYCLIN and wild type strains showed comparable sensitivity to reduction in *oma-1/2(-)* activity. In addition, depleting an unrelated gene, *wrm-1* (Rocheleau et al., 1997) by RNAi caused similar levels of embryonic lethality in all three strains, suggesting that GFP::TAF-4 transgene does not have overall an increased sensitivity to activation of RNAi pathway. Increased sensitivity

of TAF-4 overexpression to reduction in *oma-1/2* levels suggests that *oma-1/2* and *taf-4* genetically interact, in addition to their physical interaction in yeast and in pull-down assays.

Dilution of O1O2 RNAi bacteria[a]	Wildtype N2		TX903[b]		ET113[c]	
	Oma[d]	DEB[e]	Oma	DEB	Oma	DEB
1:3	100	100	100	100	100	100
1:9	100	95	100	90	100	95
1:33	30-40	50	100	67	30-50	50
1:100	0	0	70	50	<5	0
1:200	0	0	50	50	0	0
1:400	0	0	10-20	50	0	0
Non RNAi	0	0	0	0	0	0

Table 2.1: TAF-4 overexpression causes increased sensitivity to *oma-1/2* reduction
[a.] *oma-1* and *oma-2* feeding bacteria was mixed with non-RNAi bacteria HT115 to bypass sterility. Dilutions of RNAi are the dilution of equally mixed *oma-1* and *oma-2* bacteria in the final volume.
[b.] TX903 is GFP::TAF-4 transgenic strain.
[c.] ET113 is GFP::CYB-1 (Cyclin B) transgenic strain.
[d.] Percentage of adults with oocyte maturation phenotype (n>300 for all the dilutions tested).
[e.] Percentage of dead embyos of total embryos laid onto plates (n>100 when there is no 100% sterility).

VI. OMA-1 interacts with HFD of TAF-4

In our yeast two hybrid screens, all the TAF-4 clones pulled using OMA-1 N terminal bait, contained TAF-4 C terminal portion. Therefore, we were interested in finding the smaller domain of TAF-4 that is required and sufficient for OMA-1 interaction to study that minimal domain in vivo. Using again yeast two hybrid system, I narrowed down the

interaction domain of TAF-4 to 50 amino acids (from amino acid 333 to 382), which is located within histone fold domain (HFD) of TAF-4. This 50 amino acid domain of TAF-4 is both necessary and sufficient to interact with OMA-1, in yeast (Figure 2.9).

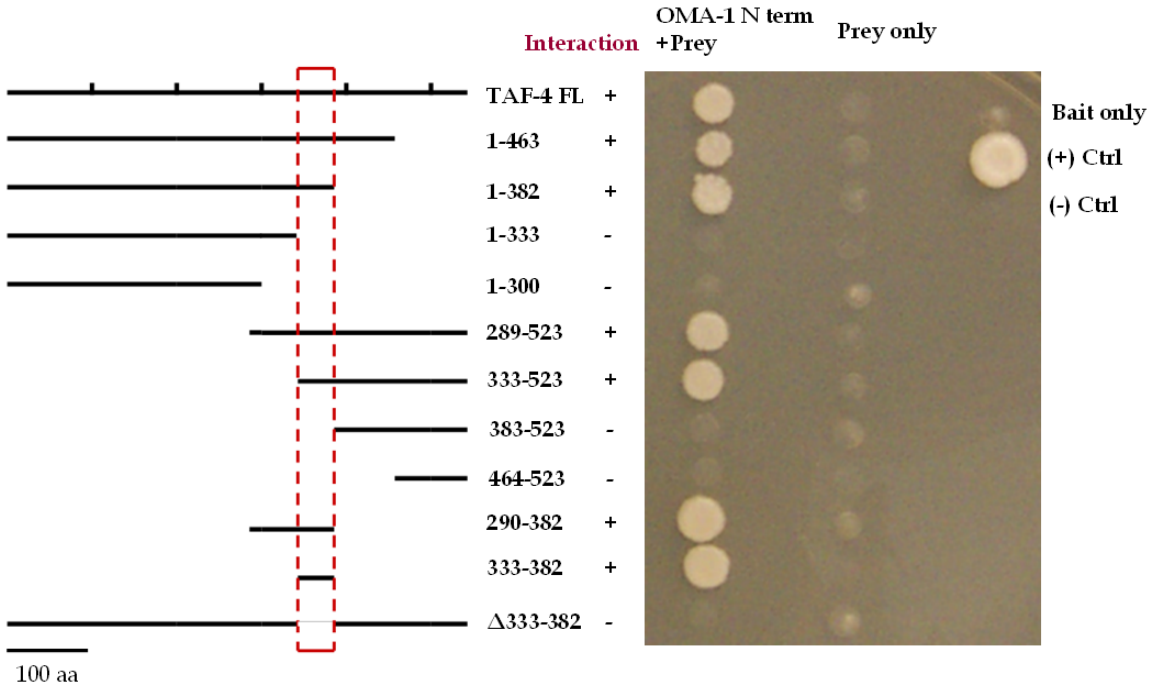


Figure 2.9. OMA-1 N terminal 117 amino acids interacts with TAF-4 HFD
Left lines depict serial truncations to full length TAF-4 that is used as prey and corresponding amino acids are shown in the middle. Red dashed box: minimal interaction domain that is both necessary and sufficient. Last two serial truncation show sufficiency and necessity for 50 amino acid domain, respectively. Bait is OMA-1 N terminal 117 amino acids.
Growth of Mav203 strain on His-, Trp-, Leu- 50mM 3AT plates indicates interaction. No growth means negative interaction.

C. elegans TAF-4 is a 523 amino acid protein with two conserved domains: N terminal TAFH/NHR1 domain (amino acids 135-220) and C terminal Histone Fold Domain (HFD, amino acids 333-382) (Figure 2.10) (Walker et al., 2001). TAFH/NHR1 domain is predicted to be important for protein-protein interactions.

Figure 2.10: TAFH and Histone fold are conserved among TAF4 proteins from different species
C.elegans TAF-4 has two identifiable domains that are conserved throughout different species, TAFH/NHR1 domain located N terminally and HFD located C terminally.

More than half of the TAFs have a histone fold domain predicted to have roles in the formation of a histone octamer like multi-subunit protein complex (Gangloff et al., 2000; Hoffmann et al., 1996; Selleck et al., 2001). Histone fold containing TAF10 does not have a nuclear localization signal and it is expressed in the cytoplasm when transfected into cell culture system. However, at least one of its histone fold interaction partners like TAF3, TAF8 or SPT7L is sufficient to move TAF10 to nuclei (Soutoglou et al., 2005), suggesting that histone fold domain of some TAFs might have role in their subcellular localization. To test if HFD of TAF-4 plays any role in its localization pattern, I deleted HFD from full length TAF-4 and generated GFP::TAF-4ΔHFD transgenic animals

(Figure 2.11). Interestingly, GFP::TAF-4 did not localize to any subcellular compartment when HFD deleted, suggesting a role for nuclear localization of TAF-4.

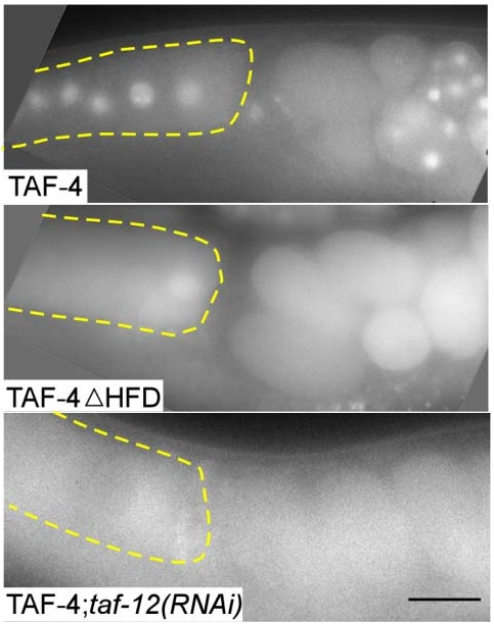


Figure 2.11. HFD of TAF-4 and HFD binding partner TAF-12 are required for nuclear localization of TAF-4
GFP fluorescence micrographs of GFP::TAF-4, GFP::TAF-4ΔHFD and GFP::TAF-4;*taf-12 (RNAi)* live adult animals. Yellow dashed lines: Oocytes of the animals and located on the right of the image are embryos located in the uterus.
Scale bar represents 25μm.

Histone fold containing TAFs have very specific binding partners to dimerize. Crystal structure of the human TAF4 HFD is solved and it specifically interacts with HFD of human TAF12 (Werten et al., 2002). I confirmed that TAF-4 and TAF-12 interaction is

conserved in *C. elegans* (data not shown) by yeast two hybrid system. To investigate a possible involvement of HFD binding partner TAF-12 in subcellular localization of GFP::TAF-4, I injected double stranded RNA (dsRNA) of *taf-12* into GFP::TAF-4 animals and analyzed GFP expression one day later. Consistent with HFD deletion lines, when HFD interaction partner was depleted, nuclear enrichment of TAF-4 was lost (Figure 2.11).

To further study subcellular localization of TAF-4, I used human HEK293T cell line. When transfected alone, TAF-4 was exclusively cytoplasmic (100% n>1000, Figure 2.12), suggesting that TAF-4 does not have a nuclear localization sequence and is probably missing the interaction partner(s) that moves TAF-4 into the nuclei in this human cell culture system. Co-expression of TAF-4 with TAF-12 resulted in nuclear localization of TAF-4 in almost all cells that expressed TAF-12 (~100%, n>300), demonstrating that TAF-12 is sufficient to move TAF-4 into the nucleus in HEK293T cells (Figure 2.12). Although HEK293T cells have their own human TAF12 protein, and TAF4 proteins are evolutionarily conserved, TAF-4 was still exclusively cytoplasmic without *C. elegans* TAF-12, suggesting that TAF-4 and TAF-12 interaction is species specific.

OMA-1 N terminus did not localize to any subcellular compartment when transfected into HEK293T cells (data not shown), therefore I tagged OMA-1 N terminus with myristylation sequence, which localized OMA-1 N terminus under the cytoplasmic membrane. When co-transfected with membrane tethered OMA-1 N terminal domain, TAF-4 was targeted to cell membrane in ~80% of the cells that co-expressed both TAF-4 and myr-OMA-1 N proteins (n>200, Figure 2.12).

OMA-1 and TAF-12 interact with the same histone fold domain of TAF-4; therefore, we asked whether OMA-1 could compete with TAF-12 for TAF-4 interaction. When all three proteins TAF-4, TAF-12 and myr-OMA-1 were transfected into HEK293T cells, membrane targeting of TAF-4 still occurred, even in the presence of TAF-12 (Figure 2.12). ~40% of the cells expressing all three proteins had membrane localized TAF-4 with low or no nuclear TAF-4 localization (n>200). We concluded that OMA-1 interacts with TAF-4 by competing with TAF-12.

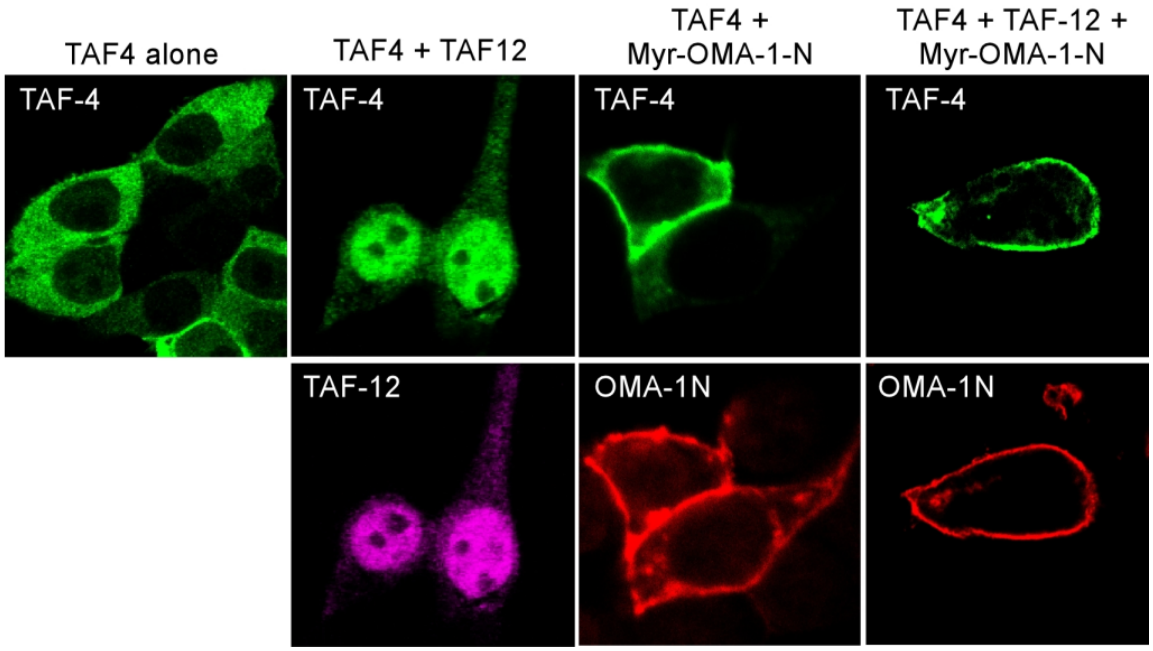


Figure 2.12. OMA-1 competes with TAF-12 for TAF-4 interaction
Immunofluorescence of HEK293T cells transfected with GFP::TAF-4 (green) alone (most left column), GFP::TAF-4 co-transfected with HA::TAF-12 (purple) (second column) or with myr-FLAG::OMA-1 N terminus (red, third column) or triple transfected with HA::TAF-12 and FLAG::OMA-1 N terminus (last column).

All the changes observed in TAF-4 localization with double or triple transfections were dependent on HFD of TAF-4, because when HFD was deleted from full length TAF-4, TAF-4 became ubiquitous throughout the cell (100% n>1000) similar to in vivo deletion transgenes (Figure 2.11), and remained so even in the presence of TAF-12 or OMA-1 (Figure 2.13).

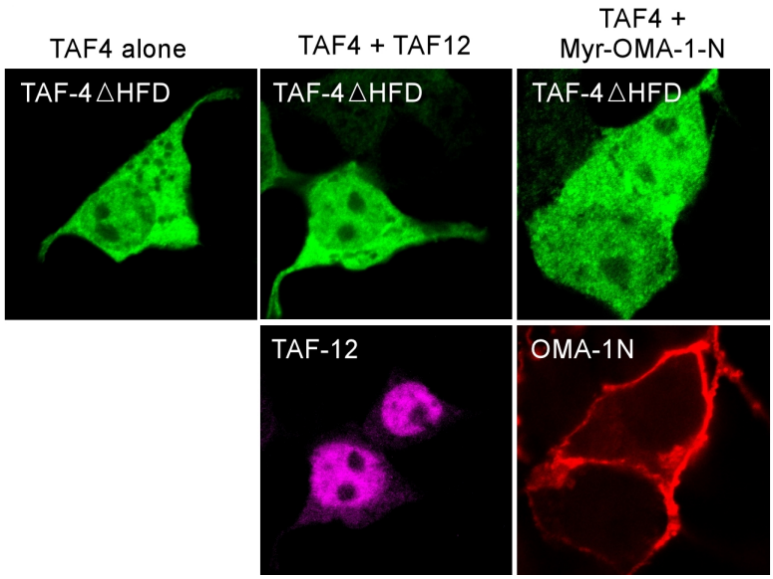


Figure 2.13. HFD is required for changes in subcellular localization of TAF-4
Immunofluorescence of TAF-4ΔHFD alone, cotransfected with TAF-12 or with myr-OMA-1 N terminus. TAF-4: green, TAF-12: purple, myr-OMA-1 N: red

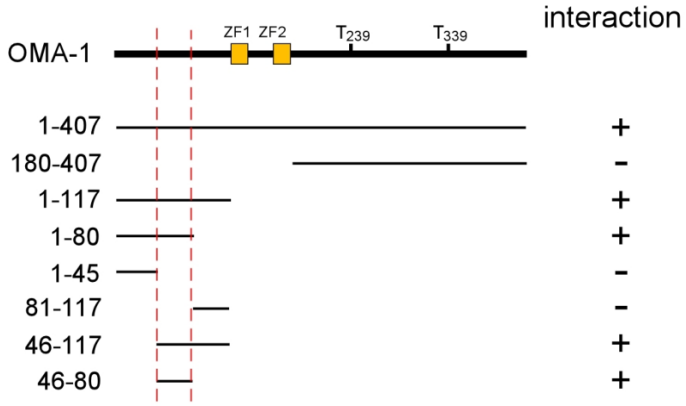
VII. TAF-4-binding domain of OMA-1 is essential for embryonic viability but not for oocyte maturation

Most N terminal 117 amino acid domain of OMA-1 was used in yeast two hybrid screen and interaction assays but to get a deeper insight into the significance of TAF-4 interaction, I further mapped the binding domain to an even smaller 35 amino acid region, from amino acid 46 to 80 (Figure 2.14A, data not shown), which was both necessary and sufficient for TAF-4 interaction.

35 amino acid TAF-4 interaction domain of OMA-1 has a predicted alpha helical structure using an online secondary structure prediction tool (Pole Bioinformatique Lyonnais Gerland). Since histone fold domains use alpha helices for the interaction, we checked if OMA-1 N terminal minimal TAF-4 interaction domain had any similarity to TAF-12 histone fold. Interestingly, smaller TAF-4 interaction domain of OMA-1, while sharing no similarity to primary sequence, showed some similarity to TAF-12 histone fold alpha helix 2 (Gangloff et al., 2001; Werten et al., 2002) considering their secondary structure predictions (Figure 2.14B).

Crystal structure of human TAF4 and TAF12 heterodimer indicated that hydrophobic core of TAF4 and TAF12 histone fold interaction surface have 13 residues with 11 hydrophobic residues and 2 small side chain residues (Figure 2.14B) (Werten et al., 2002). Aligning minimal TAF-4 interaction domain of OMA-1 (amino acids 46-80) with TAF-12 histone folds from different species, we detected hydrophobic residues in OMA-1 at 10 out of 11 positions. We believe that OMA-1 can compete with TAF-12 for TAF-4 interaction using a hydrophobic surface.

(A)



(B)

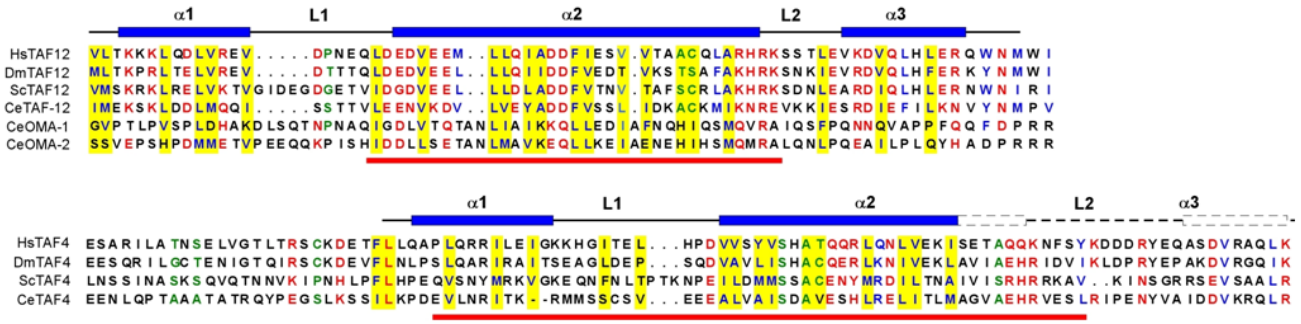


Figure 2.14. TAF-4 interaction domain of OMA-1 resembles to HFD of TAF-12 at secondary structure

A. Deletion analysis to map TAF-4 interaction domain of OMA-1 using yeast two hybrid demonstrated that 35 amino acid region from amino acid 46 to 80 is both necessary and sufficient. ZF1:zinc finger 1, ZF2: zinc finger 2, T239: MBK-2 phosphorylation site, T339: GSK-3 phosphorylation site.
Numbers on the left show amino acid positions for deletion constructs.

B. Protein sequence alignments of top: TAF-12 histone folds from human, *Drosophila, S. cerevisiae, C. elegans,* against OMA-1 and OMA-2 N terminal portions. Underlined with red is TAF-4 binding domain of OMA-1.
Bottom alignment: TAF-4 histone folds from human, *Drosophila, S. cerevisiae* and *C. elegans.* Underlined in red is OMA-1 interaction domain of TAF-4. Yellow highlighting indicates residues located in the hydrophobic core of TAF-4/TAF-12 heterodimer (Werten et al., 2002). α: alpha helix; L: loop. Amino acids conserved among TAF HFDs are highlighted; blue: hydrophobic, red: charged, green:small.

To understand the in vivo significance of OMA-1 N terminal 35 amino acids domain, its involvement in sequestering TAF-4 in the cytoplasm and in transcriptional repression, I generated transgenic animals with either wild type OMA-1::GFP or OMA-1::GFP lacking TAF-4 interaction domain (OMA-1Δ46-80::GFP) in *oma-1 (te33)* loss of function background. When *oma-2* is depleted by RNAi, it causes 100% Oma phenotype in *oma-1(te33)* loss of function strain (n>500) but OMA-1::GFP and OMA-1Δ46-80::GFP transgenes rescued the gonad phenotype in *oma-1(te33); oma-2(RNAi)* mutant background. 100% of transgenic animals were fertile (n>500) regardless of the deletion and both strains produced embryos. However, only wild type OMA-1::GFP transgene produced viable progeny and rescued embryonic lethality about 50% (n=551); OMA-1Δ46-80::GFP deletion strain could not rescue embryonic lethality (0%, n>2000), suggesting that 35 amino acid TAF-4 interaction domain is required for proper embryogenesis. Then, I analyzed the possible reason for embryonic lethality by assaying nuclear TAF-4 levels and transcriptional activity in early embryos. 83% of the 1-cell (24 of 29) and 96% of 2-cell (27 of 28) embryos from OMA-1Δ46-80::GFP; *oma-1 (te33)* deletion transgenic strain had detectable anti-Ser2P signal when *oma-2* was depleted by RNAi; while 0% of non-*oma-2 (RNAi)* embryos had ectopic transcriptional activity neither in 1-cell nor in 2-cell embryos (Figure 2.15). Wild type OMA-1::GFP transgene is not fully functional as it caused around 50% viable larvae and 50% dead embryos, when *oma-2* was depleted by RNAi. I detected some level of transcription defect when *oma-2* was depleted even in the wild type transgenic strain, most likely as a result of partial functionality; 26% of 1-cell (5 of 19) and around 50% of 2-cell (9 of 18) embryos had ectopic anti-Ser2P signal in OMA-1::GFP; *oma-2 (RNAi)* strain.

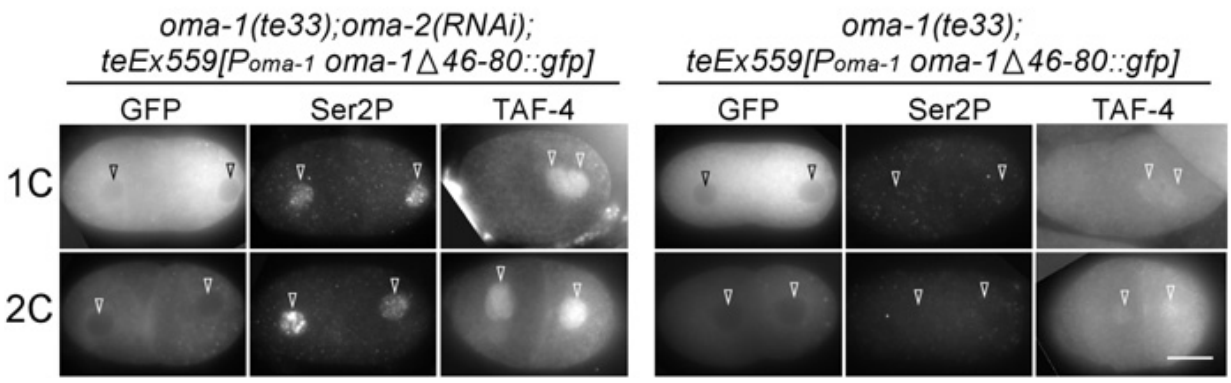


Figure 2.15. Lack of TAF-4 interaction domain of OMA-1 causes ectopic transcription and nuclear retention of TAF-4 in 1- and 2-cell embryos
Immunofluorescence images of OMA-1Δ46-80::GFP; *oma-1 (te33)* strain treated with (left panel) or without *oma-2 (RNAi)*(right panel) and stained with anti-GFP, anti-Ser2P and anti-TAF-4 antibodies. Arrowheads point to pronuclei in 1-cell (1C) and nuclei in 2-cell (2C) images.
Scale bar represents 10μm.

Transcriptional defect observed in OMA-1Δ46-80::GFP; *oma-1 (te33); oma-2 (RNAi)* strain is not due to a more rapid progression of meiotic cell cycle, because when I stained those embryos and wild type OMA-1::GFP embryos using Dapi to compare the cell cycle stages, I did not observe a significant difference between deletion transgene and the wild type one (Table 2.2). This analysis indicates that cell cycle staging and relative percentage of different embryonic stages is relatively similar in wild type and TAF-4 interaction domain deletion OMA-1 transgenes, when and *oma-2* is depleted.

	Meiosis I	Meiosis II	1-cell	2-cell	4-cell	8-cell
OMA-1::GFP; *oma-1(te33)* (n=192)	12.0	9.9	27.6	20.8	17.7	12.0
OMA-1Δ46-80::GFP; *oma-1 (te33); oma-2 RNAi* (n=215)	12.6	10.7	24.2	19.1	20.0	13.5

Table 2.2. Quantification of early embryonic stages of OMA-1::GFP strains by DAPI staining
Embryonic stages were scored based on DNA morphology. n: Number of total embryos scored, numbers for each embryonic stage is the percentile of that particular stage to total number of embryos.

