

POST-TRANSCRIPTIONAL REGULATION OF MATERNAL mRNA SHAPES  
EARLY *C. ELEGANS* EMBRYOGENESIS

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## DEDICATION

This dissertation is dedicated to my parents Cornelus Oldenbroek and Eleonora Oldenbroek and to my caring and loving husband Zebulon Burleson who were a continuous inspiration and motivation throughout my studies.

STUDY OF MATERNAL mRNA REGULATION DURING EARLY *C. ELEGANS*  
EMBRYOGENESIS

by

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by

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STUDY OF MATERNAL mRNA REGULATION DURING EARLY *C. ELEGANS*  
EMBRYOGENESIS

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Much of early embryogenesis is controlled through complex networks comprised of maternally provided factors. Oocytes are packed with protein and RNA that are ready to spring into action after fertilization to guide early embryonic development. The regulation of maternally provided factors is therefore critical and is a fundamental goal of developmental biology. During my studies, I investigated how two maternally provided mRNAs, *zif-1* and *mom-2*, are regulated post-transcriptionally through their 3' untranslated region (3' UTR) to ensure proper spatio-temporal protein expression. I discovered that seven RNA binding proteins bind directly to the *zif-1* 3' UTR in a combinatorial fashion thereby ensuring that *zif-1* is only translated in somatic

blastomeres, beginning at the four cell stage embryo. Interestingly a similar set of RNA binding proteins (nine total) regulate the spatio-temporal expression of *mom-2* in a similar fashion despite the fact that *mom-2* has a reciprocal expression pattern when compared to *zif-1*. My studies on *zif-1* and *mom-2* regulation indicate that a “code” is embedded within the 3’ UTR of mRNAs to mediate translational regulation. The precise combination of RNA binding proteins present in a particular cell at a particular time, each with the intrinsic capability of binding to regulatory sequences contained in this “code”, determines when and where mRNAs get translated.

I also investigated mechanisms by which maternal mRNAs get degraded. Zygotic transcription activation is often linked to maternal mRNA degradation, which I showed to be the case in *C. elegans* embryos. Specifically, I discovered a gene termed *vet-5* that is first transcribed in the somatic blastomeres of the four-cell embryo and is sufficient to degrade at least several maternal mRNAs when provided exogenously as dsRNA. *vet-5* maps to a highly repetitive locus and has been shown to be a target of siRNA production. Consistent with *vet-5* derived siRNA production I found that the siRNA pathway is, at least partly, required for the degradation of maternal mRNAs and that removing components of the siRNA pathway affects *vet-5* expression. Therefore, I hypothesize that siRNAs could be produced from the *vet-5* locus that target maternally provided mRNAs for degradation.