Functional analysis of full length OMA-1::GFP and TAF-4 interaction domain deleted transgene, OMA-1Δ46-80::GFP led to three important conclusions: (1) Functions of OMA-1 in the oocytes and embryos can be separated, (2) TAF-4 interaction domain of OMA-1 is essential during embryogenesis, not during oocyte maturation, (3) OMA-1 repress transcription in newly fertilized embryos by interacting with TAF-4.

Embryonic phenotypes I detect with *oma-1/2 RNAi* are more severe than OMA-1Δ46-80::GFP; *oma-1(te33); oma-2(RNAi)* strain suggesting that OMA-1/2 might have additional function(s) besides transcriptional repression in the zygote.

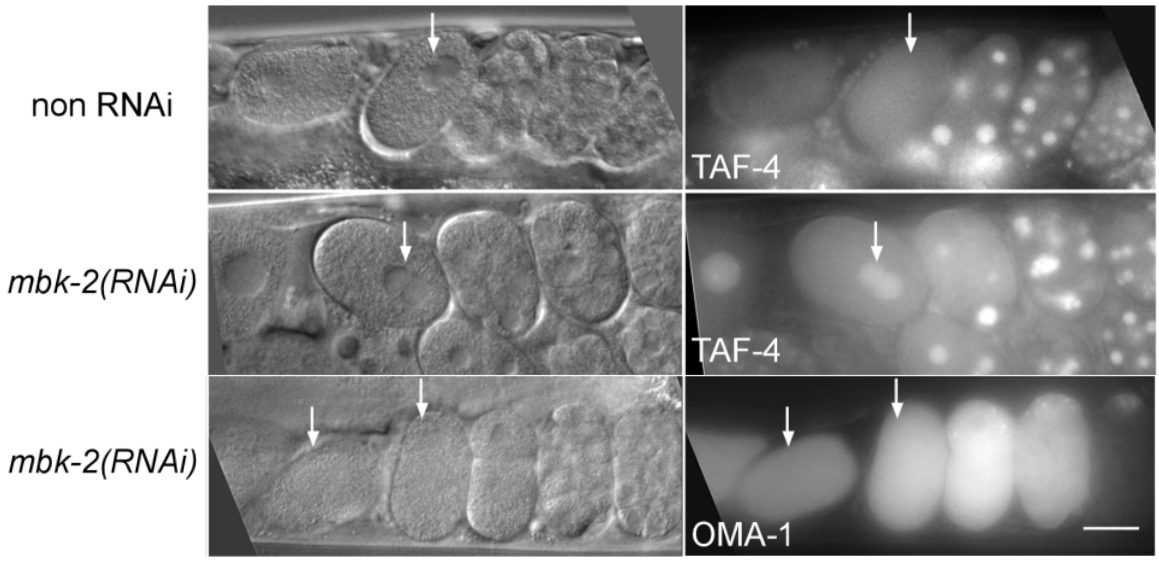
VIII. TAF-4/OMA-1 interaction is regulated by MBK-2 phosphorylation

1-cell and 2-cell stages are the only time points with reduced nuclear TAF-4 levels in the gonad and embryos of wild type *C. elegans*. Although OMA-1/2 are expressed at high levels, TAF-4 is still highly nuclear in the oocytes, suggesting that TAF-4/OMA

interaction does not occur in the oocytes but occur only in the newly fertilized embryos; suggesting that TAF-4/OMA protein interaction might require some regulation at the oocyte to embryo transition step. Another observation we had, supporting this idea was that when I was doing tissue culture experiments to see subcellular localization of TAF-4; I noticed that myr-OMA-1 N terminus could interact with TAF-4 very efficiently, but full length myr-OMA-1 could not. Therefore, we reasoned that OMA-1 N terminus may not be fully exposed in full length protein and may require some post translational modification to interact with TAF-4.

MBK-2 is a candidate regulator of the TAF-4/OMA-1 interaction because it directly phosphorylates OMA-1 at meiosis II, during oocyte to embryo transition and this phosphorylation event is required for timely degradation of OMA-1/2 (Figure 2.16A) (Nishi and Lin, 2005; Pellettieri et al., 2003; Shirayama et al., 2006). MBK-2 phosphorylation may enable OMA-1 N terminus to be more accessible for TAF-4 interaction. Three pieces of evidence support the idea that OMA-1 and TAF-4 interaction is regulated by direct phophorylation by MBK-2 at position T239 of OMA-1 protein. Firstly, *mbk-2(RNAi)* in GFP::TAF-4 embryos resulted higher nuclear levels of TAF-4 compared to wild type transgene, which correlated with ectopic transcription detected by anti-Ser2P immunostaining in 1-cell embryos (100%, n=30) (Figure 2.16A, B). Therefore, MBK-2 activity is required for transcriptional repression and cytoplasmic sequestration of TAF-4 in the newly fertilized *C. elegans* embryos. Same *mbk-2* RNAi in OMA-1::GFP strain resulted in ectopic OMA-1 beyond 1-cell stage, a control for strength of RNAi condition (Figure 2.16A).

(A)



(B)

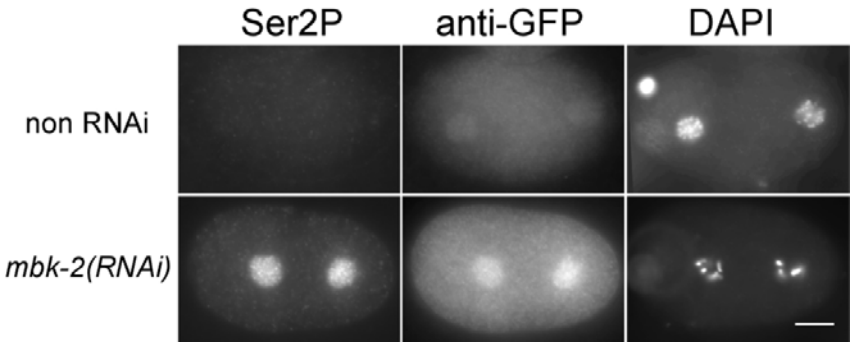


Figure 2.16. MBK-2 is required for cytoplasmic sequesterization of TAF-4 and transcriptional repression

A. DIC and fluorescence images of GFP::TAF-4 live animals treated with or without *mbk-2 RNAi* and OMA-1::GFP animals treated with *mbk-2 RNAi.* White arrows point to 1-cell embryos at pronuclear meeting stage. Embryos on the right of 1-cell are later stage embryos for comparison.
Scale bar represent 25 μm.

B. anti-Ser2P, anti-GFP and Dapi staining of GFP::TAF-4 1-cell embryos treated with and without *mbk-2 RNAi.* Nuclear level of GFP::TAF-4 correlates with ectopic transcription.
Scale bar represent 10 μm.

Secondly, *zu405* allele of OMA-1 has a proline to leucine mutation at position 240, one residue downstream of MBK-2 phosphorylation site; therefore this mutation interferes with proper phosphorylation, resulting in ectopic hypophosphorylated OMA-1 beyond 1-cell embryo (Lin, 2003; Nishi and Lin, 2005). I crossed GFP::TAF-4 strain with *zu405* and depleted *oma-2* by RNAi to remove wild type copy of OMA proteins and analyzed localization of TAF-4. Consistent with *mbk-2 RNAi,* I detected a higher nuclear TAF-4 in 1-cell embryos in *zu405* 1-cell embryos (Figure 2.17).

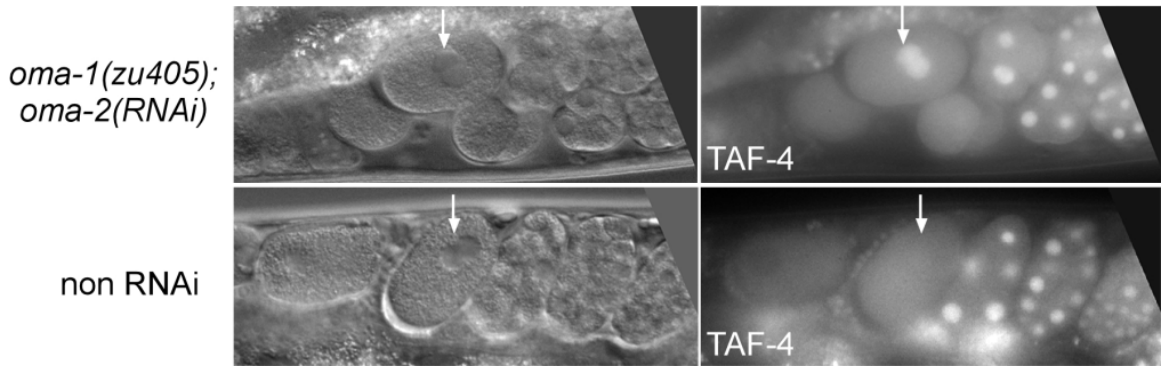


Figure 2.17. *zu405; oma-2 RNAi* 1-cell embryos have higher nuclear GFP::TAF-4 levels
DIC and GFP fluorescence images of GFP::TAF-4 strain with and without *zu405; oma-2 RNAi.* Arrows point to 1-cell embryos during pronuclear meeting stage. Embryos on the right of 1-cell are later stage embryos in the uterus of the animal.

Thirdly, co-expressing MBK-2 with myr-OMA-1 FL (full length) and GFP::TAF-4 in HEK293T cells, greatly increased membrane sequesterization of TAF-4 by myr-OMA-1 FL (48%, n=50, by Scott Robertson). Previously, I observed that full length myr-OMA-1 was not as efficient as N terminal portion in recruiting GFP::TAF-4 to the cell cortex but adding MBK-2 to the transfection enhanced OMA-1 FL and TAF-4 interaction. This effect of MBK-2 was dependent on its kinase domain since kinase dead version of MBK-2 promoted recruitment of GFP::TAF-4 to the cell membrane poorly (16%, n=50). The effect was also dependent on phosphorylation site on OMA-1 T239, because almost no enhancement was observed when myr-OMA-1 T239A was used (12%, n=50, by Scott Robertson).

Three observations mentioned above using *mbk-2 RNAi* or *zu405; oma-2 RNAi* in GFP::TAF-4 animals along with tissue culture transfection experiments using myr-OMA-1 FL indicate that MBK-2 phosphorylation regulates OMA-1/2 and TAF-4 interaction. This regulation enables nuclear reduction of TAF-4 only in 1-cell embryos where MBK-2 is activated, not during oocyte development.

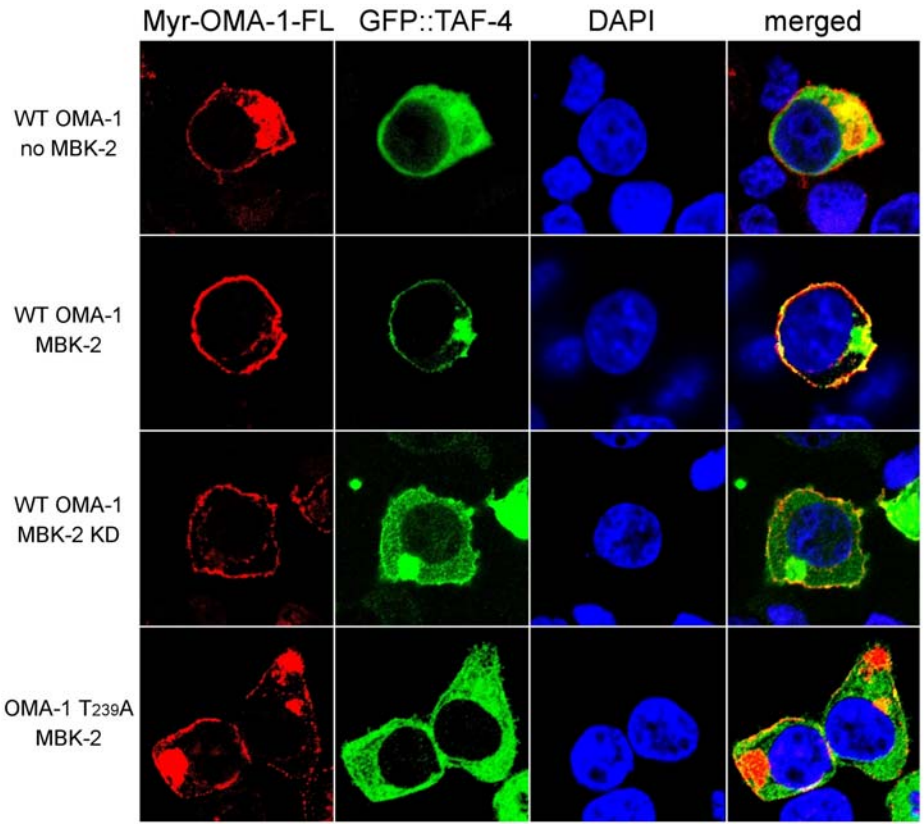


Figure 2.18. Co-expressing MBK-2 enhances GFP::TAF-4 recruitment to cell cortex by myr-OMA-1 FL in HEK293T cells (by Scott Robertson)
Confocal immunofluorescence images of HEK293T cells stained for myr-FLAG::OMA-1 FL (red), GFP::TAF-4 (green) and Dapi (blue). Right column is the merged image of all three channels. Genes transfected together with GFP::TAF-4 are shown on the left. KD: kinase dead. Overexpresion of myr-OMA-1 causes cytoplasmic aggregates that are not affected by MBK-2.

IX. Properly posphorylated Ectopic OMA-1 is sufficient to repress transcription in later stage embryos

In order to to assay sufficiency of ectopic OMA-1/2 in later stage embryos, for transcriptional repression and TAF-4 sequesterization in the cytoplasm, we checked transcriptional activity and GFP::TAF-4 localization in 1-cell embryos of mutants with

OMA-1 degradation defect. Both *oma-1(zu405)* and *mbk-2(RNAi)* embryos have OMA-1 degradation defect with OMA-1 persisting beyond 1-cell embryo, but persistent OMA-1 in these genetic backgrounds are not phosphorylated at T239, therefore they did not cause either ectopic transcription (data not shown), nor sequesterization of TAF-4 in the cytoplasm in 1-cell embryos (Figure 2.19).

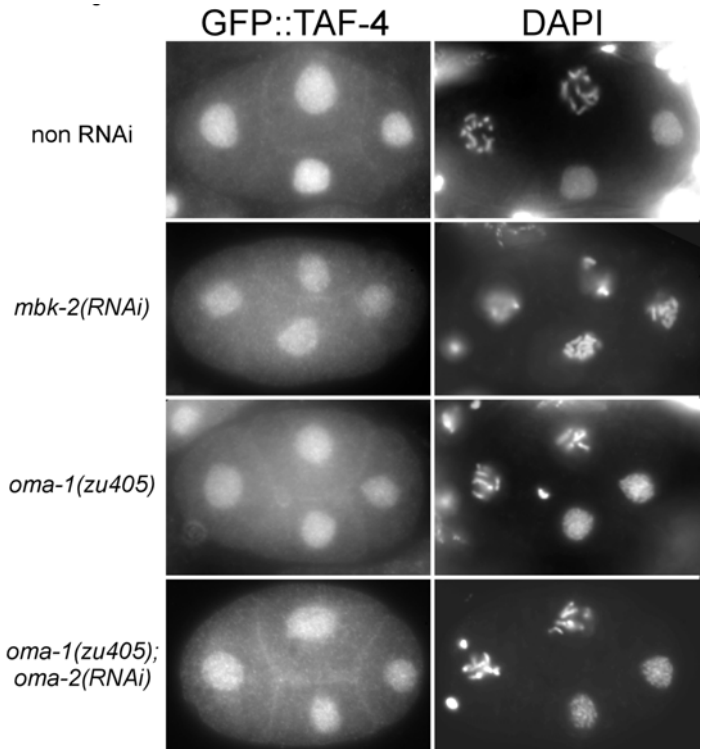


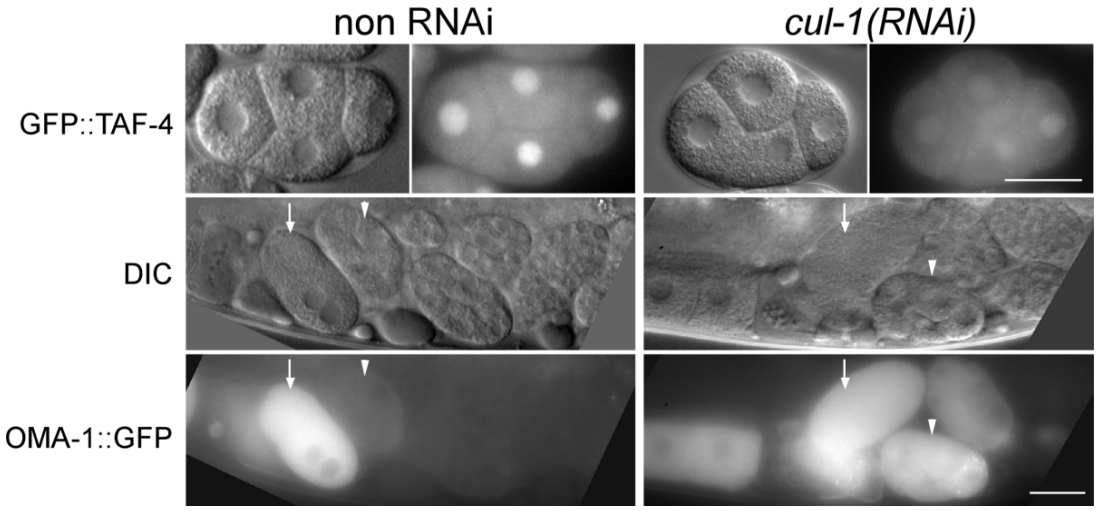
Figure 2.19. Ectopic hypophosphorylated OMA-1 cannot sequester TAF-4 in the cytoplasm
Immunofluorescence images of GFP::TAF-4 4-cell embryos stained with anti-GFP (shown on the left column) and Dapi (shown on the right column). Genotypes are indicated on the right.

cullin-RING ubiquitin ligases (CRLs) form the largest known ubiquitin ligase family. CRLs have multiple subunits with a cullin, a RING H2 finger protein, a substrate recognition subunit (SRS) and in some complexes an adaptor subunit that link SRS to the CRL complex (Bosu and Kipreos, 2008). Yuichi Nishi, a former graduate student in the lab was interested in identifying through which CRL complex OMA proteins were developmentally degraded. When he depleted *C. elegans* cullins in OMA-1::GFP strain, he observed that *cul-1* and *cul-2(RNAi)* resulted in OMA-1 degradation delay whereas *cul-3* or *cul-4(RNAi)* did not, indeed he identified that OMA-1 was degraded by a CUL-1 based CRL complex and degradation delay observed in *cul-2(-)* background is likely to be indirect (data not shown). Interestingly, in *cul-1(RNAi)* background MBK-2 dependent activities are normal and different aspects of cell fate and differentiation are not affected (Kipreos et al., 1996; Shirayama et al., 2006); therefore we predicted that ectopic OMA-1 in *cul-1(RNAi)* background would be properly phosphorylated by MBK-2 enabling us to study the effect(s) of properly phosphorylated OMA proteins beyond 1-cell stage.

I analyzed nuclear GFP::TAF-4 and transcriptional marker anti-Ser2P in *cul-1(RNAi)* embryos (Figure 2.20). OMA-1/2 are expressed at high levels in all blastomeres of 4-cell *cul-1(-)* embryos and primarily localized to P blastomeres in 12- and 16-cell stages (100%, n=14) (Figure 2.20A). I focused on 4-cell stage embryos during my analysis because OMA-1 is expressed in all blastomeres at that stage and expression was brighter compared to later stage embryos. Nuclear GFP::TAF-4 was greatly reduced in 4-cell embryos when *cul-1* was depleted by RNAi (64%, n=14) (Figure 2.20A). To investigate if reduction in nuclear TAF-4 correlates with loss of transcriptional activity, I stained *cul-1(RNAi)* embryos with anti-Ser2P antibody and observed either a very low level of

transcription or repression of transcription in all blastomeres of 4-cell stage embryos (77%, n=18) (Figure 2.20B). Transcriptional repression and reduced nuclear GFP::TAF-4 in *cul-1(-)* is dependent on OMA-1/2, because animals treated with *cul-1(RNAi)* together with *oma-2(RNAi)* in *oma-1 (te33)* null background had only 18% (n=17) of 4-cell embryos with either very low or no anti-Ser2P signal. In a control experiment, where *oma-1*instead of *oma-2* was depleted together with *cul-1* in *oma-1(te33)* mutant, around 66% (n=8) of 4-cell embryos had low or no anti-Ser2P signal (Figure 2.20B).

(A)



(B)

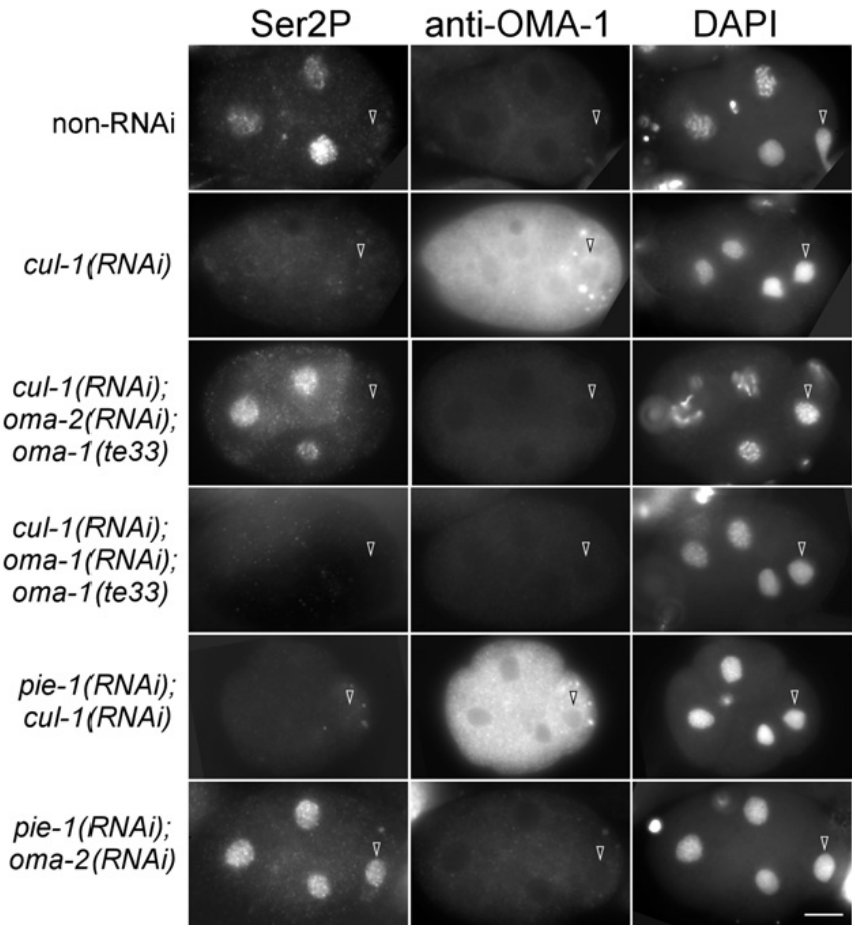


Figure 2.20. Ectopic OMA-1 when properly phosphorylated can repress transcription

A. DIC and GFP fluorescence images of 4-cell GFP::TAF-4 embryos treated with or without *cul-1 RNAi* (top panels, scale bar represents 10 μm) and live adult images of OMA-1::GFP animals with or without *cul-1 RNAi* treatment (lower two panels). White arrows point to 1-cell embryos, white arrowheads to 4-cell embryos. Scale bar represents 20 μm.

B. Immunostaining of wild type embryos treated with various RNAi combinations shown on the left, and stained with anti-Ser2P, anti-OMA-1 and Dapi. Arrowheads point to P2 blastomere of 4-cell embryo. Scale bar represents 10 μm.

PIE-1 is responsible for transcriptional repression in P blastomeres of 4-cell and older stage embryos, we asked whether ectopic OMA-1 could substitute for PIE-1 in P lineage transcriptional repression. Transcription is depressed in 100% of P2 blastomeres of 4-cell *pie-1* RNAi embryos, whereas when *pie-1* is depleted together with *cul-1* transcriptional repression is preserved in 67% (n=12) of 4-cell embryos (Figure 2.20B), suggesting that ectopic OMA-1 in *cul-1(-)* background can repress transcription in the absence of PIE-1. This result was not due to insufficient depletion of *pie-1* in double RNAi condition*;* my double RNAi control *pie-1 (RNAi); oma-2 (RNAi)* had anti-Ser2P signal in all 4-cell embryos analyzed (n=11) (Figure 2.20B). Altogether these results suggest that properly phosphorylated ectopic OMA-1/2 are sufficient to repress transcription by sequestering TAF-4 in the cytoplasm of later stage embryos and ectopic OMA-1/2 can substitute for PIE-1 in germline transcriptional repression, in the absence of functional *pie-1*.

DISCUSSION

Repression of somatic genes is evolutionarily conserved in PGCs

Vertebrate and invertebrate animals adopt two different modes of germline specification; vertebrates specify embryonic germ lineage by an inductive mechanism, while invertebrates use maternally inherited germ plasm components to set aside their germ cell precursors during embryogenesis, to ensure correct passage of their genetic material to next generations. Although there are different ways to specify embryonic germ cell precursors, global repression of somatic genes in PGCs is evolutionarily conserved in all animals to protect germ cells from inappropriate differentiation into

somatic lineages. Emerging new evidence suggests that some mechanisms to repress global transcripton in *Drosophila* and *C. elegans* germ cell precursors are conserved (Nakamura and Seydoux, 2008). *Drosophila* Pgc protein and *C. elegans* PIE-1 are not homologous, nor they have any sequence similarity, though they both repress global transcription in embryonic germ lineage around the time of onset of zygotic transcription by targeting different components of the same kinase complex: P-TEFb, evolutionarily conserved kinase complex required for transcriptional elongation by phosphorylating CTD tail of RNAPII large subunit (Hanyu-Nakamura et al., 2008; Nakamura and Seydoux, 2008; Seydoux and Dunn, 1997; Seydoux et al., 1996). Inhibiting the same P-TEFb kinase complex activity by different class of proteins in two different organisms suggests that although the players are not homologous or similar, mechanisms can be conserved between different organisms in similar biological processes.