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## LIST OF ABBREVIATIONS

ZP – Zona Pellucida

SPE – Defective Spermatogenesis

MTOC – Microtubule Organizing Center

3' UTR – 3' Untranslated Region

FBF – Fem-3 mRNA Binding Factor

GLD – Defective in Germline Development

NOS – Nanos Related

MAPK – Mitogen Activated Protein Kinase

MSP – Major Sperm Protein

GPCR – G Protein Coupled Receptor

PGC – Primordial Germ Cell

OMA – Oocyte Maturation Defective

PIE – Pharynx and Intestine in Excess

MES – Maternal Effect Sterile

RBP – RNA Binding Protein

CUL - Cullin

PTEFb – Positive Transcription Elongation Factor b

CTD – C Terminal Domain

RNAPII – RNA Polymerase II

DYRK - Dual-specificity YAK1 Related Kinase

TAF – TATA-binding protein associated Factor

HFD – Histone Fold Domain

DTC – Distal Tip Cell

GLP – Abnormal Germline Proliferation

PAR – Abnormal Partitioning of Embryonic Cytoplasm

PKC – Protein Kinase C

ZIF – Zinc Finger Interacting protein

MZT – Maternal to Zygotic Transition

AMA – Amanatin resistant

MEX – Muscle Excess

SPN – Spindle Orientation Defective

POS – Posterior Segregation

GFP – Green Fluorescent Protein

HSC – Hedgehog Signaling Complex

APC – Activated Protein C

GSK - Glycogen Synthase Kinase

MOM – More Of Mesoderm

RISC – RNA Induced Silencing Complex

TRAMP - Trf4/Air2/Mtr4p Polyadenylation complex

ERGO – Endogenous RNAi deficient arGO naute

ERI – Enhanced RNAi

DCR – Dicer Related

DRH – Dicer Related Helicase

RdRP – RNA dependent RNA Polymerase

MBP – Maltose Binding Protein

DIC – Differential Interference Contrast

EMSA – Electrophoretic Mobility Shift Essay

ZF- Zinc Finger

smFISH – Single Molecule Fluorescent In Situ Hybridization

RNP - Ribonucleoprotein

RNAi – RNA interference

SCC – Saline Sodium Citrate

IPTG - Isopropylthio- $\beta$ -Galactoside



## **CHAPTER 1: INTRODUCTION**

### **I. From oocyte to embryo**

The creation of a new individual is remarkably complex and orchestrated by numerous tightly regulated molecular events. Although the precise mechanisms underlying these events vary from species to species, the main processes that occur are universal. The creation of life begins with precursor germ cells from the mother and father that undergo differentiation and maturation to generate haploid gametes termed oocytes (also known as eggs) and sperm respectively. When haploid gametes come into contact with one another, membrane fusion occurs during a process termed fertilization, after which embryonic development begins. Maturation of the oocytes and subsequent fertilization is collectively known as the oocyte-to-embryo transition. A unique feature about the oocyte-to-embryo transition is that oocytes are maintained in a relatively dormant state, whereas embryos are developmentally active and rapidly dividing. Therefore, the mechanisms that orchestrate this event must be very tightly regulated. In the following few sections I will highlight some of the processes that occur during oocyte maturation and fertilization.

#### **1. Fertilization**

The union of two gametes, an oocyte from the mother and sperm from the father, is the hallmark of fertilization. During this process genetic information from both parents is combined to generate a zygote and development of a new individual is initiated. Once

gametes come into contact with one another, fertilization is tightly regulated in a temporal manner. It is well established in vertebrates that oocytes and sperm are incapable of surviving long on their own; therefore fertilization must occur rapidly once they enter the reproductive tract in order to prevent degeneration of the individual gametes. Many key events regulate the process of fertilization to ensure correct fusion of the gametes and eventually development of the zygote.

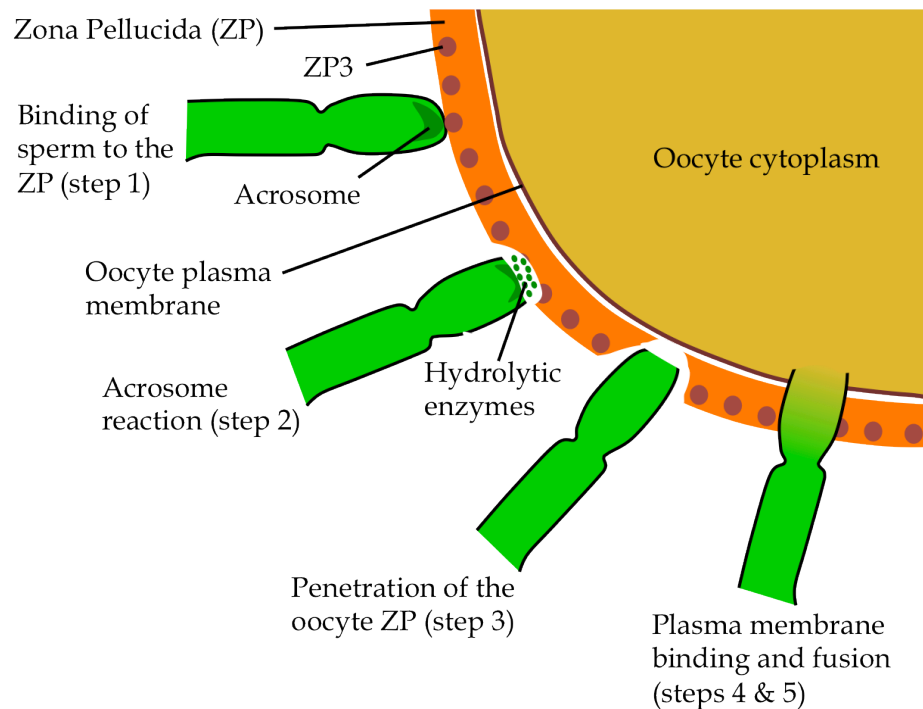
### **1.1 Vertebrate fertilization**

In vertebrates, an ovulated oocyte is derived from the XX female and sperm from the XY male. Very few ovulated oocytes are generally detected in the female oviduct and relatively few sperm, when compared to the total sperm deposited, is found at the site of fertilization. Once an oocyte and sperm get into contact with one another five precisely timed events will take place in an orchestrated manner to complete fertilization and give rise to the zygote.