Sequential transcriptional repression in *C. elegans* germ cell precursors

PIE-1 C terminal domain represses global transcription at the level of transcriptional elongation, primarily in P2, P3 and to a certain extent in P4 germline blastomeres (Seydoux et al., 1996) by interacting with Cyclin T component of P-Tefb complex (Batchelder et al., 1999; Hirose and Ohkuma, 2007; Shim et al., 2002; Zhang et al., 2003). PIE-1 contains CCCH RNA binding zinc fingers but transcriptional repression mechanism seems to be independent of zinc finger domains. However, it was not known that how transcriptional repression occured in 1-cell and 2-cell *C. elegans* embryos, before the onset of zygotic transcription.

My data demonstrates that global transcription is repressed in 1-cell and 2-cell stage of *C. elegans* embryos by two highly redundant proteins OMA-1 and OMA-2. Similar to PIE-1, OMA-1/2 have tandem CCCH RNA binding zinc finger domains but OMA proteins repress global transcription through a different mechanism than PIE-1, using a domain independent from their zinc finger domains. N terminal portion of OMA-1/2 interact with TAF-4 (TBP associated factor 4) and sequester this essential component of basal transcription machinery in the cytoplasm during 1-cell and 2-cell stages of *C. elegans* embryos (Figure 2.21).

By targeting a keystone component of RNAPII basal transcription machinery, OMA proteins provide a transient but very effective means of transcriptional repression at the initiation level, in the newly fertilized *C. elegans* embryos. Interestingly, OMA proteins are required for PIE-1 mediated transcriptional repression in later germline blastomeres by stabilizing PIE-1 protein, suggesting that OMA-1/2 are crucial for the establishment of germline fate during *C. elegans* embryogenesis.

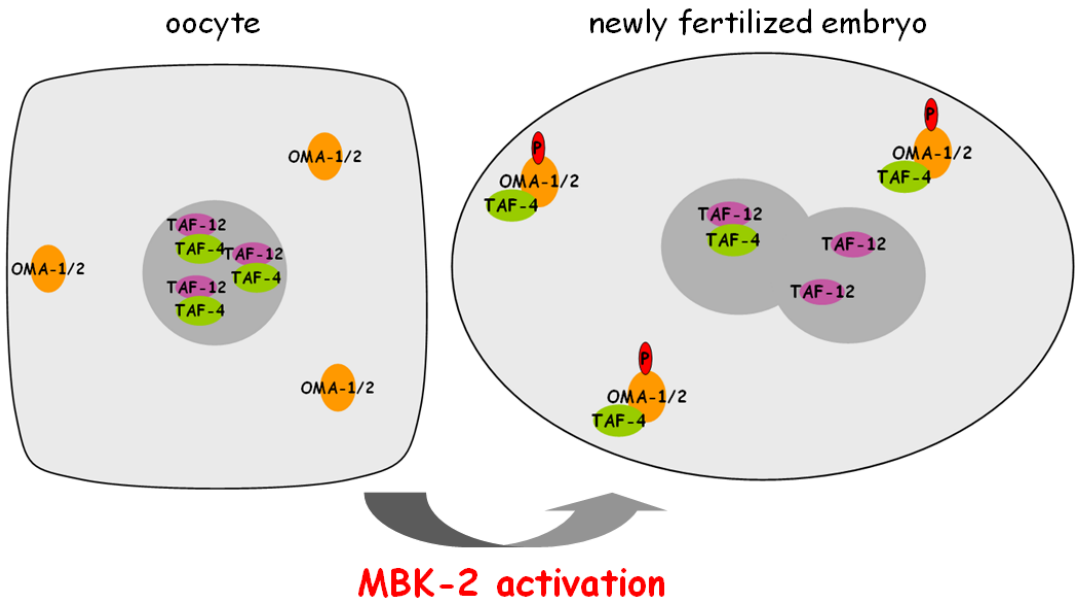


Figure 2.21. Model for transcriptional repression by OMA-1/2
C. elegans oocyte to embryo transition is schematized. Oocyte is shown on the left with nuclearly enriched TAF-4, while OMA-1 is exclusively cytoplasmic, oocyte nucleus is shown with a darker gray circle in the middle of the cell. 1-cell embryo is shown on the right image. Two large darker gray circles inside the 1-cell embryo are sperm and oocyte pronuclei during meeting stage. OMA-1/2 are still cytoplasmic but they are phosphorylated by MBK-2 during the oocyte to embryo transition, so they can interact with TAF-4 and decrease TAF-4 nuclear levels by sequestering it in the cytoplasm. TAF-4 is not exclusively cytoplasmic in 1-cell embryo.

TAF-4 as a target for global transcriptional repression

No homolog(s) of OMA proteins is known in other organisms so far, there are tandem CCCH RNA binding zinc finger proteins but they show similarity only in their zinc finger domains. However, TAF-4 is a highly conserved, essential protein required for TFIID stability and basal transcription. Therefore, targeting TAF-4 and changing its subcellular localization or some other essential component of RNAPII multisubunit

complex might be a conserved mechanism to repress global transcription transiently at initiation level in other developmental contexts when necessary.

Aberrant TAF4 may lead to pathological conditions, for example inhibiting TAF4 function by polyQ expansion domain of mutant Huntington protein leads to a neurodegenerative disorder: Huntington's disease. Mutant Huntington protein localize TAF4 to neuronal intranuclear inclusions leading to dysregulation of transcription by suppressing CREB (cAMP-responsive element binding protein)-dependent transcriptional activation that leads to neuronal cell death and neurodegenerative disorder (Dunah et al., 2002; Shimohata et al., 2000; Shimohata et al., 2001). It would be interesting to find other binding partners for TAF4 protein under both physiological and pathological conditons, which may lead to discovery of new processes that involves global transcriptional regulation.

MBK-2 phosphorylation regulates OMA-1/2 and TAF-4 interaction at oocyte to embryo transition

Although both OMA-1/2 and TAF-4 are expressed at high levels in the oocytes, the interaction occurs only during embryogenesis. My study suggests that MBK-2 phosphorylation of OMA proteins at meiosis II, during oocyte to embryo transition, regulates OMA-1/2 and TAF-4 interaction to occur only in the embryos before OMA proteins are degraded by proteasome. OMA proteins expressed in the oocytes are unphosphorylated at MBK-2 site; therefore they cannot interact with TAF-4. As a result, subcellular localization of TAF-4 is unaffected in the oocytes with a nuclearly enriched localization parttern throughout adult germline (Figure 2.21).

Regulation of OMA-1/2 activities

Inability of OMA-1/2 to interact with TAF-4 in the oocytes could be resulting from inaccessibility of OMA-1/2 N terminal TAF-4 interaction domain in the oocytes prior to MBK-2 phosphorylation. OMA-1/2 N terminal domain contains a predicted alpha-helical structure; therefore it is possible that this domain with a lot of hydrophobic residues will not be exposed to free solution. N terminal domain could be buried inside the protein or it could be occupied by some other interaction partner(s) in the oocytes. MBK-2 phosphorylation event may induce conformational switch in OMA-1/2 proteins that may provide N terminal domain for TAF-4 interaction. *oma-1 (zu405)* allele causes ~50% embryonic lethality even at permissive temperature, 16ºC; interestingly depleting *oma-2* at the same temperature exacerbates the phenotype causing 100% lethality. OMA-1 P240L protein might be dominant negative form of the protein by adversely affecting normal, wild type OMA-2 function. I tested a possible homodimerization of OMA-1 or heterodimerization with OMA-2, and my assay showed that OMA-1 or OMA-2 N terminal domains can homodimerize or heterodimerize, in yeast two hybrid system (data not shown). If homo/heterodimerization occurs in vivo as well, it can explain the dominant negative nature of *oma-1 (zu405)* allele while *oma-1(-)*null mutant does not show any aberrant phenotype with wild type looking animals. One model to explain regulation of OMA-1/2 during oocyte to embryo transition can be as follows: OMA-1 and OMA-2 N terminal domains might homo/heterodimerize in the oocytes, maybe required for oocyte maturation events; therefore N terminal portions are not available for TAF-4 interaction in the oocytes. MBK-2 phosphorylation at oocyte to embryo transition may induce structural changes in OMA proteins, dissociating the dimer and enabling

OMA/TAF-4 interaction to occur only in the embryos. It would be very interesting to find out the structural nature of MBK-2 phosphorylation.

In addition to regulation by MBK-2, another possible mechanism to prevent OMA-1 and TAF-4 interaction in the oocytes could be regulation by nuclear envelope of oocytes, which might provide a physical boundary separating OMA-1/2 from TAF-4 in different subcellular compartments before embryogenesis.

Activation of zygotic transcription after degradation of OMA proteins

Direct phosphorylations by MBK-2 and GSK-3 (Nishi and Lin, 2005; Shirayama et al., 2006) tag OMA proteins for destruction by proteasome after first mitosis of the zygote. Our model predicts that when OMA proteins disappear, TAF-4/TAF-12 interaction can resume beginning from 2-cell embryos and TAF-4 can be translocated back to nuclei towards late 2-cell stage embryos. Nuclear TAF-4 enables resumption of RNAPII transcription machinery as activation of zygotic transcription at 4-cell stage embryos. Transcription is kept repressed in 2-cell embryos, while there is only residual amount of OMA proteins expressed at this stage (Figure 2.4). In addition, the majority of TAF-4 translocates back to nuclei towards late 2-cell embryo. Time delay between degradation of OMA proteins after first mitotic division to onset of zygotic transcription at 4-cell stage of embryo could be due to combination of different reasons: (1) Residual amount of OMA proteins left in 2-cell embryo (<10% of 1-cell level, Figure 2.4) may still interact with some portion of TAF-4 protein and (2) the other free pool of TAF-4 start interacting with TAF-12, and gradually accumulate in nuclei toward late 2-cell stage. When nuclear TAF-4 levels are enough, subunits required for basal transcription

machinery can start to assemble. (3) A major rate limiting step in transcriptional initiation is the recognition and binding of TATA-box by TFIID (Chatterjee and Struhl, 1995; Klages and Strubin, 1995). Although, TAF-4 is nuclearly enriched during late 2-cell stage, it can not kick off transcription very quickly as DNA recognition is rate limiting and formation of RNAPII basal transcription machinery will take some time; therefore transcription can initiate around 4-cell stage. Another possibility is that the transcriptional activity markers used in this and in the previous studies may not be sensitive enough to detect exact timing of the onset of zygotic transcription.

OMA-1/2 are required for the establishment of *C. elegans* germline

C. elegans embryos utilize a sequential mechanism by a set of different tandem CCCH RNA binding zinc finger proteins to repress transcription in germline precursor cells, to protect germline identity from inappropriate somatic differentiation during early embryogenesis. Firstly, OMA proteins repress transcription in 1- and 2-cell stage embryos and later PIE-1 block global transcription in P blastomeres starting from 4-cell stage. My data suggest that in addition to a direct involvement in transcriptional repression in the newly fertilized embryos by interacting with and sequestering TAF-4 in the cytoplasm, OMA-1/2 can also indirectly repress transcription in later P blastomeres by regulating PIE-1 protein levels. As a result, although OMA proteins are expressed in the oocytes and only briefly in the embryo (mainly at 1-cell stage), they are redundantly required for the establishment of future germ lineage of *C. elegans* by regulating PIE-1 protein, the later germline transcriptional repressor. OMA-1/2 could be regulating maternal PIE-1 levels by different mechanisms: either by controlling *pie-1* gene

expression at transcriptional or post-transcriptional level or by protecting PIE-1 protein from premature degradation or by combination of both methods. We believe CCCH RNA binding zinc fingers of OMA proteins might have role in this process either by directly regulating *pie-1* mRNA translation or indirectly through another gene that has a role in PIE-1 degradation machinery and stability, in order to protect germline protein PIE-1 during earlier stages of germline development. Details of how OMA proteins regulate PIE-1 levels will be the subject of Chapter 3.

CHAPTER THREE

OMA1/2 protect CCCH proteins from degradation by repressing *zif-1* translation in adult *C. elegans* germline

SUMMARY

Proper establishment of germline lineage is vital for life cycles of all animal species. Germ cell precursors are set aside early during embryogenesis to protect them from inappropriate somatic differentiation. Global transcription is turned off in late oocytes of *C. elegans* germline and activated as zygotic transcriptional program at 4-cell stage of the embryo but only in somatic blastomeres. OMA-1 and OMA-2 repress global transcription in 1-cell and 2-cell stage of *C. elegans* embryos by sequestering TAF-4 in the cytoplasm, a key conserved subunit of mRNA transcription machinery. Interestingly, in addition to a direct role in transcriptional repression of newly fertilized embryos, OMA-1/2 indirectly repress transcription in later germline blastomeres by protecting PIE-1 protein from degradation (Guven-Ozkan et al., 2008). However, it was not clear how maternal proteins expressed in the oocytes and in 1-cell embryos can regulate a later germline transcriptional repressor and contribute to establishment of germline fate during *C. elegans* embryogenesis.

OMA-1/2 and PIE-1 are from the same family of tandem CCCH RNA binding zinc finger proteins but post-transcriptional regulation of mRNAs by these proteins is poorly described. Post-transcriptional control of maternally deposited mRNAs is a conserved mechanism in diverse species and is very critical during germline development and during oocyte to embryo transition before the onset of zygotic transcription. In this

chapter, I present my data demonstrating that OMA-1/2 protect PIE-1 protein along with some other CCCH RNA binding proteins from premature degradation in the oocytes by inhibiting *zif-1* mRNA post-trancriptionally. ZIF-1 is the substrate specific binding partner for PIE-1 and CCCH zinc finger protein degradation. I generated *zif-1* 3'UTR reporter and it is repressed in the pachytene region of the germline and in the oocytes, as well as in the germline blastomeres of the embryos with a pattern reciprocal to PIE-1 degradation in the embryos, which is consistent with a translational regulation of ZIF-1 activity. Repression of the *zif-1* 3'UTR reporter in the oocytes requires unphosphorylated OMA-1/2, suggesting that OMA proteins can inhibit *zif-1* mRNA translation before MBK-2 phosphorylation at meiosis II, during oogenesis. We believe that OMA-1/2 function as translational repressors of *zif-1* in the oocytes to prevent CCCH proteins from premature degradation to ensure protection of embryonic cell fate determinants and germline specific proteins until they are needed during embryogenesis. Activation of MBK-2 during oocyte to embryo transition converts roles of OMA-1/2 from being oocyte translational repressors to embryonic transcriptional repressors.

INTRODUCTION

Global transcription is shut down in maturing diakinetic oocytes, undergoing prophase of meiosis I and resumes after fertilization in somatic blastomeres of 4-cell stage of *C. elegans* embryo (Blackwell and Walker, 2006; Kelly et al., 2002; Schisa et al., 2001; Seydoux and Dunn, 1997). The lack of transcriptional activity during oocyte to embryo transition might be involved in facilitating smoother transition between maternal and zygotic transcriptional programs that are suggested to be very different (Baugh et al., 2003; Blackwell and Walker, 2006). Therefore, the most critical initial events of embryogenesis like activation of signaling pathways, localized expression of cell fate determinants and localized activation of transcription factors, which all lead to generation of a new organism rely heavily on post-transcriptional control of maternal mRNAs and proteins (Evans and Hunter, 2005). Mitotic and early meiotic germ nuclei of adult *C. elegans* germline are transcriptionally very active to produce mRNAs that are translated into protein products later, either during germline development or embryogenesis (Evans and Hunter, 2005). Post-transcriptional control of mRNA can occur at different stages as splicing, polyadenylation, mRNA stability, specific localization and translation (de Moor et al., 2005; de Moor and Richter, 2001; Lee and Schedl, 2006). mRNA binding proteins can interact with either 5' or 3' end of pre-mRNA untranslated region (UTR) to regulate mRNA stability or translation. Using a transgenic expression assay of 5' promoter and 3'UTR reporters in adult *C. elegans* germline; 3'UTR sequences were shown to be the primary cis-acting element for the expression of *C. elegans* maternal mRNAs, with the exception of sperm specific genes (Merritt et al., 2008), supporting the idea that post-

transcriptional regulation is the primary mechanism to regulate maternal mRNA expression during germline development and early embryogenesis.

Patterning of early *C. elegans* embryo involves three mechanisms (1) translational control of cell fate determinants, (2) directed movement of polarity and cell fate determinants and (3) localized stabilization or degradation of key embryonic proteins (Evans and Hunter, 2005) which all contribute to segregation of germ lineage from soma. Newly fertilized zygote of *C. elegans* is the first germline blastomere, called P0 and it gives rise to germline restricted P4 blastomere through a series of asymmetric divisions (Figure 1.3). Maternally expressed tandem CCCH RNA binding zinc finger proteins OMA-1/2 and PIE-1 repress global transcription in P lineage sequentially, using dynamic and readily reversible but different mechanisms without interfering with quick transcriptional activation in somatic blastomeres (Guven-Ozkan et al., 2008; Seydoux et al., 1996). OMA-1/2 are two maternal proteins expressed in the proximal oocytes and in the newly fertilized embryos (Figure 3.1) and they directly repress global transcriptional at the initiation level in 1- and 2-cell stage of *C. elegans* embryos by interacting with TAF-4, and sequestering it in the cytoplasm to displace TAF-4 away from nuclei and from basal transcription machinery. N terminal 35 amino acid domain of OMA-1 can interact with TAF-4 by competing with TAF-12, very specific highly conserved binding partner for TAF-4. TAF-4 interacting domain of OMA-1 is outside of its RNA binding zinc finger domains, suggesting that transcriptional repression in 1- and 2-cell stage embryos is independent of RNA binding domains (Guven-Ozkan et al., 2008). PIE-1 represses global transcription in germline precursors of the embryo starting from 4-cell stage, P2 blastomere. PIE-1 is expressed maternally starting from oocytes and it is

inherited preferentially by germline P blastomeres during embryogenesis (Figure 3.1). Strikingly, reduction of *oma-1/2* activities results in transcriptional derepression not only in newly fertilized embryos, but also in later germline blastomeres starting from P2 where PIE-1 normally is responsible for transcriptional repression. OMA proteins are developmentally and rapidly degraded right after first mitotic division of the embryo (Detwiler et al., 2001; Lin, 2003) and their degradation requires tight regulation through at least two direct phosphorylations by MBK-2 and GSK-3 kinases (Nishi and Lin, 2005; Pellettieri et al., 2003; Shirayama et al., 2006). Lack of OMA protein expression in later germline blastomeres suggests that transcriptional derepression phenotype detected in *oma-1/2 (-)* P blastomeres starting from P2 is an indirect consequence. Consistently, PIE-1 level is greatly reduced in *oma-1/2 (-)* embryos correlated with ectopic transcription in P lineage (Guven-Ozkan et al., 2008). Similar to OMA proteins, PIE-1 belongs to tandem CCCH RNA binding zinc finger family but transcriptional repression activity is independent of its zinc fingers, through its C terminal domain (Batchelder et al., 1999; Tenenhaus et al., 2001; Zhang et al., 2003). PIE-1 localizes asymmetrically to germ blastomeres (Figure 1.8) by two mechanisms: (1) directed enrichment towards posterior cell before the cell division and (2) somatic degradation by CUL-2 based degradation complex after the division (DeRenzo et al., 2003; Reese et al., 2000).

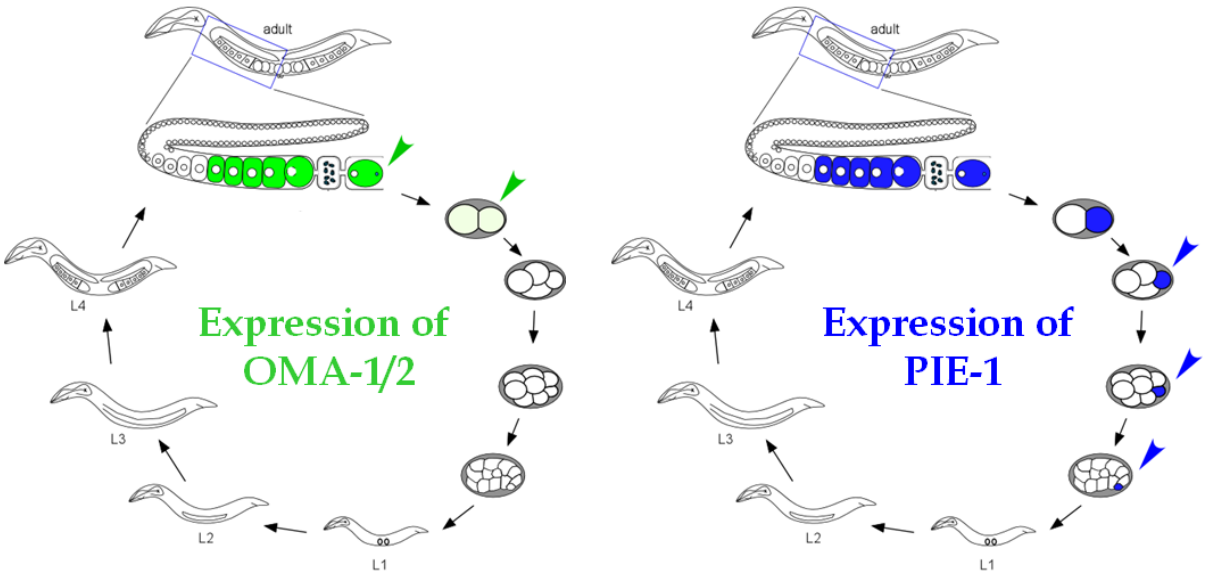


Figure 3.1. Localization of OMA-1/2 and PIE-1 during *C. elegans* germline developmental cycle
Expression patterns of OMA-1/2 and PIE-1 proteins are shown during the germline developmental cycle. Both OMA proteins and PIE-1 are maternally expressed in the proximal oocytes, but OMA proteins are degraded earlier during embryogenesis, right after first mitosis of the zygote. PIE-1 is expressed in P blastomeres and degraded around the time Z2 and Z3 (*C. elegans* PGCs) are born. OMA-1/2 are shown in green, PIE-1 in blue.

In this chapter, I present my work demonstrating that OMA proteins regulate establishment of future germline of *C. elegans* and repress global transcription in later germline blastomeres by protecting PIE-1 protein stability and preventing its premature degradation. OMA proteins repress translation of substrate specific binding partner for PIE-1 degradation *zif-1* in the oocytes. I generated a *zif-1* 3'UTR reporter to study post-transcriptional regulation of *zif-1*, and the reporter was repressed in the pachytene and proximal regions of the adult germline and was activated in anterior AB blastomeres of 4-

cell embryos and was kept repressed in the germ blastomeres and their sister cells. Depletion of *oma-1/2* by RNAi results in derepression of *zif-1* reporter in proximal oocytes, supporting the idea that OMA proteins protect PIE-1 along with four other CCCH RNA binding proteins, post-transcriptionally by inhibiting a specific component of their degradation machinery. In addition to regulation by OMA-1/2, my data shows translational repression of *zif-1* mRNA is regulated by GLD-1 in pachytene region of the germline, by POS-1 and SPN-4 in the germline precursors of the embryo and the reporter activation in the 4-cell embryo requires MEX-5 and MEX-6, two redundant anterior cell fate determinants from the same CCCH RNA binding zinc finger domain family with OMA-1/2 and PIE-1.

120 nucleotide domain of the 3'UTR is both necessary and sufficient for OMA-1/2 mediated translational repression. Computational analysis of 120 nucleotide region predicts binding elements for GLD-1, POS-1 and OMA proteins. More extensive future mutagenesis and biochemical analyses will likely to uncover the minimal elements for different RNA binding proteins.

I further show that only unphosphorylated or hypophosphorylated OMA-1/2 can repress *zif-1* in the oocytes or in the embryos when ectopically expressed. We believe that MBK-2 phosphorylation at oocyte to embryo transition converts OMA proteins from an oocyte *zif-1* translational repressor to embryonic transcriptional repressors. Requirement for different post-translational versions of OMA-1/2 for the translational and transcriptional repression activities suggest that functions of OMA-1/2 in the oocytes and in the embryos are likely to be incompatible.

EXPERIMENTAL PROCEDURES

Strains

N2 was used as the wild-type strain (Brenner, 1974). Genetic markers used are: LGI, *gld-1 (q485)*; LGIII, *unc-119(ed3)*; LGIV, *oma-1 (te33), oma-1(zu405)*; LGV, *oma-2 (te51)*. Transgenic strains were generated by complex array injection (Kelly et al., 1997) or microparticle bombardment (Praitis et al., 2001). Consistency of expression patterns were confirmed in at least two lines. Plasmids used, strain names and integrations are as follows: TX1246 (*teIs113 [$P_{pie\text{-}1}$gfp::h2b::zif-1$^{3'UTR771bp}$]*), TX1248 (*teIs114 [$P_{pie\text{-}1}$gfp::h2b::zif-1$^{3'UTR771bp}$]*), TX1240 (*teEx602 [$P_{pie\text{-}1}$gfp::h2b::zif-1$^{3'UTR304bp}$]*), TX1251 (*teEx604 [$P_{pie\text{-}1}$gfp::h2b::zif-1$^{3'UTR\Delta 4\text{-}63}$]*), $P_{pie\text{-}1}$*gfp::h2b::zif-1*$^{3'UTR\Delta 64\text{-}123}$, TX1272 (*teEx606 [$P_{pie\text{-}1}$gfp::h2b::zif-1$^{3'UTR\Delta 124\text{-}183}$]*), TX1298 (*teEx607 [$P_{pie\text{-}1}$gfp::h2b::zif-1$^{3'UTR\Delta 184\text{-}243}$]*), $P_{pie\text{-}1}$*gfp::h2b::zif-1*$^{3'UTR\Delta 244\text{-}303}$, TX1311 (*teEx610 [$P_{pie\text{-}1}$gfp::h2b::zif-1$^{3'UTR\ 64\text{-}183}$]*), TX1315 (*teEx611 [$P_{pie\text{-}1}$gfp::h2b::zif-1$^{3'UTR\ \Delta 64\text{-}183}$]*) and TX938 (*teEx400 [$P_{\ hsp16}$::oma-1 FL]*). SS747 (*bnIs1*), JH227 (*axEx73*) and JH1436 (*axEx1120*) contain $P_{pie\text{-}1}$ *gfp::pgl-1*, $P_{pie\text{-}1}$ *gfp::pie-1* and $P_{pie\text{-}1}$ *gfp::pie-1* ^{ZincFinger1} transgenes, respectively, as described before (Cheeks et al., 2004; DeRenzo et al., 2003; Reese et al., 2000).

Plasmid Construction

Most plasmids were constructed with the Gateway cloning technology. *zif-1* 771 bp 3'UTR sequence was amplified from genomic DNA by PCR and fused to Histone 2B (H2B) at C terminus to generate Gateway entry clone pRL2698. Germline expression constructs were derived from pID3.01B, *pie-1* promoter Gateway destination vector

(Reese et al., 2000). All the mutagenized fragments of *zif-1* 3'UTR are derived from pRL2698 using either PCR amplification or Quick Change site directed mutagenesis kit (Stratagene). Germline expression plasmids for complex array injections were linearized with NaeI and mixed with EcoRV digested N2 genomic DNA before injections.

RNA interference (RNAi)

RNAi target clones were cloned into L4440 RNAi feeding vector, pRL731 by Gateway cloning or recovered from Julie Ahringer's RNAi library (Kamath and Ahringer, 2003; Kamath et al., 2003). Feeding RNAi was performed as described (Timmons and Fire, 1998) using HT115 bacteria seeded on NGM plates containing 1mM IPTG. L1 larvea were fed for 2 days at 25ºC or 3 days at 20ºC.

Immunofluorescence

Immunofluorescence for *C. elegans* embryos are carried out as follows: for α -PIE-1 (Mello et al., 1996) 1/50 dilution, α -OMA-1a (Shimada et al., 2006), 1/100 dilution, α -GFP (Invitrogen, rabbit) 1/250 dilution, anti-FLAG (Sigma, F3165), 1/250 dilution used and the protocols were as described in the previous chapter. Secondary antibodies for all immunofluorescent analyses were from Invitrogen (Alexa fluor), goat anti-rabbit Alexa488 or Alexa 568, goat anti-mouse Alexa 488 or Alexa568 (1/250 dilution).

Analysis of embryos and imaging

Imaging of immunofluorescence and live animals was performed with an Axioplan microscope (Zeiss) equipped with a MicroMax-512EBFT CCD camera (Princeton Instruments) controlled by the Metamorph acquisition software (Molecular Devices).

RESULTS

I. OMA-1/2 repress transcription in later germline precursors indirectly by regulating PIE-1 levels

OMA-1/2 repress global transcription in newly fertilized embryos by sequestering TAF-4 in the cytoplasm (Guven-Ozkan et al., 2008). Interestingly, reduction of *oma-1/2* activities causes derepression of transcription in later germline blastomeres, although OMA proteins are not expressed at these stages; suggesting an indirect role in transcriptional repression beyond P2 blastomere (Figure 2.6). Correlated with ectopic transcription in germline blastomeres, PIE-1 protein level is greatly reduced when *oma-1/2* are depleted by RNAi (Figure 2.8), suggesting that an earlier embryonic transcriptional repressor regulates levels of a later transcriptional repressor protein. *oma-1/2* double mutant or strong RNAi depletion animals have oocyte maturation defect with 100% sterility (Detwiler et al., 2001), 4-cell and later embryos produced by *oma-1/2* depletion were obtained by milder RNAi conditions to be able to analyze the phenotypes in later embryonic stages. I checked the expression of PIE-1 in *oma-1/2* double mutant animal gonads to see how much PIE-1 was expressed in a more severe mutant and as

expected stronger loss of function of *oma-1/2(-)* resulted in even more dramatic reduction, with almost no PIE-1 protein in the oocytes (Figure 3.2A, B).

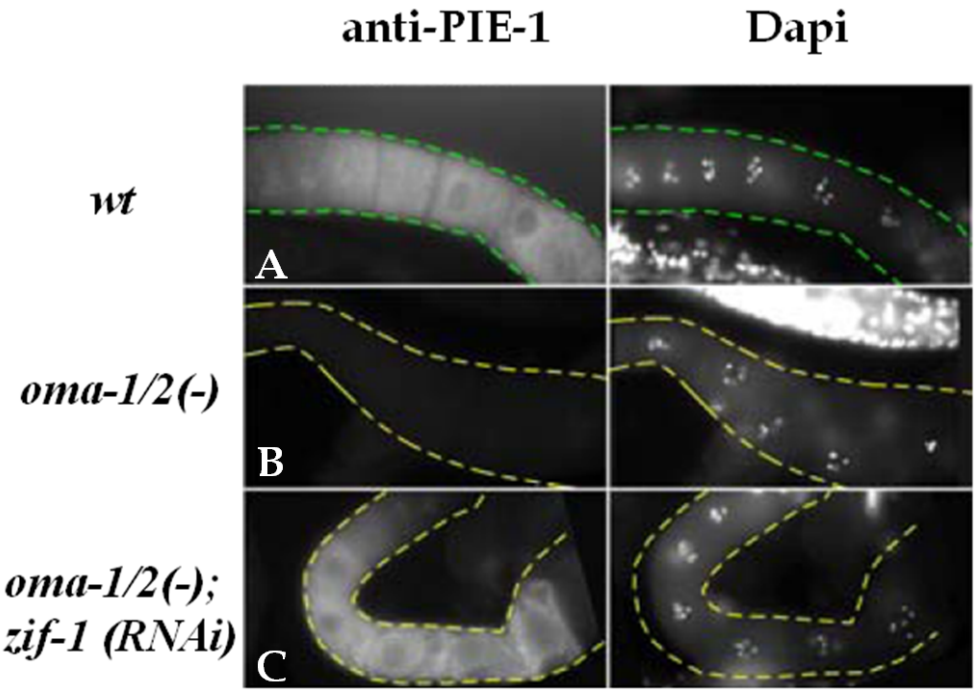


Figure 3.2. Loss of *oma-1/2* results in undetectable PIE-1 protein and can be rescued by *zif-1* RNAi
Immunofluorescence micrographs of wild type (top) and *oma-1/2* double mutant gonads with (bottom) or without *zif-1* depletion (middle), immunostained with anti-PIE-1 (left column) and Dapi (right column). Gonads are highlighted with green (wild type) or yellow (*oma-1/2*) dashed lines.

II. Blocking PIE-1 degradation restores the expression in *oma-1/2(-)* animals

To understand if loss of PIE-1 is due to its reduced gene expression or loss of protein stability, I depleted SOCS box gene *zif-1*, the substrate specific binding partner for PIE-1 degradation by RNAi in *oma-1/2* double mutant animals (Figure 3.2C). When PIE-1 degradation was blocked by *zif-1* depletion, PIE-1 protein was restored in *oma-1/2* double mutant gonads (Figure 3.2C), suggesting that OMA-1/2 regulate PIE-1 at the protein level and they are required for the stability of PIE-1 protein rather than for their gene expression.

III. PIE-1 UTRs are not required for protection by OMA-1/2

To confirm the regulation of PIE-1 by OMA proteins was at the level of protein stability, not through loss of gene expression in the gonads, I depleted *oma-1/2* in various transgenic backgrounds which uses *pie-1* 5' promoter and 3'UTR regulatory sequences; a commonly used tool to express a gene of interest maternally in *C. elegans* germline and embryos (Reese et al., 2000). PGL-1 (P granule abnormality) is a P granule component (RNA rich organelles localized to germline) and when expressed with *pie-1* regulatory sequences it is maternally expressed and localized to gonad and P blastomeres of the embryos. When *oma-1/2* is depleted in GFP::PGL-1 strain, no reduction in GFP signal was observed compared to non RNAi animals (Figure 3.3), confirming the idea that OMA-1/2 protect PIE-1 at the level of protein stability and untranslated sequences (UTRs) are not required for OMA mediated protection. Consistently other transgenes like GFP::TAF-4 and GFP::H2B (histone 2B) that use the same *pie-1* regulatory sequences did not have reduced GFP signal upon *oma-1/2* RNAi (data not shown).

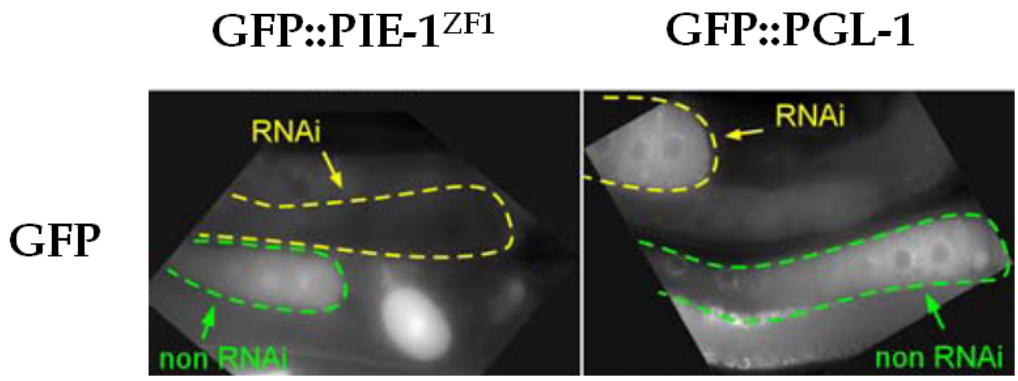
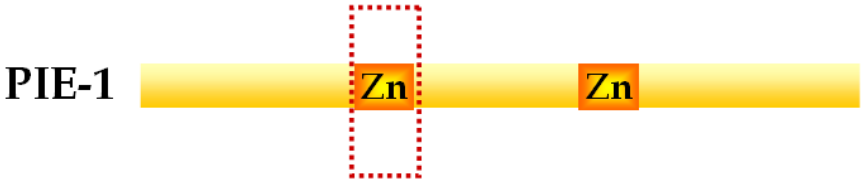


Figure 3.3. OMA-1/2 do not mediate PIE-1 protection through *pie-1* promoter or 3'UTR sequences (by Yuichi Nishi)
Fluorescencent micrographs of live GFP::PIE-1 zinc finger 1 (left) and GFP::PGL-1 (right) animals treated with (yellow dashed gonads) or without (green dashed gonads) *oma-1/2* RNAi. Both transgenes use the same promoter and 3'UTR regulatory sequences. GFP::PIE-1 ZF1 (zinc finger 1) is a fusion of first zinc finger domain of PIE-1 to GFP and used as a tool to study PIE-1 degradation in vivo.

PIE-1 degradation by ZIF-1 (zinc finger interacting factor) mediated CUL-2 based degradation complex is first detected at 4-cell stage of *C. elegans* embryo in two anterior AB blastomeres, the first time point CCCH proteins are degraded by proteasome (DeRenzo et al., 2003; Reese et al., 2000). ZIF-1 interacts with the first zinc finger of PIE-1 and when depleted by RNAi, *zif-1(-)* results in PIE-1 degradation defect. ZIF-1 interaction domain of PIE-1was fused to GFP and can be used as a tool to study PIE-1 degradation in vivo (Figure 3.4) (DeRenzo et al., 2003; Reese et al., 2000).

(A)



(B)

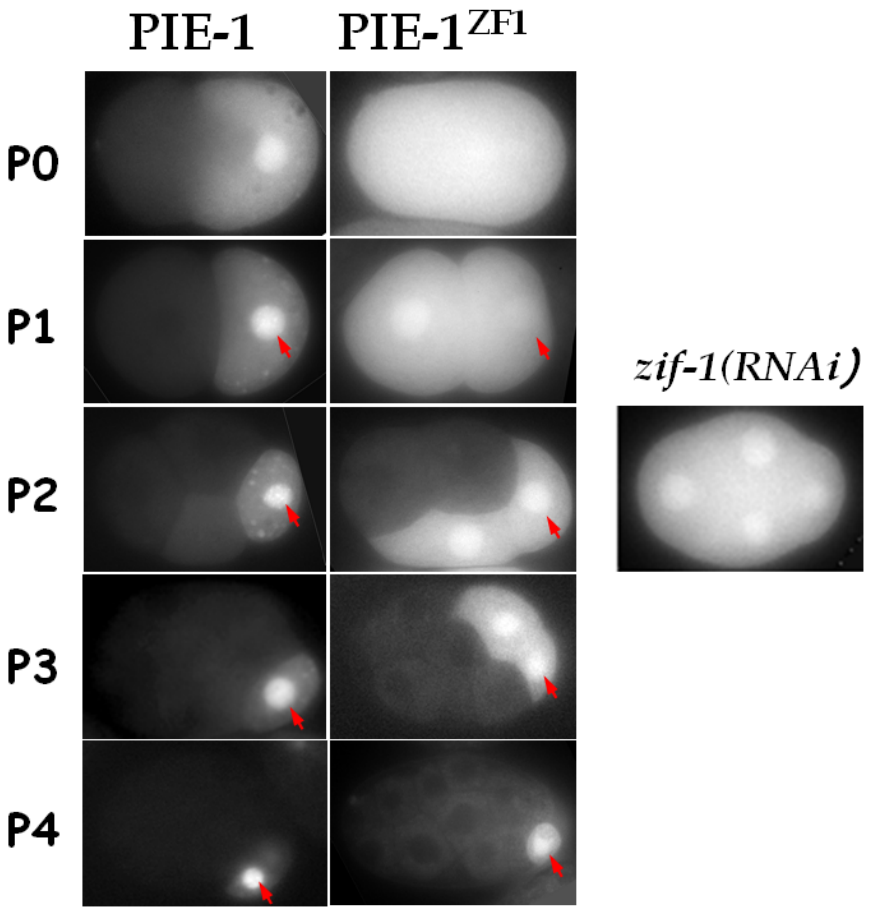


Figure 3.4. PIE-1 zinc finger 1 is degraded in anterior blastomeres starting from 4-cell embryo (Adapted from DeRenzo *et al.*, 2003)

A. PIE-1 has two CCCH RNA binding zinc finger domains. Boxed with red dashed line is the first zinc finger domain that is used to create GFP fusion transgene to study PIE-1 degradation in vivo.

B. Expression of full length PIE-1(left column) and first zinc finger domain of PIE-1 (middle column) in embryos. Red arrows point to germline precursors of the embryos from 1-cell to 28-cell stage (P0-P4). Right most image shows degradation defect of PIE-1$^{\text{zinc finger 1}}$::GFP in two anterior blastomeres of 4-cell embryo when *zif-1* is depleted by RNAi (DeRenzo et al., 2003).

Fusion protein consistently gets degraded in two anterior AB blastomeres of the 4-cell embryo (Figure 3.4B). Degradation of GFP::PIE-1 ^{ZF1} fusion protein in the two anterior blastomeres is blocked when *zif-1* or other genes in PIE-1 degradation machinery are depleted by RNAi (Figure 3.4B) (DeRenzo et al., 2003). When *oma-1/2* were depleted in GFP::PIE-1 ^{ZF1} transgene, as we would have expected the transgene expression was severely compromised, confirming that OMA proteins prevent PIE-1 degradation and first zinc finger of PIE-1 is sufficient to observe the same loss of PIE-1 expression phenotype (Figure 3.3).

IV. Ectopic OMA-1 causes PIE-1 degradation defect in somatic blastomeres

Reduction in *oma-1/2(-)* activities results in loss of PIE-1 expression severely by affecting protein stability and I tested the reverse case. Ectopic OMA-1 beyond 1-cell stage in later embryonic blastomeres expressed under the heat shock promoter resulted in failure to degrade PIE-1 in somatic blastomeres without affecting PIE-1 enrichment towards P blastomeres as PIE-1 protein was consistently higher in P blastomere compared to other somatic blastomeres (Figure 3.5). Similarly, when *zif-1* or some other gene in PIE-1 degradation pathway is lost PIE-1 enrichment in P blastomeres is unaffected and P blastomeres have higher endogenous PIE-1 compared to somatic blastomeres. Heat shock experiments suggests that OMA proteins are sufficient to

stabilize PIE-1 protein and prevent its degradation in soma but OMA proteins do not affect directed movement of PIE-1 towards P blastomeres.

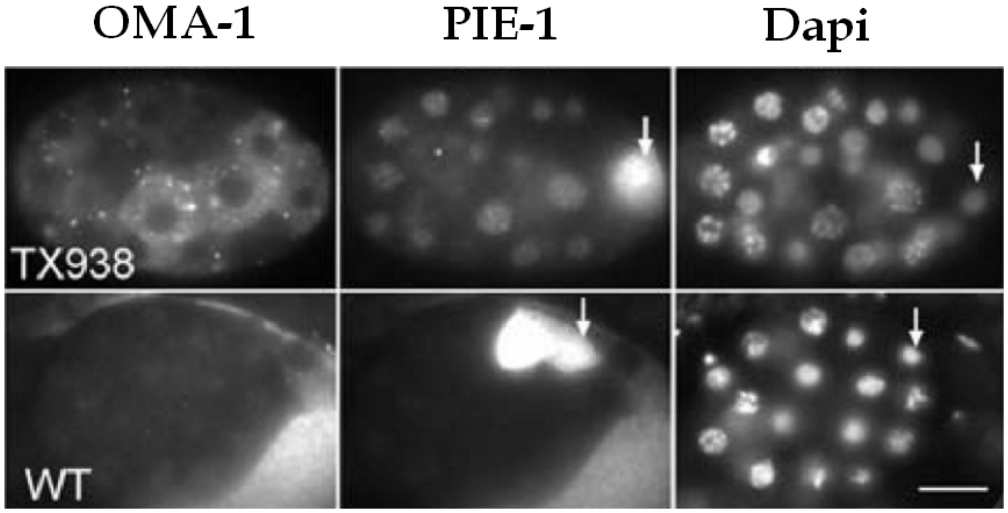


Figure 3.5. Ectopic OMA-1 causes ectopic PIE-1 in all blastomeres
Immunofluorescence micrographs of OMA-1 heat shock (top row) and wild type (bottom row) animals immunostained with anti-OMA-1 (left column), anti-PIE-1 (middle column) and Dapi. Arrows point to germline P blastomeres. Scale bar represents 10μm.

V. *zif-1* 3'UTR Reporter is repressed in the oocytes and activated in anterior blastomeres of 4-cell embryo

OMA proteins could mediate PIE-1 stability by repressing developmental expression of key protein(s) in PIE-1 destruction complex to prevent activation of PIE-1 and other CCCH protein degradation machinery prematurely in the oocytes or in the newly fertilized embryos to tightly control degradation of PIE-1 spatiotemporally (Figure 1.10).

ZIF-1 is a SOCS box protein and the substrate specific binding partner for five CCCH RNA binding protein degradation complexes. ZIF-1 has the ability to interact both with PIE-1 and Elongin C component of CUL2 based degradation complex. Therefore, regulating ZIF-1 expression would be an efficient way to specifically control PIE-1 and other CCCH zinc finger proteins, POS-1, MEX-1, MEX-5, MEX-6 stability in vivo because other components of CUL2 based degradation complex are more general factors likely to be responsible for degradation of multiple targets and not specific to CCCH protein degradation.

Tandem CCCH RNA binding zinc finger domains of OMA proteins are poorly characterized but are predicted to interact with mRNAs to control their translation; and one direct target of OMA proteins in the oocytes is *nos-2* mRNA (Jadhav et al., 2008). Therefore, we built a tempting hypothesis in which we believe that OMA proteins control PIE-1 and other CCCH zinc finger protein stability by regulating *zif-1* mRNA translation. Our hypothesis predicts that *zif-1* mRNA would be repressed where PIE-1 and CCCH proteins are expressed and *zif-1* mRNA could begin to be translated into protein product spatiotemporally in the embryos but only in the blastomeres where CCCH zinc finger proteins are normally degraded anteriorly, not in the posterior P blastomeres.

In situ hybridization using *zif-1* cDNA clone (Nematode Expression Pattern DataBase, Yuji Kohara Lab) detects *zif-1* mRNA all over the gonad as early as L2 larvae and with higher expression in adult gonads. In addition, it is uniformly distributed in all blastomeres of the early embryos (Figure 3.6). Uniform distribution of the mRNA throughout germline and embryos suggests that ZIF-1 activity is likely to be regulated post-transcriptionally to keep ZIF-1 protein expression repressed in the gonad and

germline precursors of the embryo but specifically activate *zif-1* translation in somatic blastomeres to degrade CCCH proteins during embryogenesis.

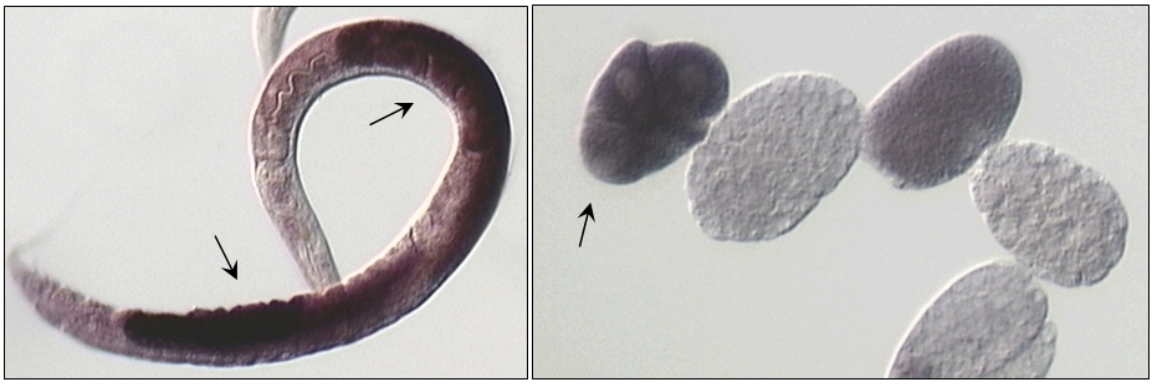


Figure 3.6.In situ hybridization of *zif-1*
(Reproduced from NEXTDB, Yuji Kohara)
In situ hybridization of *zif-1* in the adult gonad and in the embryos. Arrows point to gonads of the animals or uniform embrynonic expression.

To investigate if *zif-1* expression was regulated post-transcriptionally, I generated a germline *zif-1* 3'UTR reporter driven by *pie-1* promoter which expresses GFP::H2B fusion protein. I used 771 bases of 3'UTR region up to next downstream gene to include as much 3'UTR sequence as I could (Figure 3.7) and GFP::H2B was used as readout for translational regulation since nuclearly localized fluorescent protein makes detection and visualization easier whenever the transgene is on.

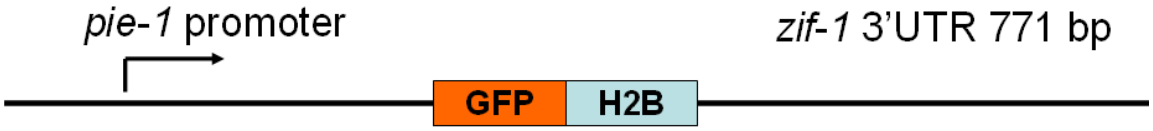
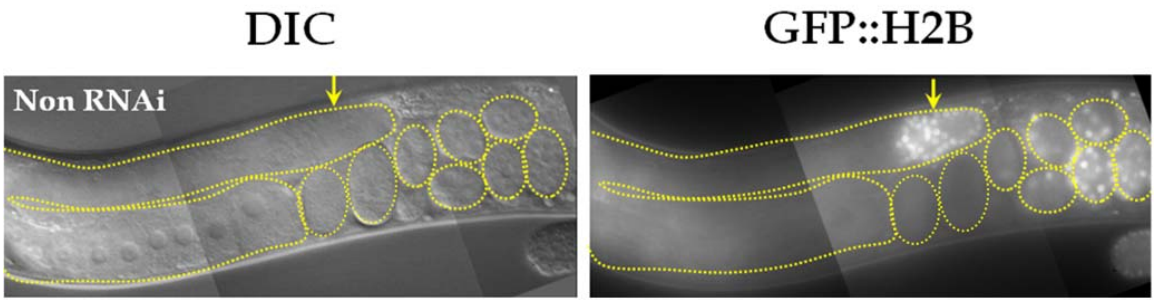


Figure 3.7. 771 bp *zif-1* 3'UTR reporter
zif-1 3'UTR reporter drives expression of GFP::H2B under *pie-1* promoter. Spatiotemporal translation of maternal *zif-1* can be studied using this nuclearly localized fluorescent reporter.

Transgenic worms for *zif-1* translational reporter were generated and showed an expression pattern reciprocal to the expression pattern of PIE-1 zinc finger 1 transgene (Figure 3.8). *zif-1* 3'UTR reporter expression is first detected distally in the mitotically dividing nuclei of the gonad and repressed in the pachytene region of the adult germline as well as in the oocytes (Figure 3.8A), probably to protect CCCH zinc finger proteins from premature degradation temporally. Translational repression of *zif-1* 3'UTR reporter in the oocytes is consistent with our hypothesis that spatiotemporal activity of ZIF-1 is regulated by post-transcriptional mechanisms and maybe by OMA-1/2.

(A)



(B)

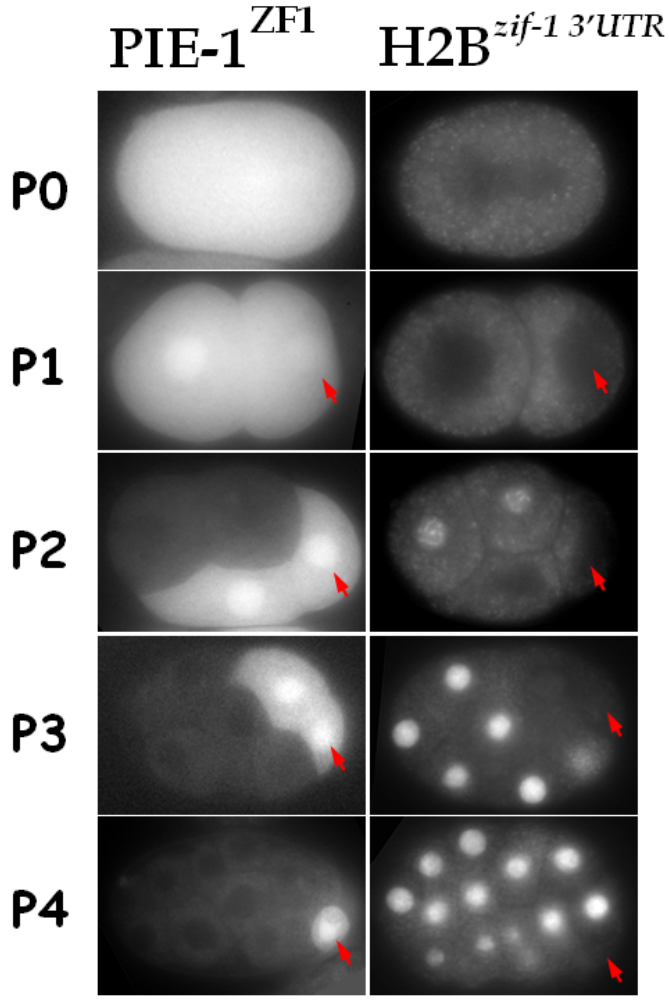


Figure 3.8. *zif-1* 3'UTR reporter shows expression pattern reciprocal to PIE-1
A. DIC (left) and fluorescent (right) images of GFP::H2B::*zif-1*$^{3'UTR}$ animals. Outlined with yellow dashed line is the germline of adult animal. Yellow arrow points to distal region of the gonad with mitotically dividing germ nuclei. Outlined in yellow dashed circles are embryos in the uterus.
B. Fluorescent images of GFP::PIE-1$^{\text{zinc finger 1}}$ and GFP::H2B::*zif-1*$^{3'UTR}$ fusion proteins during embryogenesis, starting from 1-cell to 28-cell stage embryo. Two transgenes show reciprocal expression pattern. Shown on the left is the stage of P blastomeres in the corresponding row. Red arrows point to P blastomeres.