The first event in vertebrate fertilization is the species-specific binding of sperm to the extracellular coat of the oocyte, also known as the zona pellucida (ZP) (Figure 1.1). Although there are examples of hybrid animals, such as the mule, which is comprised of one gamete from a horse and one gamete from a donkey, it is generally well documented that the binding of sperm to the ZP of a different species does not occur *in vitro*. Species specificity arises due to the expression of specific sperm receptors in oocytes and oocyte binding proteins in sperm. The mammalian ZP is not particularly complex and is comprised of polysaccharides and only three main glycoproteins, ZP1, ZP2, and ZP3 (Wassarman 1988; Dietl 1989). These three glycoproteins account for nearly all of the

extra-cellular mass of the ZP (Bleil and Wassarman 1980). ZP3, through several lines of evidence, has been designated as the sperm receptor (Wassarman 1990; Wassarman 1999). Sperm can recognize and bind to specific O-linked oligosaccharides near the carboxy terminus of ZP3 (Kinloch et al. 1995; Chen et al. 1998). It is therefore believed that the structure of the ZP3 oligosaccharides account, at least in part, for the species-specific binding of sperm to oocytes. Many different sperm-specific oocyte-binding proteins have also been discovered that are implicated in the species-specific binding of sperm to oocytes (Snell et al. 1996; McLeskey 1998; Wassarman 1999)

The second event is the acrosome reaction (Figure 1.1). The acrosome is a relatively large organelle present in the anterior part of the sperm head. The plasma membrane of the acrosome is capable of binding to thousands of copies of ZP3 in the ZP (Bleil et al. 1983; Florman et al. 1999; O'toole et al. 2000). Upon binding to ZP3, the membrane surrounding the sperm acrosome will fuse with the oocyte ZP, leading to formation of hybrid membrane vesicles and exposure of the contents within the acrosome (Yanagimachi et al. 1994). These contents include numerous enzymes that are responsible for breaking through the oocyte ZP leading to the third event of fertilization: penetration of the oocyte ZP by sperm (Figure 1.1). The penetration by sperm is achieved through a combination of sperm motility and enzymatic hydrolysis (Yanagimachi et al. 1994; Bedford et al. 1998). After the sperm have penetrated the ZP, they must bind to the plasma membrane of the oocyte (the fourth event) and finally fuse with it (the fifth event) (Figure 1.1). Both of these final steps of fertilization require several transmembrane proteins on sperm and integrin  $\alpha 6 \beta 1$  receptors on oocytes (Snell et al. 1996; Wassarman 1999; McLeskey et al. 1998)



**Figure 1.1: Mammalian fertilization.**

Cartoon illustrating the five steps of mammalian fertilization. Green structures are sperm and orange structure is an oocyte. The first step is binding of sperm to the ZP, followed by the acrosome reaction (step 2) leading to exposure hydrolytic enzymes. Finally the sperm penetrates the oocyte ZP (step 3) followed by plasma membrane binding (step 4) and fusion (step 5).

## 1.2 The self-fertilizing *C. elegans* hermaphrodite

Although *Caenorhabditis elegans* (*C. elegans*), similarly to mammals, contains two different sexes, they have some very significant differences. The two sexes in *C. elegans* are comprised of males and self-fertilizing hermaphrodites. The hermaphrodite condition is a result of modifications of the basic male/female sex-determination system, which permits both spermatogenesis and oogenesis to occur within the female germline. Unlike most species where the primary sex-determining signal is the presence of a

dominant sex chromosome, like the Y chromosome in mammals, sex determination in *C. elegans* is regulated by X chromosome dosage. Diploid animals with two X chromosomes, XX, are hermaphrodites whereas diploid animals with only one X chromosome, XO, are males. In addition, it has been shown that X chromosome ratio determines sex over absolute number of X chromosomes; tetraploid animals with three X chromosomes are female, whereas tetraploid animals with only two X chromosomes are male (Madl and Herman 1979). XO Males arise spontaneously at a low frequency (0.1%) due to non-disjunction in the hermaphrodite germline and at higher frequency as a result of male/hermaphrodite matings.