Translation of *zif-1* 3'UTR reporter is activated starting from two anterior AB blastomeres of 4-cell stage embryo and GFP::H2B expression is detected in most blastomeres except P blastomere and its sister blastomere during later stages of embryogenesis (Figure 3.8B). Expression of *zif-1* 3'UTR reporter is reciprocal to PIE-1 zinc finger 1 GFP reporter during embryogenesis, which marks PIE-1 degradation zones in the embryo (Figure 3.8B).

VI. OMA-1/2 repress *zif-1* translation in the oocytes

To test if *zif-1* translational repression in proximal oocytes requires OMA-1/2, I depleted *oma-1/2* by RNAi in the *zif-1* 3'UTR reporter. A very bright derepression of GFP::H2B was detected in *oma-1/2* double mutant (Figure 3.9), supporting the hypothesis that OMA-1/2 repress *zif-1* translation in the oocytes to prevent premature degradation of PIE-1 along with other CCCH zinc finger proteins for gametogenesis and embryogenesis to proceed normally. Derepressed GFP::H2B signal in proximal oocytes of *oma-1/2(-)* animals were even brighter than the signal in the distal region of the gonad. The molecular reason for this very strong derepression is not clear but might involve a

synergistic effect of loss of multiple RNA binding proteins as *oma-1/2(-)* removal results in premature degradation of PIE-1 and likely four other CCCH RNA binding zinc finger proteins. All known targets of ZIF-1 dependent degradation as PIE-1, POS-1, MEX-1, MEX-5/6 have essential functions; therefore spatiotemporal activation of ZIF-1 function is very critical for proper embryogenesis.

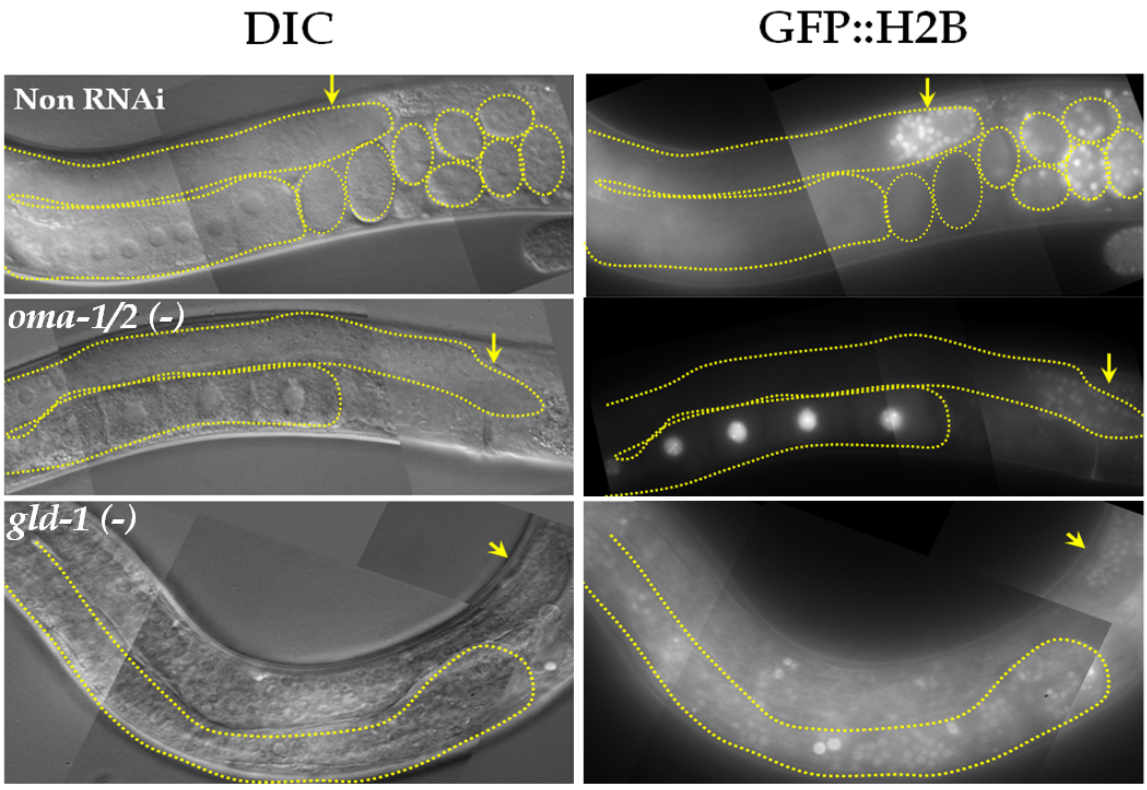


Figure 3.9. *zif-1* 3'UTR reporter is repressed in meiotic germ cells by OMA-1/2 and GLD-1
DIC and fluorescent images of GFP::H2B::*zif-1*$^{3'UTR}$ animals crossed into *oma-1(te33); oma-1(te51)* double loss of function and *gld-1 (q485)* null strains. Yellow dashed lines show the outline of the gonad. Yellow arrow points to the mitotically dividing distal germ nuclei.

VII. GLD-1 represses *zif-1* 3'UTR reporter in the pachytene region of germline

OMA-1/2 proteins are expressed temporally starting from proximal oocytes; but *zif-1* 3'UTR reporter is still repressed before proximal oocyte formation during the pachytene region of the germline suggesting that repression in this region requires some other factor(s).

GLD-1 is a sequence specific KH domain RNA binding protein and functions as translational repressor in the center region of the *C. elegans* germline and some direct mRNA targets of GLD-1 are already known (Jan et al., 1999; Jones et al., 1996; Lee and Schedl, 2001; Lee and Schedl, 2004; Ryder et al., 2004). Protein instability and repression of GLD-1 translation restricts its expression to pachytene region of the germline (Lee and Schedl, 2001). Expression pattern of GLD-1 and already known function of GLD-1 as translational repressor of mRNAs prompted us to look at *zif-1* 3'UTR reporter in *gld-1 (-)* mutant animals and as we predicted we detected derepression of the reporter in *gld-1(q485)* strong loss of function mutants (Figure 3.9). Although *gld-1 (-)* mutant gonads look uniform with many tumorous germ nuclei, somehow the derepression of *zif-1* 3'UTR reporter showed lower penetrance in the pachytene region and more obvious stronger effect in the more proximally located germ nuclei. It is not clear why derepression phenotype is stronger in the proximal region compared to earlier germ nuclei. One reason could be accumulation of GFP signal over time, towards later stages of germ nuclei.

VIII. MEX-5/6, SPN-4 and POS-1 are required for spatial regulation of *zif-1* 3'UTR reporter during embryogenesis

zif-1 3'UTR reporter is active in mitotically dividing zone of the *C. elegans* germline and translational repression of *zif-1* mRNA involves GLD-1 in the pachytene region and OMA-1/2 in the proximal oocytes to protect PIE-1 and CCCH zinc finger proteins from premature degradation during adult germline development. We were interested in finding the factor(s) activating translation of *zif-1* mRNA in the anterior blastomere of the embryos to initiate production of functional ZIF-1 protein to degrade CCCH zinc finger proteins spatially specifically in the anterior of the embryo, also the factor(s) that keep *zif-1* untranslated in the germline precursors. There are some known RNA binding proteins expressed in different regions of *C. elegans* embryo. Therefore, I took a candidate approach and analyzed *zif-1* 3'UTR reporter expression in RNAi backgrounds of those genes to test possible regulation.

zif-1 3'UTR is activated in the two anterior AB blastomeres of 4-cell stage embryo and it is known that MEX-5/6 are CCCH RNA binding proteins required for the anterior cell fate specification of the *C. elegans* embryo. However, no direct mRNA target for MEX-5/6 has been identified yet. When depleted by RNAi, *mex-5/6(-)* resulted in loss of *zif-1* 3'UTR reporter expression throughout embryogenesis (Figure 3.10), consistent with a previous report demonstrating somatic degradation defect of PIE-1, POS-1 and MEX-1 in *mex-5/6* RNAi embryos possibly as a consequence of unactivated ZIF-1 translation and degradation machinery (Schubert et al., 2000).

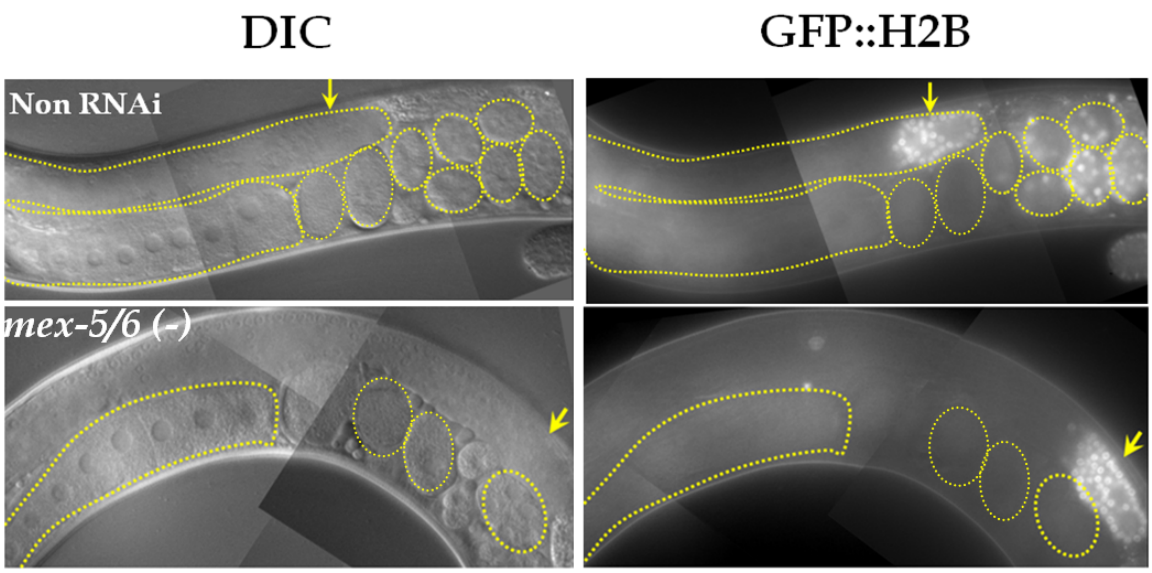


Figure 3.10. MEX-5/6 are required for activation of *zif-1* 3'UTR reporter in the embryos

DIC and fluorescent micrographs of GFP::H2B::*zif-1*$^{3'UTR}$ animals with and without *mex-5/6* depletion. Yellow dashed lines show the outline of the germline. Yellow arrow points to the mitotically dividing distal germ nuclei and yellow dashed circles outlines embryos with no GFP signal.

Then, I tested some candidates for the repression of *zif-1* 3'UTR reporter in the germline precursors of *C. elegans* embryos after OMA-1/2 is degraded. POS-1 is itself target of ZIF-1 mediated degradation but it has CCCH type RNA binding zinc fingers and a strong candidate for post-transcriptional regulation of gene expression specifically in P blastomeres. POS-1 is expressed weakly in 1-cell embryo but it shows strong expression in P blastomeres until P4. POS-1 positively regulates translation of Notch ligand *apx-1* and negatively regulates translation of Notch receptor *glp-1* in germline precursor cells (Ogura et al., 2003; Tabara et al., 1999). When depleted by RNAi, *pos-1(-)* resulted in

derepression of *zif-1* 3'UTR reporter expression in P3 and P4 blastomeres (Figure 3.11). POS-1 is expressed in earlier P blastomeres but with this assay, I could not detect derepression earlier than P3, either POS-1 represses *zif-1* mRNA starting from P3 or the transgene I used could have a delayed expression of the fusion reporter making it harder to detect lower or delayed expression.

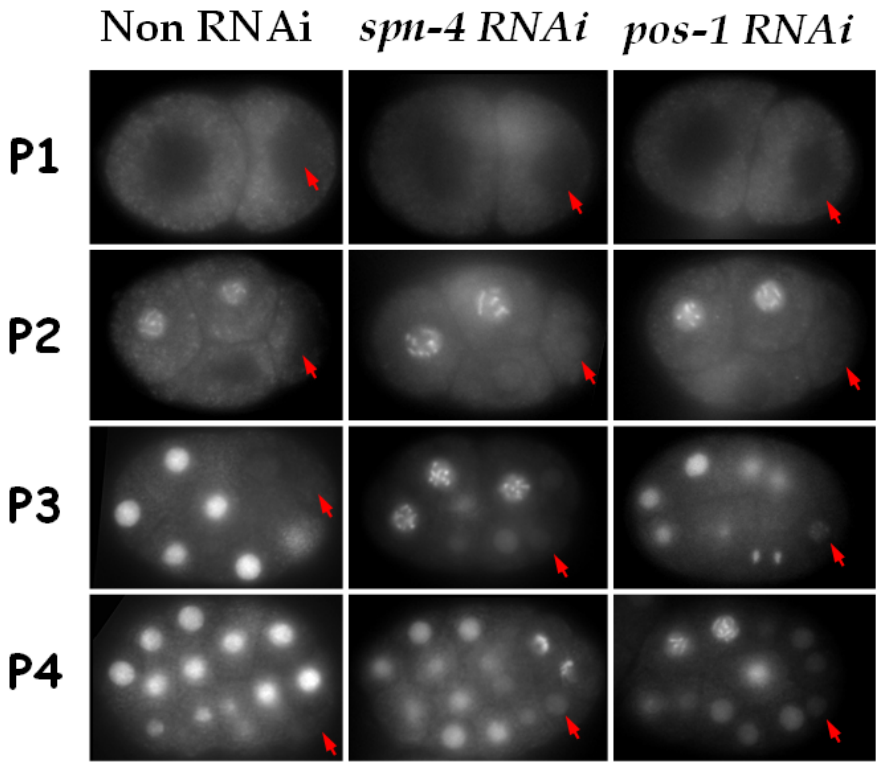


Figure 3.11. SPN-4 and POS-1 repress *zif-1* 3'UTR reporter in P blastomeres of the embryo
Fluorescent images for GFP::H2B::*zif-1*$^{3'UTR}$ embryos with no RNAi treatment (first column), with *spn-4* depletion (middle column) and with *pos-1* depletion. Stages of P blastomeres are shown on the left and P blastomeres are marked with red arrows.

POS-1 interacts with RRM type RNA binding protein SPN-4 and SPN-4 can associate with *glp-1* mRNA and regulate its translation in the embryos (Ogura et al., 2003). I tested possible involvement of SPN-4 in *zif-1* mRNA regulation and I detected derepression of *zif-1* 3'UTR reporter in P blastomeres as early as P2 (Figure 3.11). SPN-4 is expressed in the oocytes and in earlier stage embryos, *zif-1* 3'UTR reporter derepression starting from P2, 4-cell stage embryo suggests that SPN-4 somehow repress *zif-1* mRNA later stages starting from P2 or the reporter I used has delayed expression.

IX. 120 bp region of 3'UTR is required for translational repression in the germline and activation in the embryo

Analysis *zif-1* 3'UTR regulation in different genetic backgrounds were done using a 771 nucleotide fragment of the 3'UTR sequence that starts with *zif-1* stop codon and includes the downstream sequence up to next gene in the genome. We wanted to find a smaller region that is required for translational repression of *zif-1* by OMA-1/2 in the proximal oocytes. Sanger Center 3'UTR determination project annotates *zif-1* 3'UTR as 304 nucleotides. Therefore, I started my domain mapping analysis by using this 304 nucleotide smaller region to make a GFP::H2B reporter (Figure 3.12), to see if it is sufficient to get an expression pattern similar to 771bp transgene.

304 nucleotide *zif-1* 3'UTR reporter shows expression pattern similar to wild type except that expression in the mitotically dividing region was very low otherwise it was repressed in the pachytene region and proximal oocytes and activation started at 4-cell stage in AB blastomeres (data not shown). 304 nucleotide long 3' UTR region still contained the OMA-1/2 dependent response element since *oma-1/2* RNAi resulted in

derepression of GFP signal in proximal oocytes in this transgene (data not shown). Having identified a smaller fragment for the reporter expression, I divided 300 nucleotide region into five domains and deleted each 60 nucleotides individually from the larger 771 nucleotide fragment (Figure 3.12).

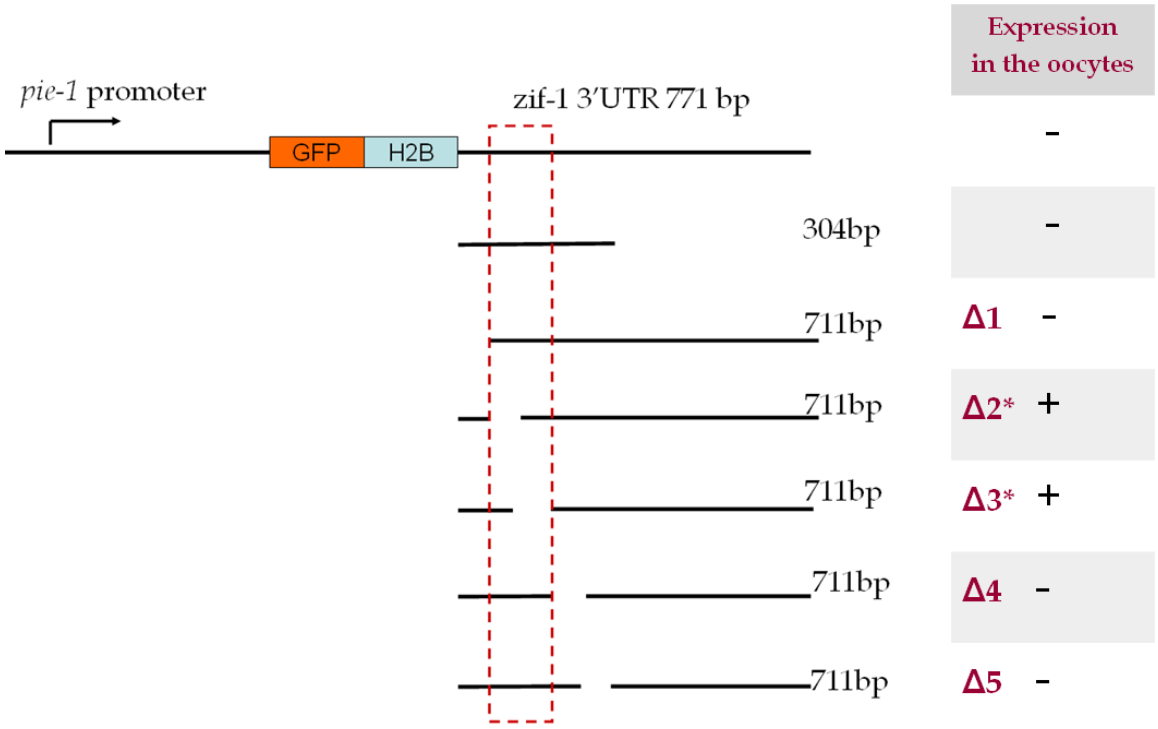


Figure 3.12. *zif-1* 3'UTR deletion analysis
Different deletion constructs of *zif-1* 3'UTR driving expression of GFP::H2B fusion protein under *pie-1* promoter. Top construct shows the longest 3'UTR fragment with 771 nucleotide sequence. Sizes of the other constructs are written on the right and deletion numbers for serial truncations are shown on the left with Δ sign and the deletions with expression pattern different than wild type are shown with an asterisk (Deletions #2 and #3). Red dashed box demonstrates the required domain for translational repression of *zif-1* in the proximal oocytes.

Out of five deletions, only two of them demonstrated expression patterns different than 771 nucleotide fragment shown with an asterisk sign in Figure 3.12. Deletion construct #2 deletes the second 60 nucleotide fragment downstream of *zif-1* stop and it still maintains the expression of the reporter in the mitotic zone of the gonad with repressed expression in pachytene region. However, there is a slight derepression in the proximal oocytes that could be visualized only when signal was overexposed (Figure 3.13) and interestingly embryonic expression of the reporter is lost in all stages of the embryos similar to *mex-5/6* RNAi embryos (Figures 3.10 and 3.13).

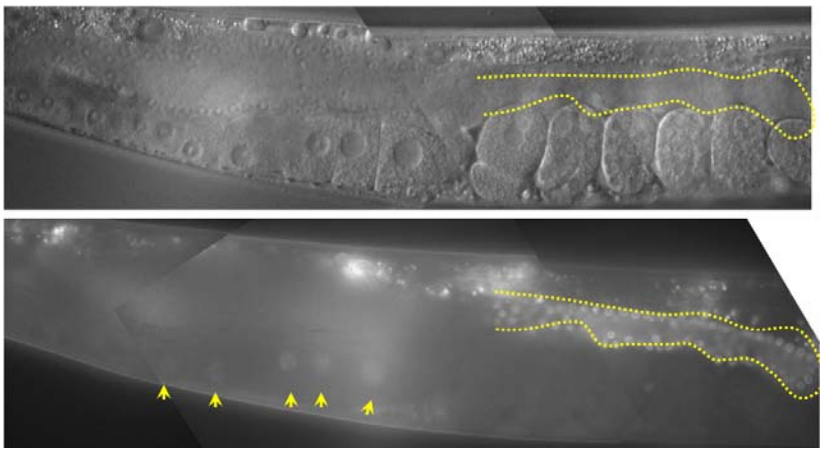


Figure 3.13. Deletion of nucleotides from 64 to 123 of *zif-1* 3'UTR reporter
DIC (top) and fluorescent micrographs of GFP::H2B::zif-1$^{3'UTR\ \Delta 64\text{-}123}$ animals. Yellow dashed lines show the outline of mitotic zone of the gonad, yellow arrows point to derepressed GFP signal in proximal oocytes. Embryos in the uterus do not have any reporter expression.

Deletion #3 construct has truncation of nucleotides from 124 to 183 of the longer *zif-1* 3'UTR fragment and it is the other construct showed an expression pattern different than 771 nucleotide 3'UTR construct. Expression of the reporter was detected in the mitotic zone and it is repressed in the early pachytene zone but interestingly, I detected slight derepression of the reporter in the late pachytene stage of the gonad and in the early oocytes, but somehow derepression was not very clear in the later most matured oocytes (Figure 3.14). This lack of derepression could be due to overall weak expression level of the transgene as well. Expression of the reporter in the embryos was detectable and similar to 771 nucleotide 3'UTR reporter (data not shown).

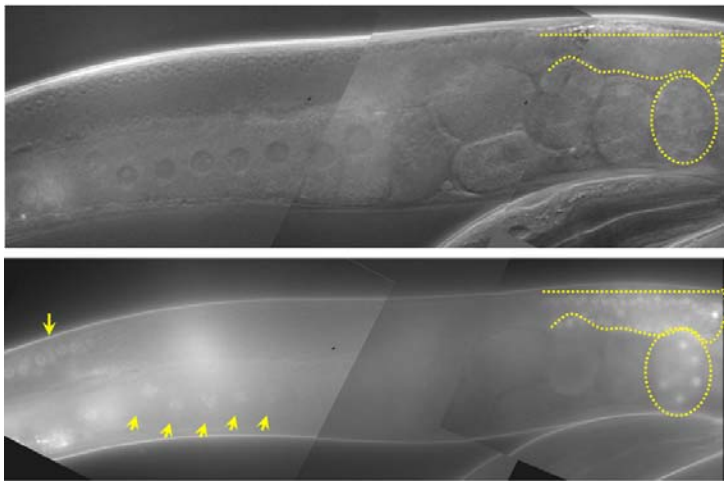


Figure 3.14. Deletion of nucleotides from 124 to 183 of *zif-1* 3'UTR reporter
DIC (top) and fluorescent images of GFP::H2B::zif-1$^{3'UTR\Delta 124\text{-}183}$ animals. Yellow dashed lines show the outline of mitotic zone of the gonad, yellow arrows point to derepressed signal in late pachytene region and early oocytes. Embryos in the uterus show wild type like expression pattern.

Remaining deletion constructs of *zif-1* 3'UTR: deletions #1, #4 and #5 had expression patterns similar to non deleted 771bp fragment using transgenic assay, suggesting that 120 nucleotide region of 3'UTR that is 60 bp downstream of *zif-1* coding stop codon is necessary for overall translational regulation and OMA-1/2 mediated repression in the oocytes. However, derepression of the reporter in both constructs #2 and #3 are weaker compared to GFP signal in mitotic zone and also a lot weaker than derepression signal in *oma-1/2* RNAi oocytes in the longest 771 bp 3'UTR reporter. Very strong derepression in *oma-1/2* RNAi could be due to secondary effects coming from premature degradation of other CCCH proteins in this background or some other indirect effects of losing *oma* functions. Alternatively, constructs #2 and 3 may have partial deletions of the OMA binding element. To see if I could get a higher derepression signal using a combined larger deletion, I deleted regions #2 and #3 simultaneously and generated a 651 nucleotide long, 120 nucleotide deletion construct that is 60 bp downstream of *zif-1* stop codon (Figure 3.15). 120 nucleotide deletion construct had brighter derepression signal in the proximal oocytes as predicted but interestingly, signal was derepressed in late pachytene nuclei as well, suggesting that this 120 nucleotide region of *zif-1* 3'UTR is necessary for OMA-1/2 and maybe for GLD-1 mediated translational repression in the adult germline. 120 nucleotide deletion reporter maintained the expression in mitotic zone of the gonad but signal disappears in the early pachytene nuclei (Figure 3.15), it is not clear why derepression is observed only in later pachytene region, because GLD-1 is expressed throughout pachytene zone.