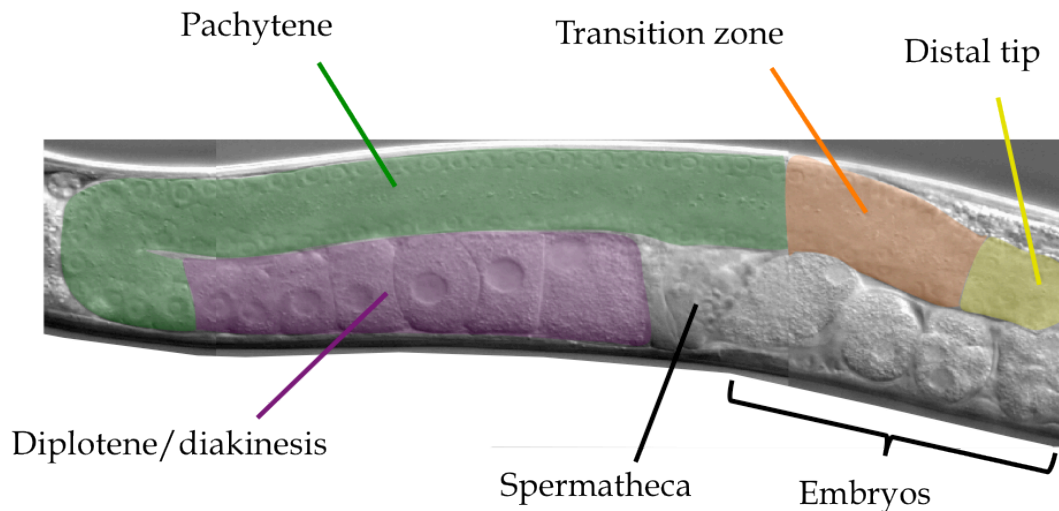
The process of fertilization in *C. elegans* hermaphrodites occurs at the spermatheca where sperm is produced and stored (Figure 1.2). Contractions of the oviduct force an oocyte into the spermatheca leading to membrane fusion of a single sperm with the oocyte (ward and carrel 1979). Although it is well studied in *C. elegans* that a vast majority of factors required for early embryogenesis are maternally deposited into the oocyte (will be discussed later), sperm has also been shown to have several essential roles in the embryo.

First of all, embryos are incapable of completing meiosis in the absence of a sperm-supplied factor called SPE-11. Embryogenesis is compromised when the *spe-11* gene is mutated, even though the production of sperm is not affected (hill et al. 1989). Secondly, centrosomes in *C. elegans* are paternally inherited. Oocyte meiotic spindles are incapable of functioning as microtubule organizing centers (MTOCs) due the lack of centrioles (Albertson and Thomson 1993). Therefore, in the absence of sperm-supplied centrosomes, mitosis cannot occur (Albertson 1984). Finally, an unfertilized *C. elegans*

oocyte has no predetermined axis. The site of sperm entry establishes polarity of the future embryo (Goldstein and Hird 1996). Sperm enters the side of the oocyte that first penetrates the spermatheca, which will become the posterior end of the embryo. The oocyte nucleus resides on the opposite end of the fertilized oocyte thereby marking it to become the anterior end of the future embryo.

## 2. Differentiation and maturation of oocytes in *C. elegans*

Oocyte maturation appears in an assembly like fashion in the *C. elegans* germline. The *C. elegans* hermaphrodite contains a pair of gonadal arms where the oocytes reside, develop and differentiate. The gonad arms can be divided into four distinct regions: the mitotically dividing distal tip, the transition zone, the pachytene zone, and the diplotene/diakinesis zone (Figure 1.2).



**Figure 1.2: One *C. elegans* gonad arm**

The distal tip contains the mitotic germline stem cells and is in the most distal region of the gonad, followed by the transition zone where alignment of homolog occurs during meiosis I. In the pachytene region homologs recombine and progress further into diplotene/diakinesis of prophase I until they arrest at metaphase II. Metaphase arrest is lifted at the spermatheca where oocytes are fertilized by sperm allowing them to complete meiosis II and begin embryonic mitotic division.

Oocytes are initially derived from mitotically dividing proliferating germline stem cells in the distal tip of the gonad arm. Two nearly identical proteins, FBF-1 and FBF-2 (FBF) are required to keep these cells in a mitotically dividing state (Crittenden et al. 2002). Whereas four additional key regulators, GLD-1, GLD-2, GLD-3, and NOS-3, are required to promote entry into meiosis (Kadyk and Kimble, 1998; Eckmann et al. 2004; Hansen et al. 2004; Hansen et al. 2004). The details of these events will be described later. Upon entry into meiosis I at the transition zone, homologous chromosomes will partially condense, pair through a synaptonemal complex, and align in a side-by-side configuration (Kleckner 1996). The alignment of homologs will be completed before the pachytene stage (Goldstein and Slaton 1982). During the pachytene stage the homologs recombine before progressing further into the diplotene/diakinesis stage. The transition from pachytene to diplotene/diakinesis requires the RAS/MAPK signaling pathway (Church et al. 1995).

MAPK activation requires secretion of a hormone termed major sperm protein (MSP) (Miller et al. 2001; McCarter et al. 1999). Without MSP oocytes arrest at the pachytene stage during meiotic prophase I. Meiotic oocyte arrest is also observed in mammals where it has been shown that a surge of luteinizing hormone from the pituitary is required for oocytes to progress past meiotic prophase I (Peng et al. 1991; Epigg et al.

1997). *C. elegans* sperm secrete MSP through a vesicle budding mechanism to generate an extra-cellular gradient. Oocytes sense MSP signal through an MSP/EPH receptor and several G-protein-coupled receptors (GPCRs) (Miller et al. 2003; Govindan et al. 2006; Cheng et al. 2008). Upon reception of MSP signal and subsequent activation of MAPK, prophase I cell cycle arrest is lifted and oocytes will proceed into the diakinesis stage of maturation.

The diakinesis-stage oocytes continue to develop in the proximal gonad arm where muscle-like cells, termed gonadal sheath cells, aid in their maturation (McCarter et al. 1997; Hall et al. 1999; Miller et al. 2003). During the diakinesis stage nuclear envelope breakdown, rearrangement of the cortical cytoskeleton, and meiotic spindle assembly takes place. In the absence of sperm, oocytes will eventually arrest again at metaphase of meiosis II. Metaphase arrest is lifted upon fertilization thereby allowing for the completion of meiosis II and the start of embryonic mitotic division (Mehlmann 2005).

## **II. Germline establishment in *C. elegans***

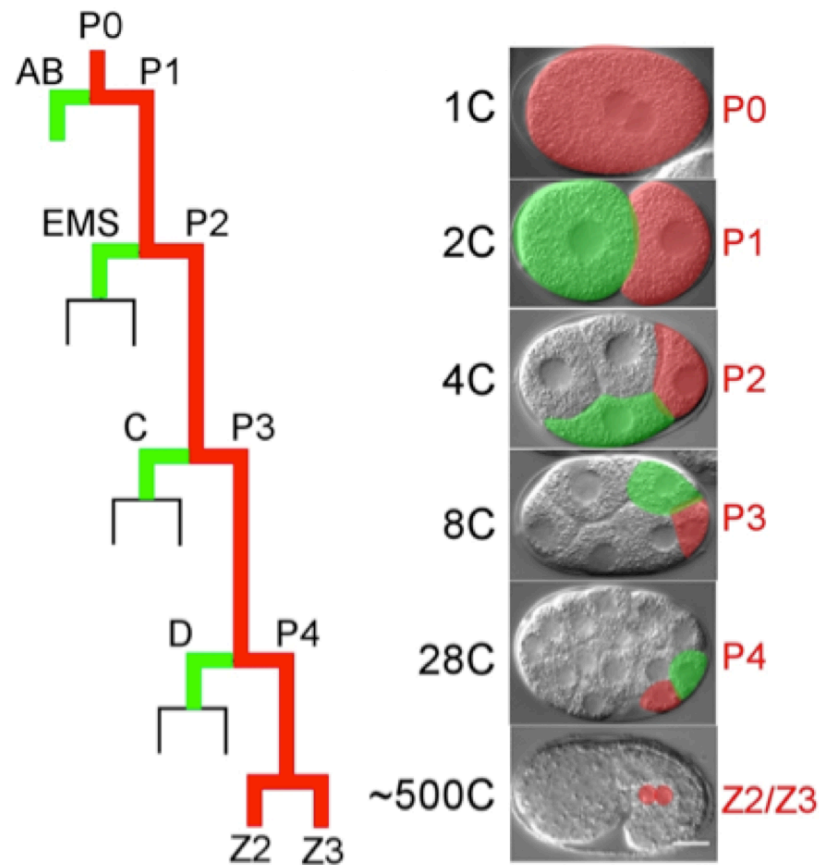
The entire *C. elegans* germline is derived from one germline precursor cell in the early embryo that gives rise to two primordial germ cells (PGCs). The two PGCs cease cell division and begin proliferating after the embryo hatches thereby generating a pool of germline stem cells (reviewed in Seydoux and Strome 1999). Germline stem cells remain restricted to mitotic proliferation, as supported by a somatic gonadal niche, during early larval stages. During later larval stages and in the adult animal, the germline stem cells leave the niche and begin differentiation to eventually form the mature gametes. The