60 nucleotide deletion transgene downstream of 60 nucleotides of *zif-1* stop codon (deletion #2) resulted in loss of embryonic signal, consistently 120 nucleotide deletion

had very low signal in the embryos most likely coming from persisting H2B::GFP protein from derepressed signal in the oocytes and weak embryonic signal does not increase over time in the embryos (Figure 3.15). The two deletion transgenes suggest that *zif-1* 3'UTR activation element is likely to be located within 120 nucleotide region, a possible MEX-5/6 binding site and most probably within the first half.

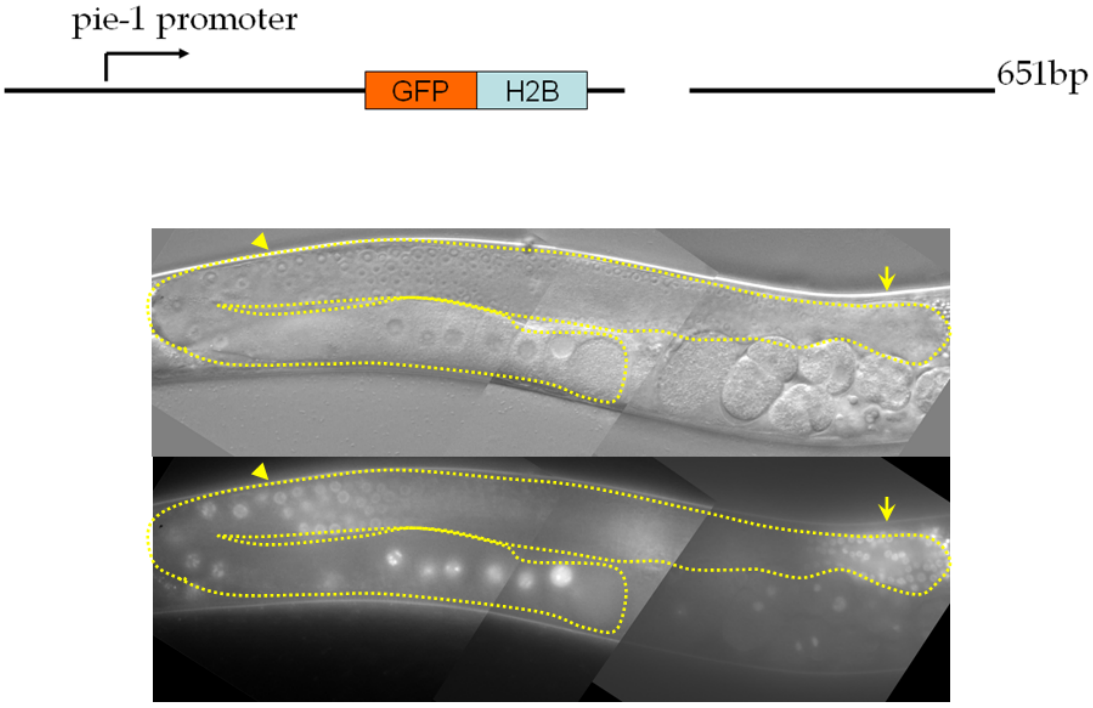
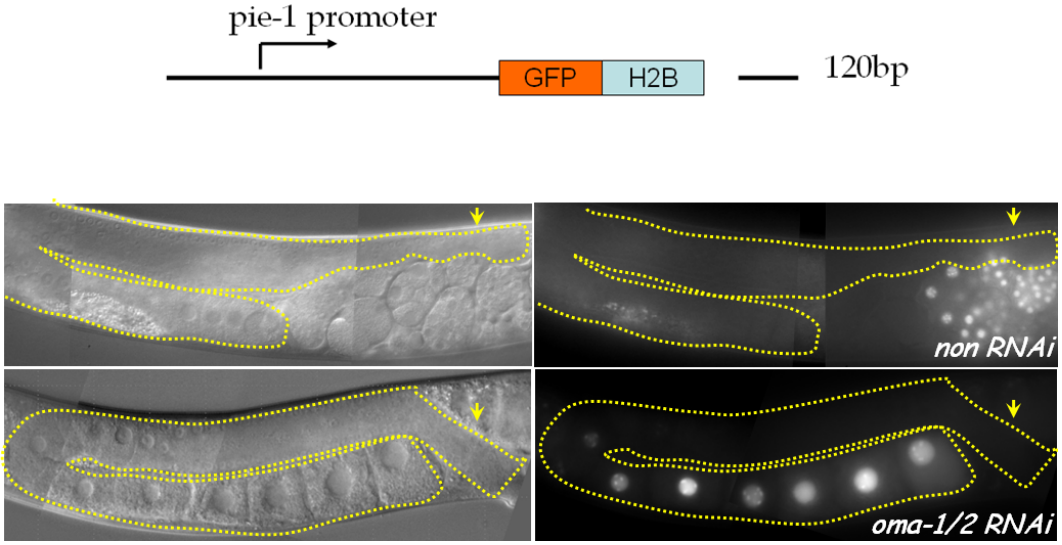


Figure 3.15. 120 bp deletion of *zif-1* 3'UTR reporter causes a stronger derepression in the oocytes
120 bp region 60bp downstream of *zif-1* stop codon is deleted in the original 771bp long *zif-1* 3'UTR reporter as schematized in top image. DIC and GFP fluorescent images for the deletion transgene is shown with a high derepressed signal in the oocytes as well as in late pachytene gonad (shown with yellow arrowhead). Mitotically dividing distal nuclei are shown with yellow arrow.

To test the sufficiency of this 120 nucleotide element in translational repression of *zif-1* in the oocytes and to see if it has an expression pattern similar to 771 nucleotide region of the *zif-1* 3'UTR, I generated smaller 120bp 3'UTR transgene that drove expression of H2B::GFP again using *pie-1* promoter construct (Figure 3.16A). 120 nucleotide transgene is repressed throughout the gonad including distal mitotically dividing region, pachytene zone and proximal oocytes. To determine if the lack of transgene expression in the proximal oocytes are due to OMA-1/2 mediated repression, I depleted *oma-1/2* by RNAi and observed a high level of derepressed GFP signal in the oocytes (Figure 3.16A), suggesting that 120 nucleotide domain has an OMA-1/2 mediated response sequence.

Minimal *zif-1* 3'UTR transgene using 120 nucleotide region showed activation of embryonic signal and I analyzed the signal more carefully in the embryos (Figure 3.16B). Embryonic signal could be detected as early as 2-cell stage embryo with a relatively weak expression but signal gets stronger in AB anterior blastomeres of 4-cell embryo and relatively weaker derepression was observed in the P blastomere and its sister. In the later stage embryos, signal was detected strongly in most somatic blastomeres and weakly in the P blastomeres and their sister cell (Figure 3.16B), suggesting that 120 base domain has the *zif-1* 3'UTR activation element for embryonic expression. P blastomere repression element(s) is likely to be outside of 120 base region since 120bp is not sufficient to fully repress *zif-1* 3'UTR expression.

(A)



(B)

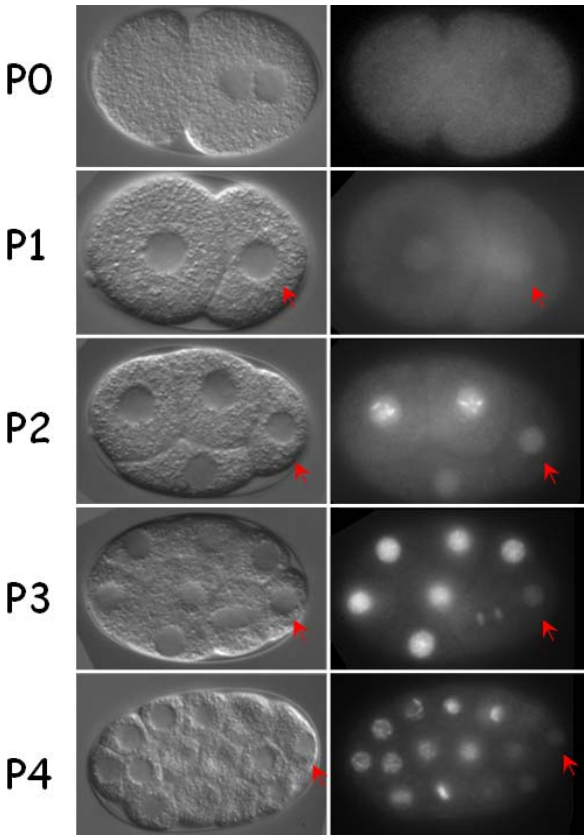


Figure 3.16. 120 bp domain of *zif-1* 3'UTR reporter is sufficient for OMA mediated repression in the oocytes
A. Schematized on the top is the construct used (GFP::H2B::zif-1$^{3'UTR\ 64-183}$) to generate 120 nucleotide *zif-1* 3'UTR reporter, to test the sufficiency of this region for post-transcriptional regulation of *zif-1* mRNA.
Shown below are DIC (left) and GFP fluorescent (right) images for the expression pattern of the transgene in the adult *C. elegans.* Non RNAi animals are shown on the top panel and *oma-1/2* RNAi on the lower panel. Yellow dashed lines outline the gonads, yellow arrows point to mitotically dividing distal germ nuclei.
B. Embryos of GFP::H2B::zif-1$^{3'UTR\ 64-183}$ animals starting from 1-cell stage, marked with red arrows are the P blastomeres, stage of which is indicated on the left of the images.

X. Hypophosphorylated ectopic OMA-1 repress *zif-1* ectopically causing PIE-1 degradation defect

Direct phosphorylation of OMA proteins by MBK-2 kinase at the oocyte to embryo transition is required for TAF-4 interaction in the embryos; therefore we believe that this phosphorylation event converts OMA proteins' functions to properly fit for their embryonic functions. Since OMA proteins expressed in the oocytes are critical for translational repression of *zif-1* mRNA, we tested whether ectopically expressed hypophosphorylated or phosphorylated OMA proteins could continue to repress *zif-1* translation and result in PIE-1 degradation defect. I have tested *zif-1* reporter expression in different genetic backgrounds which have ectopic OMA-1 beyond 1-cell stage similar to ectopic OMA-1 experiments described in Chapter 2. MBK-2 phosphorylation is required for timely degradation of OMA-1/2; therefore *mbk-2* RNAi embryos have OMA-1/2 protein degradation defect with primarily unphosphorylated or hypophosphorylated form. *zu405* allele of OMA-1 has a proline to leucine mutation at a residue one amino acid downstream of MBK-2 phosphorylation site, so *zu405* embryos when depleted of functional *oma-2* by RNAi possess only hypophosphorylated ectopic

OMA-1 beyond 1-cell stage embryo, similar to *mbk-2* RNAi embryos. When I analyzed *zif-1* 3'UTR reporter expression in these backgrounds, embryonic signal of the reporter was lost (Figure 3.17) throughout embryogenesis and consistent with a translational repression of *zif-1* mRNA; *mbk-2* RNAi and *oma-1 (zu405)*; *oma-1 (RNAi)* embryos have PIE-1 degradation defect detected by GFP::PIE-1^{ZF1} transgene (Pellettieri et al., 2003) (Figure 3.17). OMA protein degradation is defective in *cul-1* and *gsk-3* RNAi backgrounds as well. Contrary to *mbk-2* RNAi or *zu405* embryos, in *cul-1(-)* background MBK-2 dependent activities are normal therefore ectopic OMA is believed to be properly phosphorylated at residue 239, MBK-2 phosphorylation site. *zif-1* 3'UTR reporter was expressed like wild type in *cul-1* RNAi embryos and consistent with activation of *zif-1* reporter in the AB blastomeres, PIE-1 degradation occur normally in *cul-1(-)* background suggesting that hypophosphorylated OMA-1/2 can repress *zif-1* translation, when OMA-1 is phosphorylated at meiosis II by MBK-2, this post-translational modification inhibits *zif-1* translational repression activity.

MBK-2 phosphorylation of OMA-1/2 is hypothesized to prime them for GSK-3 kinase phosphorylation and both these phosphorylations are required for timely degradation of OMA proteins (Nishi and Lin, 2005; Shirayama et al., 2006). When depleted by RNAi *gsk-3 (-)* results in ectopic OMA-1 beyond 1-cell stage and I tested if this phosphorylation event is required for repression of *zif-1* reporter. Reporter activation did not occur in *gsk-3 (-)* background and PIE-1 degradation was defective; therefore similar to MBK-2, GSK-3 phosphorylation event seems to be required to inhibit *zif-1* translational repression function of OMA-1/2. Since MBK-2 phosphorylation is believed to precede GSK-3, in *gsk-3(-)* background MBK-2 phosphorylation is predicted to occur

normally but somehow it is not sufficient to inhibit *zif-1* translational repression activity of OMA-1/2, as a result PIE-1 protein persist in somatic blastomeres. It is not clear how GSK-3 phosphorylation is required for the inhibition of oocyte function of OMA-1/2 or what kind of relation this phosphorylation event has to MBK-2 phosphorylation.

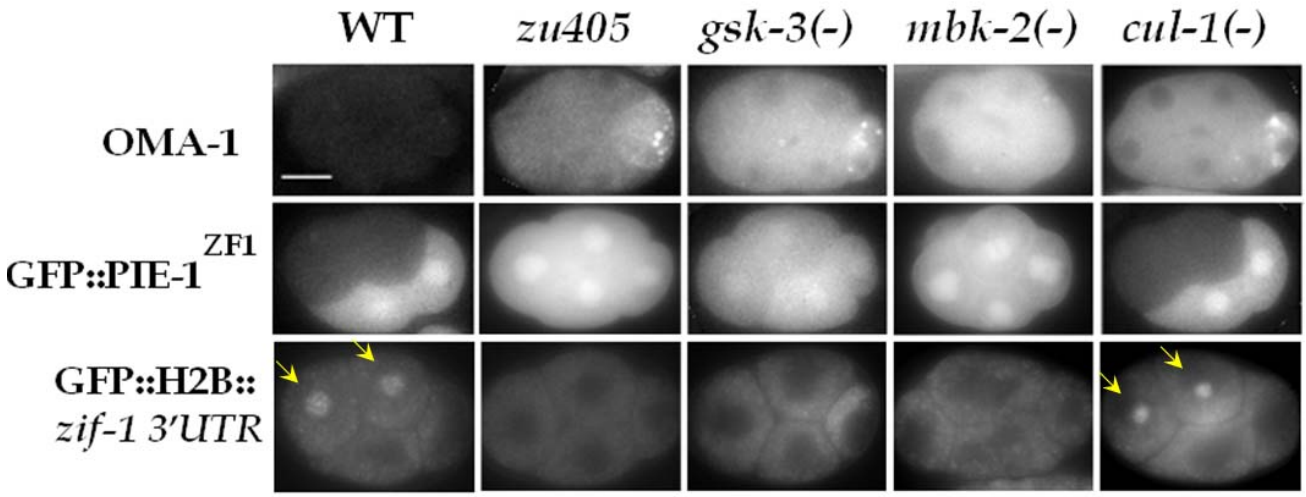


Figure 3.17 Hypophosphorylated ectopic OMA-1 represses *zif-1* translation and results in PIE-1 degradation defect in soma (some of the GFP::PIE-1^{ZF1} images are from Yuichi Nishi's analysis)
Anti-OMA-1 or OMA-1::GFP (top panel), GFP::PIE-1^{ZF1} (middle panel) and GFP::H2B::*zif-1*$^{3'UTR}$ (lower panel) images of 4-cell embryos from wild type and different genetic backgrounds with ectopic OMA-1 beyond 1-cell stage. Yellow arrows point to two anterior AB blastomeres where *zif-1* reporter is active.

DISCUSSION

Post-transcriptional control of mRNA is critical in the germline and early embryos

Fertilization triggers transformation of a relatively quiescent egg into an actively dividing and differentiating embryo. During oocyte to embryo transition, dramatic changes occur; like completion of meiosis and initiation of mitosis along with turnover of oocyte proteins and expression of zygote specific proteins, leading to establishment of embryonic polarity and proper embryogenesis. mRNA transcription is repressed in diakinetic oocytes and in the newly fertilized *C. elegans* embryos; therefore translational control of maternal mRNAs seems to be an important mechanism to regulate gene expression to coordinate oocyte to embryo transition and establishment of the initial events of the zygote. Using a transgenic assay, 3'UTR sequences are shown to be the primary cis-element to regulate gene expression during germline development and early embryogenesis of *C. elegans* (Merritt et al., 2008). 3'UTR sequences of maternal mRNAs contain multiple elements that are required for the spatiotemporal gene expression. Translational control of mRNAs is an emerging field in *C. elegans* and evidence suggest that relatively small number of RNA binding proteins form regulatory complexes to interact with 3'UTR elements of mRNAs to regulate their expression (Evans and Hunter, 2005).

Post-transcriptional regulation and disease

Post-transcriptional regulation of gene expression is not only used in the germ lineage and early patterning of the embryo, but also during later developmental mechanisms like

stem-cell proliferation, sex determination, neurogenesis or erythropoiesis (Kuersten and Goodwin, 2003). Misregulated mRNAs can lead to pathological conditions like cancer (Lukong and Richard, 2007), fragile X syndrome (Siomi et al., 1994; Siomi et al., 1993) or polycystic kidney disease (Bouvrette et al., 2008). FMRP (fragile X mental retardation protein) is an RNA binding protein that regulates local transport and translation of subset of mRNAs at synapses to affect protein synthesis dependent long term potentiation (LTP), memory and synaptic plasticity. Absence of functional FMRP protein leads to excess and dysregulated mRNA translation which is one cause of inherited autism (Bassell and Warren, 2008; Costa-Mattioli et al., 2009). Understanding the details of post-transcriptional regulation of mRNA will broaden our knowledge of not only developmental control of oogenesis and embryogenesis but also will shed light into general mechanisms of mRNA regulation which may cause pathological conditions, when dysregulated.

OMA-1/2 are CCCH type RNA binding zinc finger proteins required for the establishment of germ lineage

OMA-1 and OMA-2 are two tandem CCCH RNA binding zinc finger proteins redundantly required for *C. elegans* oocyte maturation (Detwiler et al., 2001), for transcriptional repression in newly fertilized embryos and in germ cell precursors of later stage embryos (Guven-Ozkan et al., 2008). OMA proteins are RNA binding proteins expressed during the critical time point of oocyte to embryo transition where translational regulation of mRNAs is the primary mechanism to control gene expression. One identified direct mRNA target of OMA proteins in the oocytes is *nos-2*: *C. elegans* germ

cell regulator homologous to *Drosophila* nanos. *nos-2* mRNA is repressed by multiple RNA binding proteins during different stages of germline and embryo development and its translation is specifically activated in P4 blastomere of embryonic germ lineage (D'Agostino et al., 2006; Jadhav et al., 2008). OMA-1/2 directly bind to *nos-2* 3'UTR and repress its translation in the oocytes (Jadhav et al., 2008). OMA proteins play crucial role in establishment of future germline of the *C. elegans* embryo not only by regulating germ cell regulator *nos-2* expression, but also by stabilizing germline specific proteins PIE-1, POS-1, MEX-1 as well as embryonic cell fate determinants MEX-5/6 in the oocytes (Chapter 2, (Guven-Ozkan et al., 2008)). Here, we identified *zif-1* mRNA as another OMA target for translational repression in oocytes to regulate germline establishment of *C. elegans* by inhibiting premature CCCH RNA binding protein degradation. Further biochemical work will elucidate the mechanism of *zif-1* repression by OMA proteins whether they directly associate with *zif-1* 3'UTR or indirectly in a part of RNA binding complex or by some other mechanisms. Understanding the mechanisms of mRNA translational regulation by OMA-1/2 will broaden our knowledge of how oocyte to embryo transition is regulated and how germline identity is protected at molecular level.

zif-1 translation is regulated spatiotemporally by multiple RNA binding proteins

I started the project by investigating possible regulation of *zif-1* translation by OMA proteins and I generated a 3'UTR reporter to show that OMA proteins repress the reporter in the oocytes, but interestingly *zif-1* 3'UTR is not only repressed in the oocytes but also in earlier pachytene region of the germline and in the germline precursors of the embryo and the reporter was activated in two anterior blastomeres of the 4-cell embryo. I took a

candidate approach to test the involvement of other possible RNA binding proteins, interestingly some of the candidates were shown to repress or activate *zif-1* mRNA. GLD-1 represses *zif-1* 3'UTR in the pachytene region of the gonad and there is a putative GLD-1 binding element (GRE) ACTAAT in the 120 nucleotide domain of *zif-1* 3'UTR which is required for germline repression (Figure 3.18) (Marin and Evans, 2003). GRE is not a very specific element for regulation of gene expression and GLD-1 has a lot of mRNA targets; therefore it is believed that GLD-1 interacts with other RNA binding proteins to further provide specificity. Yuichi Nishi showed that GLD-1 and OMA-1 can interact in yeast, although these two proteins do not have overlapping expression regions, this result suggests that KH domain and CCCH RNA binding zinc finger proteins can physically interact and they may be cooperating in RNA binding. It will be interesting to find RNA binding complexes that regulate *zif-1* translation, whether GLD-1 interacts with a CCCH protein or whether OMA-1 interacts with a KH domain protein, like MEX-3 which is expressed in the same zone with OMA proteins in the oocytes.

zif-1 3'UTR reporter is activated in two AB anterior blastomeres of 4-cell embryo consistent with initiation of PIE-1 degradation in those blastomeres during embryogenesis. Our candidate analysis uncovered two redundant anterior blastomere cell fate determinants, MEX-5/6 as activators of *zif-1* translation in the embryos, in addition we identified RRM protein: SPN-4 (Gomes et al., 2001) and CCCH protein: POS-1 as translational repressors in different stages of germline precursors of the embryo. RNA/protein interaction assays will reveal the level all these identified regulators act whether they can directly associate with *zif-1* 3'UTR; if they do, sequence elements

required for their regulation and possible cooperations between different classes of RNA binding proteins can be studied more extensively in the future.

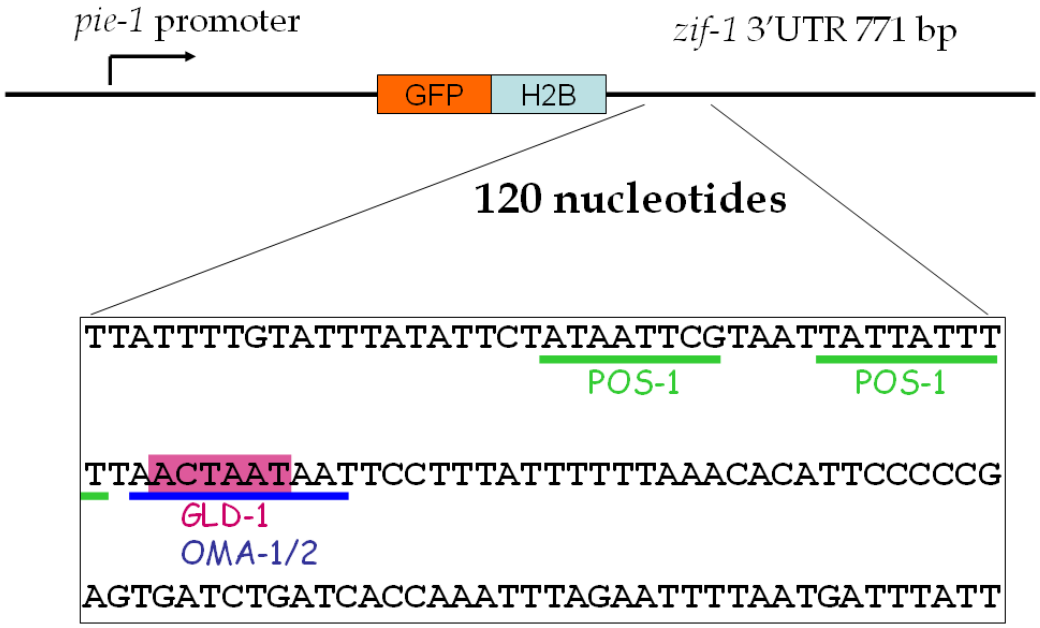
glp-1 and *zif-1* 3'UTR regulation show parallels

Interestingly, *C. elegans* Notch receptor GLP-1 shows an expression pattern strikingly similar to the one for *zif-1* 3'UTR. GLP-1 protein is expressed in the distal germline and repressed in the pachytene region as well as in the oocyte of the germline and activated in the anterior AB blastomere of 2-cell embryo. 3'UTR sequence of *glp-1* is sufficient to regulate its spatiotemporal expression indicating that *glp-1* expression is regulated by post-transcriptional mechanisms (Evans et al., 1994; Evans and Hunter, 2005). GLD-1 represses *glp-1* 3'UTR in the pachytene region of germline, as well as in the posterior blastomeres of the embryos together with POS-1. SPN-4 is required for translational activation of *glp-1* in the anterior of the embryo. Although there are differences between *glp-1* and *zif-1* 3'UTR regulations as the derepression phenotype by POS-1 can be detected earlier than the one I detected with *zif-1* and SPN-4 seems to be required for anterior activation rather than posterior repression, there are common players between the two genes' translational regulation suggesting that OMA-1/2 may have a role in *glp-1* 3'UTR repression or some other RNA binding proteins we found that regulates *zif-1* 3'UTR can be tested in *glp-1* 3'UTR regulation. In addition, sequence elements that are important in *glp-1* translational expression could be conserved and may help us to uncover more 3'UTRs computationally that are regulated by OMA-1/2.

120 nucleotide region of *zif-1* 3'UTR

Mutational analysis using 771 nucleotide *zif-1* 3'UTR revealed that 120 nucleotide region, 60 nucleotides downstream of *zif-1* stop codon is both necessary and sufficient for OMA-1/2 mediated repression in the oocytes. Interestingly, this region of 3'UTR seems to contain putative elements for RNA binding proteins that are involved in *zif-1* 3'UTR regulation. Putative GLD-1 binding element, GRE sequence: ACTAAT is located within this 120 nucleotide region of *zif-1* 3'UTR (Figure 3.18). Binding elements for other RNA binding proteins are poorly characterized but based on few known target mRNAs, two possible POS-1 binding sites and one OMA interaction site is predicted (Figure 3.18) (Farley et al., 2008; Jadhav et al., 2008; Marin and Evans, 2003). Smaller nucleotide substitutions within 120 nucleotide region of *zif-1* 3'UTR will reveal whether these predicted sites are responsible for post-transcriptional regulation of *zif-1* mRNA and could be used for in vitro RNA/protein assays. Alignment of 120 nucleotide domain of *C. elegans zif-1* 3'UTR with *zif-1* 3'UTR sequences of two other closely related *Caenorhabditis* species: *C. briggsae, C. remanei* revealed that there are nucleotide conservations between three nematode species and makes the strategy to further dissect functional elements of *zif-1* 3'UTR a promising one. When OMA-1/2 binding sequence element of *zif-1* 3'UTR is identified, it will enable its comparison to *nos-1* 3'UTR response element and may provide identification of an OMA-1/2 consensus sequence and more OMA-1/2 mRNA targets can be discovered to better understand the nature of oocyte maturation defect caused by *oma-1; oma-2* double mutant animals.

(A)



(B)

```
C. elegans      TTA-TTTTGTATTTAT--ATTCTATAATTCGTAATTATTATTTT-T
C. briggsae     GTAATTCCCTATTTTTGAATACTGTAATACTCTAAATTATTTATGT
C. remanei      GTA-TTATTTATTTTC--GTT-TGTAAT-CTCAATAATCTCTGTAT

C. elegans      AACTAATAATTCC---TTTATTTTT--TAAACACATTCCCCCGAGT
C. briggsae     AATTAATAATTCCCCTTTCACCTACACCTAGTGTCCTTTTTTATCT
C. remanei      AATTAATGATCCC---TGTATATTTATTTACCCCAGTCATCCAGTT

C. elegans      ------GATCTGATCACCAAATTTAGAATTTTAATGATTTATT--
C. briggsae     -TATTTGATCTGTTTAATAATTTTCCAAAATTCTCTAAAAATTCT
C. remanei      ATACTAGATTCTTTTAATAATTCTGAAATTCAATTTACAAAGTCT
```

Figure 3.18. Putative GLD-1, OMA and POS-1 binding sites in *zif-1* 3'UTR

A. 120 nucleotide domain of *zif-1* 60 nucleotides downstream of its stop codon is necessary and sufficient for OMA mediated repression in oocytes. This sequence element contains putative GLD-1, OMA and POS-1 sites which were shown to repress *zif-1* 3'UTR reporter at different time points during development.

B. Alignment of 120 nucleotide domain of *C. elegans zif-1* 3'UTR sequence with *C. briggsae* and *C. remanei* 3'UTR sequences.
Underlined in green: putative POS-1 site, blue: putative OMA-1/2 site and shaded or underlined in purple: putative GLD-1 binding site.

MBK-2 phosphorylation inhibits *zif-1* 3'UTR repression by OMA-1/2

We previously identified that OMA phosphorylation by MBK-2 at meiosis II is required for OMA-1/2 and TAF-4 interaction. Unphosphorylated OMA proteins are unable to bind to TAF-4, as a result TAF-4 stays nuclear during oocyte development despite high levels of OMA proteins. Here, we identified that the same phosphorylation event causes inhibition of *zif-1* 3'UTR repression, suggesting that unphosphorylated OMA proteins can repress *zif-1* translation in the oocytes of *C. elegans* and upon phosphorylation during oocyte to embryo transition OMA proteins can no longer serve as *zif-1* 3'UTR repressors. DYRK family kinase MBK-2 coordinates proper oocyte to embryo transition by phosphorylating its target proteins (Pellettieri et al., 2003). Based on the current data we present, our model predicts that MBK-2 phosphorylation of OMA-1/2 causes dramatic changes in the function(s) of OMA-1/2, switching them from translational repressors of oocyte development to transcriptional repressors of embryos (Figure 3.19). GSK-3 is another kinase that phosphorylates OMA proteins but the exact timing for this phosphorylation event is not clear yet, but previously suggested to occur after MBK-2 phosphorylation (Nishi and Lin, 2005). Interestingly, this latter phosphorylation event seems to inhibit *zif-1* translational repression by OMA proteins as well. How MBK-2 and GSK-3 phosphorylations inhibit *zif-1* translational repression is not clear yet. Do these kinases cause structural change in OMA proteins and make zinc

fingers inaccessible for *zif-1* 3'UTR interaction or do they result in change in OMA proteins interaction partners to switch their oocyte function(s) to embryonic ones? Studying MBK-2 and GSK-3 phosphorylations both in vivo and in vitro using RNA/protein assays is likely to illuminate the regulation of OMA-1/2 functions during oocyte to embryonic switch.

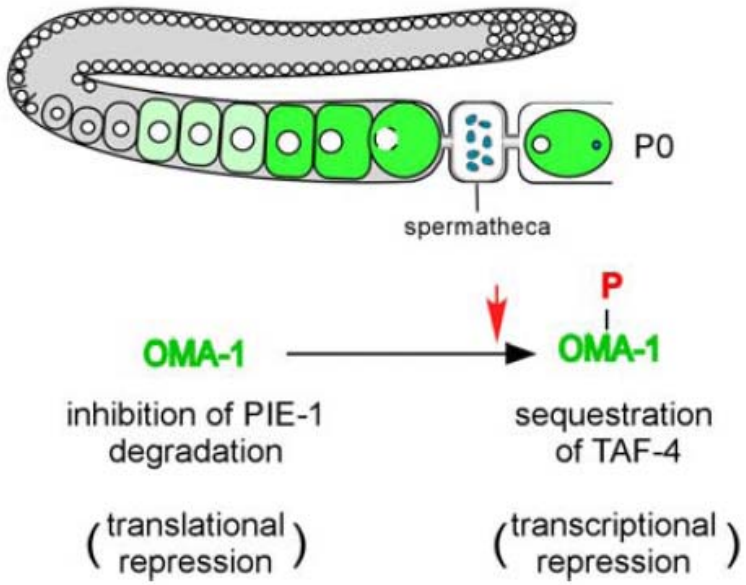


Figure 3.19. Model: MBK-2 phoshorylation converts OMA-1/2 from oocyte translational regulators to embryonic transcriptional repressors
OMA proteins are phosphorylated by MBK-2 at meiosis II stage of newly fertilized embryo and this phosphorylation event is required for TAF-4 interaction as a result for transcriptional repression in the embryos. However, evidence suggest that MBK-2 phosphorylation inhibits the *zif-1* translational repression function of OMAs. Oocyte to embryo transition regulator MBK-2 seems to regulate switch of OMA functions from oocyte to embryo.

CHAPTER FOUR

CONCLUSIONS AND FUTURE DIRECTIONS

Dual roles for OMA proteins

Previous studies identified requirement for OMA-1 and OMA-2 during *C. elegans* oocyte maturation and regulation of their proteosomal degradation. However, molecular functions of OMA-1/2 were not known, although they were predicted to regulate mRNA metabolism by their two CCCH RNA binding zinc finger domains. My studies assigned OMA-1/2 proteins two different functions during oocyte development and in the newly fertilized embryos and the two functions are likely incompatible and differentially regulated by DYRK kinase MBK-2. OMA proteins repress *zif-1* 3'UTR expression in the oocytes and they repress global transcription in the newly fertilized embryos through a newly identified mechanism, by interacting with and sequestering TAF-4 in the cytoplasm. Oocyte function of OMA is required for the protection of germline specific proteins from premature degradation and embryonic function is required for germline transcriptional repression. Interestingly, my studies suggest that the two very important roles OMAs play during oocyte to embryonic transition are carried out by two distinct domains. N terminal predicted alpha helical domain is crucial for transcriptional repression but CCCH domains are required for *zif-1* RNA binding during oocyte development. Both PIE-1 and OMA-1/2 were shown to have domains that accomplish very different molecular functions but these domains contribute to a common goal: protection of germline lineage. My studies identified that *C. elegans* uses multifunctional proteins OMA-1/2 to regulate different aspects of germline development, probably to

better coordinate the events. Interestingly, post-translational modifications are sufficient to either inactivate or activate different functions. OMA proteins provide a nice model system to study different functional modules that co-exist in the same protein which can be quickly switched to another one by phoshporylation.

OMA-1/2 repress *zif-1* 3'UTR in the oocytes

My studies identified *zif-1* 3'UTR as translational target of OMA-1/2 during oocyte development. OMA proteins are required for the stability of five maternal CCCH zinc finger proteins: PIE-1, POS-1, MEX-1, MEX-5 and MEX-6 to protect them from ZIF-1 mediated proteasomal degradation (Figure 4.1). OMA proteins are phosphorylated during oocyte to embryo transition by at least two kinases, MBK-2 and GSK-3; my data suggest that the unphosphorylated oocyte form of OMAs can repress translation of *zif-1* 3'UTR. We propose that the phosphorylated embryonic form of OMAs can no longer repress *zif-1* 3'UTR. Data I present on *zif-1* translation by OMA proteins is just the beginning of an interesting story. There are still a lot of unanswered questions remaining. It is not known whether OMA proteins interact with *zif-1* 3'UTR directly or regulate its expression indirectly. How MBK-2 and GSK-3 phosphorylations seem to inhibit the repression at molecular level, could this inhibition be studied in vitro? What is the structural basis for OMA-1 phosphorylations? What is the sequence element for OMA binding within 120 nucleotide *zif-1* 3'UTR? Once identified, can OMA binding consensus element lead to discovery of more mRNA targets? What other mRNA targets do OMAs have, do they have any role in oocyte maturation phenotype of the double mutant? And what is the involvement of MBK-2 and GSK-3 phosphorylations in their regulation?

In addition to studying mRNA regulation by OMA-1/2, this project can lead to another direction: more detailed identification of *zif-1* post-transcriptional regulators. First of all, other than 3'UTR reporter data, endogenous ZIF-1 expression pattern is not known yet, it is not known whether the endogenous protein has a similar expression to the one I identified in my studies. I showed that several RNA binding proteins are required for *zif-1* 3'UTR expression but we do not know if those proteins associate with *zif-1* 3'UTR directly; if so, the sequence elements are not defined yet. Among the identified *zif-1* 3'UTR regulators, I think MEX-5/6 seems to be interesting for further analysis. Yuichi Nishi and Eric Rogers, former graduate students from our lab showed that MBK-2 kinase phosphorylates MEX-5/6, but the molecular function of this post-translational modification is not known. I believe that phosphorylated MEX-5/6 might interact with *zif-1* 3'UTR, opposite to the one we observed with OMA proteins. All the unanswered questions stemming from my *zif-1* analysis seems very interesting and promising.

OMA proteins repress global transcription in embryos

OMA proteins are directly phosphorylated by MBK-2 after oocyte development and right before their destruction, to serve an essential function in newly fertilized embryos and to protect germ cells from inappropriate differentiation. MBK-2 phosphorylation enables OMA proteins to interact with TAF-4 which results in global transcriptional repression in 1-cell and 2-cell stages of *C. elegans* embryos. My studies identified that N terminal portion of OMA-1 interacts with histone fold domain of TAF-4, the domain required for subcellular localization of TAF-4. It would be interesting to identify the

molecular details of this interaction as it is not known how MBK-2 phosphorylation regulates the interaction and what kind of structural changes occur in OMA proteins. TAF-4 is an evolutionarily conserved protein across species and essential for RNAPII mediated mRNA transcription. Therefore, targeting TAF-4 is a very efficient mechanism to repress global transcription transiently in the newly fertilized embryos. It would be interesting to see if regulation of global transcription by TAF-4 is a conserved mechanism in other contexts as well.

OMA proteins indirectly repress transcription in later stage P blastomeres through repressing *zif-1* translation and protecting PIE-1 from premature degradation (Figure 4.1). It is still not known how transcriptional repression is achieved in diakinetic oocytes, which could be related to OMA proteins or totally independent of their functions; but for the completeness of the transcriptional repression and oocyte to embryo transition picture, it would be very interesting to identify the transcriptional repressor(s) and the mechanism(s) acting at those stages of development.

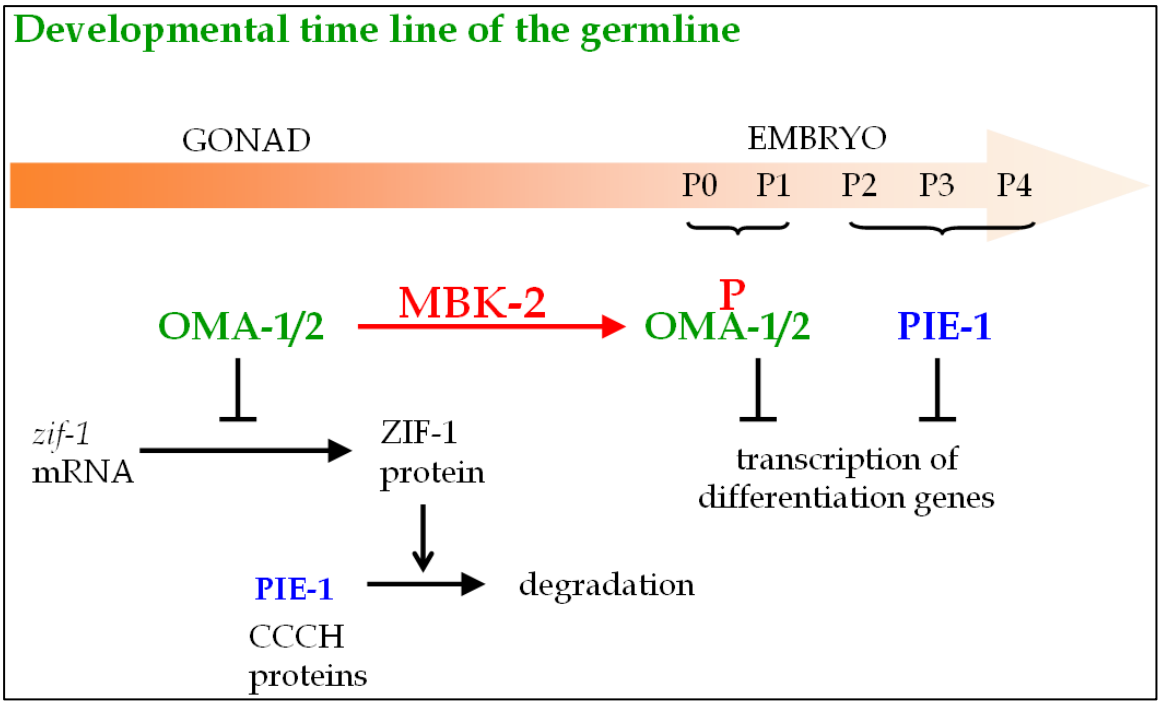


Figure 4.1. Dual roles for OMA proteins during oogenesis and embryogenesis
This figure summarizes the overall findings of my study. We identified *zif-1* 3'UTR as translational target for unphosphorylated OMA-1/2 in the oocytes to prevent *zif-1* translation into functional ZIF-1 protein to protect PIE-1 and four other CCCH proteins from premature degradation.
Phosphorylation by MBK-2 during meiosis converts OMAs from oocyte regulators to short living embryonic transcriptional repressors by interaction with TAF-4. When OMAs are degraded, PIE-1 represses global transcription in germline precursors of *C. elegans* through a different but readily reversible mechanism.

BIBLIOGRAPHY

Albright, S. R., Tjian, R., 2000. TAFs revisited: more data reveal new twists and confirm old ideas. Gene. 242**,** 1-13.

Ancelin, K., Lange, U. C., Hajkova, P., Schneider, R., Bannister, A. J., Kouzarides, T., Surani, M. A., 2006. Blimp1 associates with Prmt5 and directs histone arginine methylation in mouse germ cells. Nat Cell Biol. 8**,** 623-30.

Ariz, M., Mainpal, R., Subramaniam, K., 2009. C. elegans RNA-binding proteins PUF-8 and MEX-3 function redundantly to promote germline stem cell mitosis. Dev Biol. 326**,** 295-304.

Bassell, G. J., Warren, S. T., 2008. Fragile X syndrome: loss of local mRNA regulation alters synaptic development and function. Neuron. 60**,** 201-14.

Batchelder, C., Dunn, M. A., Choy, B., Suh, Y., Cassie, C., Shim, E. Y., Shin, T. H., Mello, C., Seydoux, G., Blackwell, T. K., 1999. Transcriptional repression by the Caenorhabditis elegans germ-line protein PIE-1. Genes Dev. 13**,** 202-12.

Baugh, L. R., Hill, A. A., Slonim, D. K., Brown, E. L., Hunter, C. P., 2003. Composition and dynamics of the Caenorhabditis elegans early embryonic transcriptome. Development. 130**,** 889-900.

Bentley, D. L., 2005. Rules of engagement: co-transcriptional recruitment of pre-mRNA processing factors. Curr Opin Cell Biol. 17**,** 251-6.

Biondi, R. M., Nebreda, A. R., 2003. Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions. Biochem J. 372**,** 1-13.

Blackwell, T. K., Walker, A. K., 2006. Transcription mechanisms. WormBook. 1-16.

Bosu, D. R., Kipreos, E. T., 2008. Cullin-RING ubiquitin ligases: global regulation and activation cycles. Cell Div. 3**,** 7.

Bouvrette, D. J., Price, S. J., Bryda, E. C., 2008. K homology domains of the mouse polycystic kidney disease-related protein, Bicaudal-C (Bicc1), mediate RNA binding in vitro. Nephron Exp Nephrol. 108**,** e27-34.

Brenner, S., 1974. The genetics of Caenorhabditis elegans. Genetics. 77**,** 71-94.

Buratowski, S., Hahn, S., Guarente, L., Sharp, P. A., 1989. Five intermediate complexes in transcription initiation by RNA polymerase II. Cell. 56**,** 549-61.

Carballo, E., Lai, W. S., Blackshear, P. J., 1998. Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. Science. 281**,** 1001-5.

Chatterjee, S., Struhl, K., 1995. Connecting a promoter-bound protein to TBP bypasses the need for a transcriptional activation domain. Nature. 374**,** 820-2.

Cheeks, R. J., Canman, J. C., Gabriel, W. N., Meyer, N., Strome, S., Goldstein, B., 2004. C. elegans PAR proteins function by mobilizing and stabilizing asymmetrically localized protein complexes. Curr Biol. 14**,** 851-62.

Ciosk, R., DePalma, M., Priess, J. R., 2006. Translational regulators maintain totipotency in the Caenorhabditis elegans germline. Science. 311**,** 851-3.

Costa-Mattioli, M., Sossin, W. S., Klann, E., Sonenberg, N., 2009. Translational control of long-lasting synaptic plasticity and memory. Neuron. 61**,** 10-26.

Cowan, C. R., Hyman, A. A., 2004. Centrosomes direct cell polarity independently of microtubule assembly in C. elegans embryos. Nature. 431**,** 92-6.

Crittenden, S. L., Bernstein, D. S., Bachorik, J. L., Thompson, B. E., Gallegos, M., Petcherski, A. G., Moulder, G., Barstead, R., Wickens, M., Kimble, J., 2002. A conserved RNA-binding protein controls germline stem cells in Caenorhabditis elegans. Nature. 417**,** 660-3.

Cuenca, A. A., Schetter, A., Aceto, D., Kemphues, K., Seydoux, G., 2003. Polarization of the C. elegans zygote proceeds via distinct establishment and maintenance phases. Development. 130**,** 1255-65.

D'Agostino, I., Merritt, C., Chen, P. L., Seydoux, G., Subramaniam, K., 2006. Translational repression restricts expression of the C. elegans Nanos homolog NOS-2 to the embryonic germline. Dev Biol. 292**,** 244-52.

de Moor, C. H., Meijer, H., Lissenden, S., 2005. Mechanisms of translational control by the 3' UTR in development and differentiation. Semin Cell Dev Biol. 16**,** 49-58.

de Moor, C. H., Richter, J. D., 2001. Translational control in vertebrate development. Int Rev Cytol. 203**,** 567-608.

DeRenzo, C., Reese, K. J., Seydoux, G., 2003. Exclusion of germ plasm proteins from somatic lineages by cullin-dependent degradation. Nature. 424**,** 685-9.

Deshpande, G., Calhoun, G., Schedl, P., 2004. Overlapping mechanisms function to establish transcriptional quiescence in the embryonic Drosophila germline. Development. 131**,** 1247-57.

Detwiler, M. R., Reuben, M., Li, X., Rogers, E., Lin, R., 2001. Two zinc finger proteins, OMA-1 and OMA-2, are redundantly required for oocyte maturation in C. elegans. Dev Cell. 1**,** 187-99.

Dow, M. R., Mains, P. E., 1998. Genetic and molecular characterization of the caenorhabditis elegans gene, mel-26, a postmeiotic negative regulator of mei-1, a meiotic-specific spindle component. Genetics. 150**,** 119-28.

Draper, B. W., Mello, C. C., Bowerman, B., Hardin, J., Priess, J. R., 1996. MEX-3 is a KH domain protein that regulates blastomere identity in early C. elegans embryos. Cell. 87**,** 205-16.

Dunah, A. W., Jeong, H., Griffin, A., Kim, Y. M., Standaert, D. G., Hersch, S. M., Mouradian, M. M., Young, A. B., Tanese, N., Krainc, D., 2002. Sp1 and TAFII130 transcriptional activity disrupted in early Huntington's disease. Science. 296**,** 2238-43.

Eckmann, C. R., Crittenden, S. L., Suh, N., Kimble, J., 2004. GLD-3 and control of the mitosis/meiosis decision in the germline of Caenorhabditis elegans. Genetics. 168**,** 147-60.

Ephrussi, A., Lehmann, R., 1992. Induction of germ cell formation by oskar. Nature. 358**,** 387-92.

Evans, T. C., Crittenden, S. L., Kodoyianni, V., Kimble, J., 1994. Translational control of maternal glp-1 mRNA establishes an asymmetry in the C. elegans embryo. Cell. 77**,** 183-94.

Evans, T. C., Hunter, C. P., 2005. Translational control of maternal RNAs. WormBook. 1-11.

Extavour, C. G., Akam, M., 2003. Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. Development. 130**,** 5869-84.

Farley, B. M., Pagano, J. M., Ryder, S. P., 2008. RNA target specificity of the embryonic cell fate determinant POS-1. RNA. 14**,** 2685-97.

Farley, B. M., Ryder, S. P., 2008. Regulation of maternal mRNAs in early development. Crit Rev Biochem Mol Biol. 43**,** 135-62.

Francis, R., Barton, M. K., Kimble, J., Schedl, T., 1995a. gld-1, a tumor suppressor gene required for oocyte development in Caenorhabditis elegans. Genetics. 139**,** 579-606.

Francis, R., Maine, E., Schedl, T., 1995b. Analysis of the multiple roles of gld-1 in germline development: interactions with the sex determination cascade and the glp-1 signaling pathway. Genetics. 139**,** 607-30.

Furukawa, T., Tanese, N., 2000. Assembly of partial TFIID complexes in mammalian cells reveals distinct activities associated with individual TATA box-binding protein-associated factors. J Biol Chem. 275**,** 29847-56.

Gangloff, Y. G., Romier, C., Thuault, S., Werten, S., Davidson, I., 2001. The histone fold is a key structural motif of transcription factor TFIID. Trends Biochem Sci. 26**,** 250-7.

Gangloff, Y. G., Werten, S., Romier, C., Carre, L., Poch, O., Moras, D., Davidson, I., 2000. The human TFIID components TAF(II)135 and TAF(II)20 and the yeast SAGA components ADA1 and TAF(II)68 heterodimerize to form histone-like pairs. Mol Cell Biol. 20**,** 340-51.

Ghosh, D., Seydoux, G., 2008. Inhibition of transcription by the Caenorhabditis elegans germline protein PIE-1: genetic evidence for distinct mechanisms targeting initiation and elongation. Genetics. 178**,** 235-43.

Goldstein, B., Hird, S. N., 1996. Specification of the anteroposterior axis in Caenorhabditis elegans. Development. 122**,** 1467-74.

Goldstein, B., Macara, I. G., 2007. The PAR proteins: fundamental players in animal cell polarization. Dev Cell. 13**,** 609-22.

Gomes, J. E., Encalada, S. E., Swan, K. A., Shelton, C. A., Carter, J. C., Bowerman, B., 2001. The maternal gene spn-4 encodes a predicted RRM protein required for mitotic spindle orientation and cell fate patterning in early C. elegans embryos. Development. 128**,** 4301-14.

Greenstein, D., 2005. Control of oocyte meiotic maturation and fertilization. WormBook. 1-12.

Grunstein, M., 1997. Histone acetylation in chromatin structure and transcription. Nature. 389**,** 349-52.

Guedes, S., Priess, J. R., 1997. The C. elegans MEX-1 protein is present in germline blastomeres and is a P granule component. Development. 124**,** 731-9.

Guven-Ozkan, T., Nishi, Y., Robertson, S. M., Lin, R., 2008. Global transcriptional repression in C. elegans germline precursors by regulated sequestration of TAF-4. Cell. 135**,** 149-60.

Hansen, D., Wilson-Berry, L., Dang, T., Schedl, T., 2004. Control of the proliferation versus meiotic development decision in the C. elegans germline through regulation of GLD-1 protein accumulation. Development. 131**,** 93-104.

Hanyu-Nakamura, K., Sonobe-Nojima, H., Tanigawa, A., Lasko, P., Nakamura, A., 2008. Drosophila Pgc protein inhibits P-TEFb recruitment to chromatin in primordial germ cells. Nature. 451**,** 730-3.

Hao, Y., Boyd, L., Seydoux, G., 2006. Stabilization of cell polarity by the C. elegans RING protein PAR-2. Dev Cell. 10**,** 199-208.

Hayashi, K., de Sousa Lopes, S. M., Surani, M. A., 2007. Germ cell specification in mice. Science. 316, 394-6.

Hirose, Y., Ohkuma, Y., 2007. Phosphorylation of the C-terminal domain of RNA polymerase II plays central roles in the integrated events of eucaryotic gene expression. J Biochem. 141, 601-8.

Hirsh, D., Oppenheim, D., Klass, M., 1976. Development of the reproductive system of Caenorhabditis elegans. Dev Biol. 49, 200-19.

Hoffmann, A., Chiang, C. M., Oelgeschlager, T., Xie, X., Burley, S. K., Nakatani, Y., Roeder, R. G., 1996. A histone octamer-like structure within TFIID. Nature. 380, 356-9.

Hubbard, E. J., Greenstein, D., 2005. Introduction to the germ line. WormBook. 1-4.

Hunter, C. P., Kenyon, C., 1996. Spatial and temporal controls target pal-1 blastomere-specification activity to a single blastomere lineage in C. elegans embryos. Cell. 87, 217-26.

Jadhav, S., Rana, M., Subramaniam, K., 2008. Multiple maternal proteins coordinate to restrict the translation of C. elegans nanos-2 to primordial germ cells. Development. 135, 1803-12.

Jan, E., Motzny, C. K., Graves, L. E., Goodwin, E. B., 1999. The STAR protein, GLD-1, is a translational regulator of sexual identity in Caenorhabditis elegans. EMBO J. 18, 258-69.

Jones, A. R., Francis, R., Schedl, T., 1996. GLD-1, a cytoplasmic protein essential for oocyte differentiation, shows stage- and sex-specific expression during Caenorhabditis elegans germline development. Dev Biol. 180, 165-83.

Kadyk, L. C., Kimble, J., 1998. Genetic regulation of entry into meiosis in Caenorhabditis elegans. Development. 125, 1803-13.

Kallies, A., Nutt, S. L., 2007. Terminal differentiation of lymphocytes depends on Blimp-1. Curr Opin Immunol. 19, 156-62.

Kamath, R. S., Ahringer, J., 2003. Genome-wide RNAi screening in Caenorhabditis elegans. Methods. 30, 313-21.

Kamath, R. S., Fraser, A. G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., Welchman, D. P., Zipperlen, P., Ahringer, J., 2003. Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature. 421, 231-7.

Kelly, W. G., Schaner, C. E., Dernburg, A. F., Lee, M. H., Kim, S. K., Villeneuve, A. M., Reinke, V., 2002. X-chromosome silencing in the germline of C. elegans. Development. 129**,** 479-92.

Kelly, W. G., Xu, S., Montgomery, M. K., Fire, A., 1997. Distinct requirements for somatic and germline expression of a generally expressed Caernorhabditis elegans gene. Genetics. 146**,** 227-38.

Kim, E., Du, L., Bregman, D. B., Warren, S. L., 1997. Splicing factors associate with hyperphosphorylated RNA polymerase II in the absence of pre-mRNA. J Cell Biol. 136**,** 19-28.

Kimble, J., Crittenden, S. L., 2005. Germline proliferation and its control. WormBook. 1-14.

Kipreos, E. T., Lander, L. E., Wing, J. P., He, W. W., Hedgecock, E. M., 1996. cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family. Cell. 85**,** 829-39.

Klages, N., Strubin, M., 1995. Stimulation of RNA polymerase II transcription initiation by recruitment of TBP in vivo. Nature. 374**,** 822-3.

Komarnitsky, P., Cho, E. J., Buratowski, S., 2000. Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. Genes Dev. 14**,** 2452-60.

Kouzarides, T., 2002. Histone methylation in transcriptional control. Curr Opin Genet Dev. 12**,** 198-209.

Kuersten, S., Goodwin, E. B., 2003. The power of the 3' UTR: translational control and development. Nat Rev Genet. 4**,** 626-37.

Kurimoto, K., Yabuta, Y., Ohinata, Y., Shigeta, M., Yamanaka, K., Saitou, M., 2008. Complex genome-wide transcription dynamics orchestrated by Blimp1 for the specification of the germ cell lineage in mice. Genes Dev. 22**,** 1617-35.

Kurz, T., Pintard, L., Willis, J. H., Hamill, D. R., Gonczy, P., Peter, M., Bowerman, B., 2002. Cytoskeletal regulation by the Nedd8 ubiquitin-like protein modification pathway. Science. 295**,** 1294-8.

Larocque, D., Richard, S., 2005. QUAKING KH domain proteins as regulators of glial cell fate and myelination. RNA Biol. 2**,** 37-40.

Lawson, K. A., Dunn, N. R., Roelen, B. A., Zeinstra, L. M., Davis, A. M., Wright, C. V., Korving, J. P., Hogan, B. L., 1999. Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes Dev. 13**,** 424-36.

Lee, M. H., Schedl, T., 2001. Identification of in vivo mRNA targets of GLD-1, a maxi-KH motif containing protein required for C. elegans germ cell development. Genes Dev. 15**,** 2408-20.

Lee, M. H., Schedl, T., 2004. Translation repression by GLD-1 protects its mRNA targets from nonsense-mediated mRNA decay in C. elegans. Genes Dev. 18**,** 1047-59.

Lee, M. H., Schedl, T., 2006. RNA-binding proteins. WormBook. 1-13.

Lemon, B., Tjian, R., 2000. Orchestrated response: a symphony of transcription factors for gene control. Genes Dev. 14**,** 2551-69.

Lin, R., 2003. A gain-of-function mutation in oma-1, a C. elegans gene required for oocyte maturation, results in delayed degradation of maternal proteins and embryonic lethality. Dev Biol. 258**,** 226-39.

Liu, J., Vasudevan, S., Kipreos, E. T., 2004. CUL-2 and ZYG-11 promote meiotic anaphase II and the proper placement of the anterior-posterior axis in C. elegans. Development. 131**,** 3513-25.

Lukong, K. E., Richard, S., 2007. Targeting the RNA-binding protein Sam68 as a treatment for cancer? Future Oncol. 3**,** 539-44.

Lyczak, R., Gomes, J. E., Bowerman, B., 2002. Heads or tails: cell polarity and axis formation in the early Caenorhabditis elegans embryo. Dev Cell. 3**,** 157-66.

Mahowald, A. P., 2001. Assembly of the Drosophila germ plasm. Int Rev Cytol. 203**,** 187-213.

Mains, P. E., Kemphues, K. J., Sprunger, S. A., Sulston, I. A., Wood, W. B., 1990. Mutations affecting the meiotic and mitotic divisions of the early Caenorhabditis elegans embryo. Genetics. 126**,** 593-605.

Marin, V. A., Evans, T. C., 2003. Translational repression of a C. elegans Notch mRNA by the STAR/KH domain protein GLD-1. Development. 130**,** 2623-32.

Martinho, R. G., Kunwar, P. S., Casanova, J., Lehmann, R., 2004. A noncoding RNA is required for the repression of RNApolII-dependent transcription in primordial germ cells. Curr Biol. 14**,** 159-65.

Matsui, T., Segall, J., Weil, P. A., Roeder, R. G., 1980. Multiple factors required for accurate initiation of transcription by purified RNA polymerase II. J Biol Chem. 255**,** 11992-6.

McCarter, J., Bartlett, B., Dang, T., Schedl, T., 1999. On the control of oocyte meiotic maturation and ovulation in Caenorhabditis elegans. Dev Biol. 205**,** 111-28.

McNally, F. J., Vale, R. D., 1993. Identification of katanin, an ATPase that severs and disassembles stable microtubules. Cell. 75**,** 419-29.

Mello, C. C., Draper, B. W., Krause, M., Weintraub, H., Priess, J. R., 1992. The pie-1 and mex-1 genes and maternal control of blastomere identity in early C. elegans embryos. Cell. 70**,** 163-76.

Mello, C. C., Schubert, C., Draper, B., Zhang, W., Lobel, R., Priess, J. R., 1996. The PIE-1 protein and germline specification in C. elegans embryos. Nature. 382**,** 710-2.

Merritt, C., Rasoloson, D., Ko, D., Seydoux, G., 2008. 3' UTRs are the primary regulators of gene expression in the C. elegans germline. Curr Biol. 18**,** 1476-82.

Miller, M. A., Nguyen, V. Q., Lee, M. H., Kosinski, M., Schedl, T., Caprioli, R. M., Greenstein, D., 2001. A sperm cytoskeletal protein that signals oocyte meiotic maturation and ovulation. Science. 291**,** 2144-7.

Mootz, D., Ho, D. M., Hunter, C. P., 2004. The STAR/Maxi-KH domain protein GLD-1 mediates a developmental switch in the translational control of C. elegans PAL-1. Development. 131**,** 3263-72.

Munro, E., Nance, J., Priess, J. R., 2004. Cortical flows powered by asymmetrical contraction transport PAR proteins to establish and maintain anterior-posterior polarity in the early C. elegans embryo. Dev Cell. 7**,** 413-24.

Munro, E. M., 2006. PAR proteins and the cytoskeleton: a marriage of equals. Curr Opin Cell Biol. 18**,** 86-94.

Nakajima, N., Horikoshi, M., Roeder, R. G., 1988. Factors involved in specific transcription by mammalian RNA polymerase II: purification, genetic specificity, and TATA box-promoter interactions of TFIID. Mol Cell Biol. 8**,** 4028-40.

Nakamura, A., Amikura, R., Mukai, M., Kobayashi, S., Lasko, P. F., 1996. Requirement for a noncoding RNA in Drosophila polar granules for germ cell establishment. Science. 274**,** 2075-9.

Nakamura, A., Seydoux, G., 2008. Less is more: specification of the germline by transcriptional repression. Development. 135**,** 3817-27.

Nishi, Y., Lin, R., 2005. DYRK2 and GSK-3 phosphorylate and promote the timely degradation of OMA-1, a key regulator of the oocyte-to-embryo transition in C. elegans. Dev Biol. 288**,** 139-49.

Nishi, Y., Rogers, E., Robertson, S. M., Lin, R., 2008. Polo kinases regulate C. elegans embryonic polarity via binding to DYRK2-primed MEX-5 and MEX-6. Development. 135**,** 687-97.

Ogura, K., Kishimoto, N., Mitani, S., Gengyo-Ando, K., Kohara, Y., 2003. Translational control of maternal glp-1 mRNA by POS-1 and its interacting protein SPN-4 in Caenorhabditis elegans. Development. 130**,** 2495-503.

Ohinata, Y., Payer, B., O'Carroll, D., Ancelin, K., Ono, Y., Sano, M., Barton, S. C., Obukhanych, T., Nussenzweig, M., Tarakhovsky, A., Saitou, M., Surani, M. A., 2005. Blimp1 is a critical determinant of the germ cell lineage in mice. Nature. 436**,** 207-13.

Ostareck-Lederer, A., Ostareck, D. H., 2004. Control of mRNA translation and stability in haematopoietic cells: the function of hnRNPs K and E1/E2. Biol Cell. 96**,** 407-11.

Pagano, J. M., Farley, B. M., McCoig, L. M., Ryder, S. P., 2007. Molecular basis of RNA recognition by the embryonic polarity determinant MEX-5. J Biol Chem. 282**,** 8883-94.

Patturajan, M., Schulte, R. J., Sefton, B. M., Berezney, R., Vincent, M., Bensaude, O., Warren, S. L., Corden, J. L., 1998. Growth-related changes in phosphorylation of yeast RNA polymerase II. J Biol Chem. 273**,** 4689-94.

Pellettieri, J., Reinke, V., Kim, S. K., Seydoux, G., 2003. Coordinate activation of maternal protein degradation during the egg-to-embryo transition in C. elegans. Dev Cell. 5**,** 451-62.

Peterlin, B. M., Price, D. H., 2006. Controlling the elongation phase of transcription with P-TEFb. Mol Cell. 23**,** 297-305.

Peterson, C. L., Laniel, M. A., 2004. Histones and histone modifications. Curr Biol. 14**,** R546-51.

Peterson, M. G., Tanese, N., Pugh, B. F., Tjian, R., 1990. Functional domains and upstream activation properties of cloned human TATA binding protein. Science. 248**,** 1625-30.

Pintard, L., Kurz, T., Glaser, S., Willis, J. H., Peter, M., Bowerman, B., 2003. Neddylation and deneddylation of CUL-3 is required to target MEI-1/Katanin for degradation at the meiosis-to-mitosis transition in C. elegans. Curr Biol. 13**,** 911-21.

Pitt, J. N., Schisa, J. A., 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**,** 315-33.

Praitis, V., Casey, E., Collar, D., Austin, J., 2001. Creation of low-copy integrated transgenic lines in Caenorhabditis elegans. Genetics. 157**,** 1217-26.

Priess, J. R., Thomson, J. N., 1987. Cellular interactions in early C. elegans embryos. Cell. 48**,** 241-50.

Quintin, S., Mains, P. E., Zinke, A., Hyman, A. A., 2003. The mbk-2 kinase is required for inactivation of MEI-1/katanin in the one-cell Caenorhabditis elegans embryo. EMBO Rep. 4**,** 1175-81.

Raz, E., 2005. Germ cells: sex and repression in mice. Curr Biol. 15**,** R600-3.

Reese, K. J., Dunn, M. A., Waddle, J. A., Seydoux, G., 2000. Asymmetric segregation of PIE-1 in C. elegans is mediated by two complementary mechanisms that act through separate PIE-1 protein domains. Mol Cell. 6**,** 445-55.

Richter, J. D., Klann, E., 2009. Making synaptic plasticity and memory last: mechanisms of translational regulation. Genes Dev. 23**,** 1-11.

Rocheleau, C. E., Downs, W. D., Lin, R., Wittmann, C., Bei, Y., Cha, Y. H., Ali, M., Priess, J. R., Mello, C. C., 1997. Wnt signaling and an APC-related gene specify endoderm in early C. elegans embryos. Cell. 90**,** 707-16.

Ryder, S. P., Frater, L. A., Abramovitz, D. L., Goodwin, E. B., Williamson, J. R., 2004. RNA target specificity of the STAR/GSG domain post-transcriptional regulatory protein GLD-1. Nat Struct Mol Biol. 11**,** 20-8.

Saitou, M., Barton, S. C., Surani, M. A., 2002. A molecular programme for the specification of germ cell fate in mice. Nature. 418**,** 293-300.

Samuels, M., Fire, A., Sharp, P. A., 1982. Separation and characterization of factors mediating accurate transcription by RNA polymerase II. J Biol Chem. 257**,** 14419-27.

Schaner, C. E., Deshpande, G., Schedl, P. D., Kelly, W. G., 2003. A conserved chromatin architecture marks and maintains the restricted germ cell lineage in worms and flies. Dev Cell. 5**,** 747-57.

Schaner, C. E., Kelly, W. G., 2006. Germline chromatin. WormBook. 1-14.

Schauer, I. E., Wood, W. B., 1990. Early C. elegans embryos are transcriptionally active. Development. 110**,** 1303-17.

Schisa, J. A., Pitt, J. N., Priess, J. R., 2001. Analysis of RNA associated with P granules in germ cells of C. elegans adults. Development. 128**,** 1287-98.

Schubert, C. M., Lin, R., de Vries, C. J., Plasterk, R. H., Priess, J. R., 2000. MEX-5 and MEX-6 function to establish soma/germline asymmetry in early C. elegans embryos. Mol Cell. 5**,** 671-82.

Seki, Y., Yamaji, M., Yabuta, Y., Sano, M., Shigeta, M., Matsui, Y., Saga, Y., Tachibana, M., Shinkai, Y., Saitou, M., 2007. Cellular dynamics associated with the genome-wide epigenetic reprogramming in migrating primordial germ cells in mice. Development. 134**,** 2627-38.

Selleck, W., Howley, R., Fang, Q., Podolny, V., Fried, M. G., Buratowski, S., Tan, S., 2001. A histone fold TAF octamer within the yeast TFIID transcriptional coactivator. Nat Struct Biol. 8**,** 695-700.

Seydoux, G., Braun, R. E., 2006. Pathway to totipotency: lessons from germ cells. Cell. 127**,** 891-904.

Seydoux, G., 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**,** 2191-201.

Seydoux, G., Fire, A., 1994. Soma-germline asymmetry in the distributions of embryonic RNAs in Caenorhabditis elegans. Development. 120**,** 2823-34.

Seydoux, G., Fire, A., 1995. Whole-mount in situ hybridization for the detection of RNA in Caenorhabditis elegans embryos. Methods Cell Biol. 48**,** 323-37.

Seydoux, G., Mello, C. C., Pettitt, J., Wood, W. B., Priess, J. R., Fire, A., 1996. Repression of gene expression in the embryonic germ lineage of C. elegans. Nature. 382**,** 713-6.

Seydoux, G., Strome, S., 1999. Launching the germline in Caenorhabditis elegans: regulation of gene expression in early germ cells. Development. 126**,** 3275-83.

Shaffer, A. L., Lin, K. I., Kuo, T. C., Yu, X., Hurt, E. M., Rosenwald, A., Giltnane, J. M., Yang, L., Zhao, H., Calame, K., Staudt, L. M., 2002. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. Immunity. 17**,** 51-62.

Shim, E. Y., Walker, A. K., Shi, Y., Blackwell, T. K., 2002. CDK-9/cyclin T (P-TEFb) is required in two postinitiation pathways for transcription in the C. elegans embryo. Genes Dev. 16**,** 2135-46.

Shimada, M., Yokosawa, H., Kawahara, H., 2006. OMA-1 is a P granules-associated protein that is required for germline specification in Caenorhabditis elegans embryos. Genes Cells. 11**,** 383-96.

Shimohata, T., Nakajima, T., Yamada, M., Uchida, C., Onodera, O., Naruse, S., Kimura, T., Koide, R., Nozaki, K., Sano, Y., Ishiguro, H., Sakoe, K., Ooshima, T., Sato, A., Ikeuchi, T., Oyake, M., Sato, T., Aoyagi, Y., Hozumi, I., Nagatsu, T., Takiyama, Y., Nishizawa, M., Goto, J., Kanazawa, I., Davidson, I., Tanese, N.,

Takahashi, H., Tsuji, S., 2000. Expanded polyglutamine stretches interact with TAFII130, interfering with CREB-dependent transcription. Nat Genet. 26**,** 29-36.

Shimohata, T., Onodera, O., Tsuji, S., 2001. Expanded polyglutamine stretches lead to aberrant transcriptional regulation in polyglutamine diseases. Hum Cell. 14**,** 17-25.

Shirayama, M., Soto, M. C., Ishidate, T., Kim, S., Nakamura, K., Bei, Y., van den Heuvel, S., Mello, C. C., 2006. The Conserved Kinases CDK-1, GSK-3, KIN-19, and MBK-2 Promote OMA-1 Destruction to Regulate the Oocyte-to-Embryo Transition in C. elegans. Curr Biol. 16**,** 47-55.

Siomi, H., Choi, M., Siomi, M. C., Nussbaum, R. L., Dreyfuss, G., 1994. Essential role for KH domains in RNA binding: impaired RNA binding by a mutation in the KH domain of FMR1 that causes fragile X syndrome. Cell. 77**,** 33-9.

Siomi, H., Siomi, M. C., Nussbaum, R. L., Dreyfuss, G., 1993. The protein product of the fragile X gene, FMR1, has characteristics of an RNA-binding protein. Cell. 74**,** 291-8.

Soutoglou, E., Demeny, M. A., Scheer, E., Fienga, G., Sassone-Corsi, P., Tora, L., 2005. The nuclear import of TAF10 is regulated by one of its three histone fold domain-containing interaction partners. Mol Cell Biol. 25**,** 4092-104.

Srayko, M., Buster, D. W., Bazirgan, O. A., McNally, F. J., Mains, P. E., 2000. MEI-1/MEI-2 katanin-like microtubule severing activity is required for Caenorhabditis elegans meiosis. Genes Dev. 14**,** 1072-84.

Stitzel, M. L., Pellettieri, J., Seydoux, G., 2006. The C. elegans DYRK Kinase MBK-2 Marks Oocyte Proteins for Degradation in Response to Meiotic Maturation. Curr Biol. 16**,** 56-62.

Strome, S., Wood, W. B., 1982. Immunofluorescence visualization of germ-line-specific cytoplasmic granules in embryos, larvae, and adults of Caenorhabditis elegans. Proc Natl Acad Sci U S A. 79**,** 1558-62.

Subramaniam, K., Seydoux, G., 1999. nos-1 and nos-2, two genes related to Drosophila nanos, regulate primordial germ cell development and survival in Caenorhabditis elegans. Development. 126**,** 4861-71.

Sulston, J. E., Schierenberg, E., White, J. G., Thomson, J. N., 1983. The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev Biol. 100**,** 64-119.

Surani, M. A., 2007. Germ cells: the eternal link between generations. C R Biol. 330**,** 474-8.

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

Tam, P. P., 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**,** 124-32.

Tanaka, T., Nakamura, A., 2008. The endocytic pathway acts downstream of Oskar in Drosophila germ plasm assembly. Development. 135**,** 1107-17.

Tanaka, T. S., Lopez de Silanes, I., Sharova, L. V., Akutsu, H., Yoshikawa, T., Amano, H., Yamanaka, S., Gorospe, M., Ko, M. S., 2006. Esg1, expressed exclusively in preimplantation embryos, germline, and embryonic stem cells, is a putative RNA-binding protein with broad RNA targets. Dev Growth Differ. 48**,** 381-90.

Tenenhaus, C., Schubert, C., Seydoux, G., 1998. Genetic requirements for PIE-1 localization and inhibition of gene expression in the embryonic germ lineage of Caenorhabditis elegans. Dev Biol. 200**,** 212-24.

Tenenhaus, C., Subramaniam, K., Dunn, M. A., 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**,** 1031-40.

Tenlen, J. R., Molk, J. N., London, N., Page, B. D., Priess, J. R., 2008. MEX-5 asymmetry in one-cell C. elegans embryos requires PAR-4- and PAR-1-dependent phosphorylation. Development. 135**,** 3665-75.

Timinszky, G., Bortfeld, M., Ladurner, A. G., 2008. Repression of RNA polymerase II transcription by a Drosophila oligopeptide. PLoS ONE. 3**,** e2506.

Timmons, L., Fire, A., 1998. Specific interference by ingested dsRNA. Nature. 395**,** 854.

Turner, C. A., Jr., Mack, D. H., Davis, M. M., 1994. Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. Cell. 77**,** 297-306.

Varnum, B. C., Ma, Q. F., Chi, T. H., Fletcher, B., Herschman, H. R., 1991. The TIS11 primary response gene is a member of a gene family that encodes proteins with a highly conserved sequence containing an unusual Cys-His repeat. Mol Cell Biol. 11**,** 1754-8.

Vincent, S. D., Dunn, N. R., Sciammas, R., Shapiro-Shalef, M., Davis, M. M., Calame, K., Bikoff, E. K., Robertson, E. J., 2005. The zinc finger transcriptional repressor Blimp1/Prdm1 is dispensable for early axis formation but is required for specification of primordial germ cells in the mouse. Development. 132**,** 1315-25.

Walker, A. K., Blackwell, T. K., 2003. A broad but restricted requirement for TAF-5 (human TAFII100) for embryonic transcription in Caenorhabditis elegans. J Biol Chem. 278**,** 6181-6.

Walker, A. K., Boag, P. R., Blackwell, T. K., 2007. Transcription reactivation steps stimulated by oocyte maturation in C. elegans. Dev Biol. 304**,** 382-93.

Walker, A. K., Rothman, J. H., Shi, Y., Blackwell, T. K., 2001. Distinct requirements for C.elegans TAF(II)s in early embryonic transcription. EMBO J. 20**,** 5269-79.

Walker, A. K., Shi, Y., Blackwell, T. K., 2004. An extensive requirement for transcription factor IID-specific TAF-1 in Caenorhabditis elegans embryonic transcription. J Biol Chem. 279**,** 15339-47.

Wallenfang, M. R., Seydoux, G., 2000. Polarization of the anterior-posterior axis of C. elegans is a microtubule-directed process. Nature. 408**,** 89-92.

Ward, S., Carrel, J. S., 1979. Fertilization and sperm competition in the nematode Caenorhabditis elegans. Dev Biol. 73**,** 304-21.

Werten, S., Mitschler, A., Romier, C., Gangloff, Y. G., Thuault, S., Davidson, I., Moras, D., 2002. Crystal structure of a subcomplex of human transcription factor TFIID formed by TATA binding protein-associated factors hTAF4 (hTAF(II)135) and hTAF12 (hTAF(II)20). J Biol Chem. 277**,** 45502-9.

Wilhelm, J. E., Smibert, C. A., 2005. Mechanisms of translational regulation in Drosophila. Biol Cell. 97**,** 235-52.

Wright, K. J., Marr, M. T., 2nd, Tjian, R., 2006. TAF4 nucleates a core subcomplex of TFIID and mediates activated transcription from a TATA-less promoter. Proc Natl Acad Sci U S A. 103**,** 12347-52.

Xie, X., Kokubo, T., Cohen, S. L., Mirza, U. A., Hoffmann, A., Chait, B. T., Roeder, R. G., Nakatani, Y., Burley, S. K., 1996. Structural similarity between TAFs and the heterotetrameric core of the histone octamer. Nature. 380**,** 316-22.

Ying, Y., Liu, X. M., Marble, A., Lawson, K. A., Zhao, G. Q., 2000. Requirement of Bmp8b for the generation of primordial germ cells in the mouse. Mol Endocrinol. 14**,** 1053-63.

Ying, Y., Qi, X., Zhao, G. Q., 2001. Induction of primordial germ cells from murine epiblasts by synergistic action of BMP4 and BMP8B signaling pathways. Proc Natl Acad Sci U S A. 98**,** 7858-62.

Ying, Y., Zhao, G. Q., 2001. Cooperation of endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 in primordial germ cell generation in the mouse. Dev Biol. 232**,** 484-92.

Zalokar, M., 1976. Autoradiographic study of protein and RNA formation during early development of Drosophila eggs. Dev Biol. 49**,** 425-37.

Zhang, F., Barboric, M., Blackwell, T. K., Peterlin, B. M., 2003. A model of repression: CTD analogs and PIE-1 inhibit transcriptional elongation by P-TEFb. Genes Dev. 17**,** 748-58.