germline stem cell niche also aids to establish polarity within the maturing germ line as the mitotically dividing germline stem cells reside in the most distal end and maturing meiotic oocytes localize to the proximal end of the gonad. The process from embryonic PGC to mature gamete is under tight regulation through the action of many different factors and pathways, some of which I will discuss in the next few sections.

## **1. Germline Specification**

Upon fertilization of an oocyte, the newly formed zygote, termed P0, will rapidly undergo four asymmetric division to give rise the sole germline precursor, P4 (Strome 2005) (Figure 1.3). Each division gives rise to a smaller germline blastomere and a larger somatic sister blastomere. The germline blastomeres P0-P4 are termed the P-lineage and remain transcriptionally repressed to prevent differentiation into somatic tissues. The somatic sister blastomeres, on the other hand, undergo rapid transcriptional activation and lineage-specific differentiation. Precise mechanisms operate throughout early development to rapidly reverse the transcriptional repressive effect, observed in the P-lineage, in the somatic blastomeres upon each cell division (Seydoux et al. 1996; Seydoux and Dunn 1997). Although the germline is transcriptionally repressed, the P-lineage contains epigenetic marks that are characteristic of transcriptional competent chromatin (Schaner et al. 2003). Transcriptional repression in the P-lineage is achieved through the action of two groups of proteins: OMA-1 and OMA-2 are required in P0 and P1, whereas PIE-1 is required in P2-P4 (Figure 1.4).



**Figure 1.3: Specification of the *C. elegans* germline (figure adapted from Guven-Ozkan et al 2008).**

On the left are the four asymmetric divisions beginning with the 1-cell embryo (P0) to give rise to the two PGCs Z2 and Z3. The germline precursors P0 to P4 are annotated in red and their somatic sisters in green. On the right are differential interference contrast (DIC) images. Embryos are oriented with the anterior pole to the left. The scale bar represents 10  $\mu\text{m}$ .

### 1.1 Regulation of chromatin in the P-lineage

Screens for maternal-effect sterile mutants carried out over 20 years ago revealed a group of genes called the *mes* genes. Hermaphrodites homozygous for *mes* mutations produce offspring that show germline degeneration midway through larval development

(Capowski et al. 1991). *mes* genes operate in the maternal germ line and the early embryonic P-lineage and are believed to regulate patterns of gene expression in the germline descendants. MES-2, MES-3, and MES-6 form a complex whereas MES-4 functions alone (Xu et al. 2001). MES-2 and MES-4 both contain SET domains, which have been associated with methylating histone tails (Holdeman et al. 1998; Bender et al. 2004). MES-4 is responsible for dimethylating H3K36 and MES-2 trimethylates H3K27 (Bender et al. 2004; Cao and Zhang 2004). Both of these epigenetic modifications are repressive marks thereby causing the chromatin to be organized into a silenced state. This is consistent with the observation that germline blastomeres are transcriptionally competent but are actively being restrained from differentiation-promoting transcription.

## **1.2 Transcriptional repression in P2-P4 by PIE-1**

PIE-1 is a CCCH-type zinc finger RNA binding protein (RBP) and has been the paradigm for global transcriptional repression in the germline precursors P2-P4 (Figure 1.4). PIE-1 is ubiquitously expressed in maturing oocytes and the 1-cell embryo, which is also the first germline precursor blastomere P0 (Mello et al. 1996). Upon each cell division PIE-1 becomes enriched in the germline blastomere through at least two mechanisms: enrichment in the cytoplasm that will become the germline daughter blastomere prior to division, and rapid degradation in the somatic daughter blastomere after division (Reese et al. 2000; DeRenzo et al. 2003). Degradation of PIE-1 is accomplished by a CUL-2 containing E3 ligase, which is targeted to PIE-1 through one of its subunits, ZIF-1. Upon each cell division, PIE-1 becomes localized primarily to the nucleus where it represses transcriptional initiation and elongation (Seydoux and Dunn

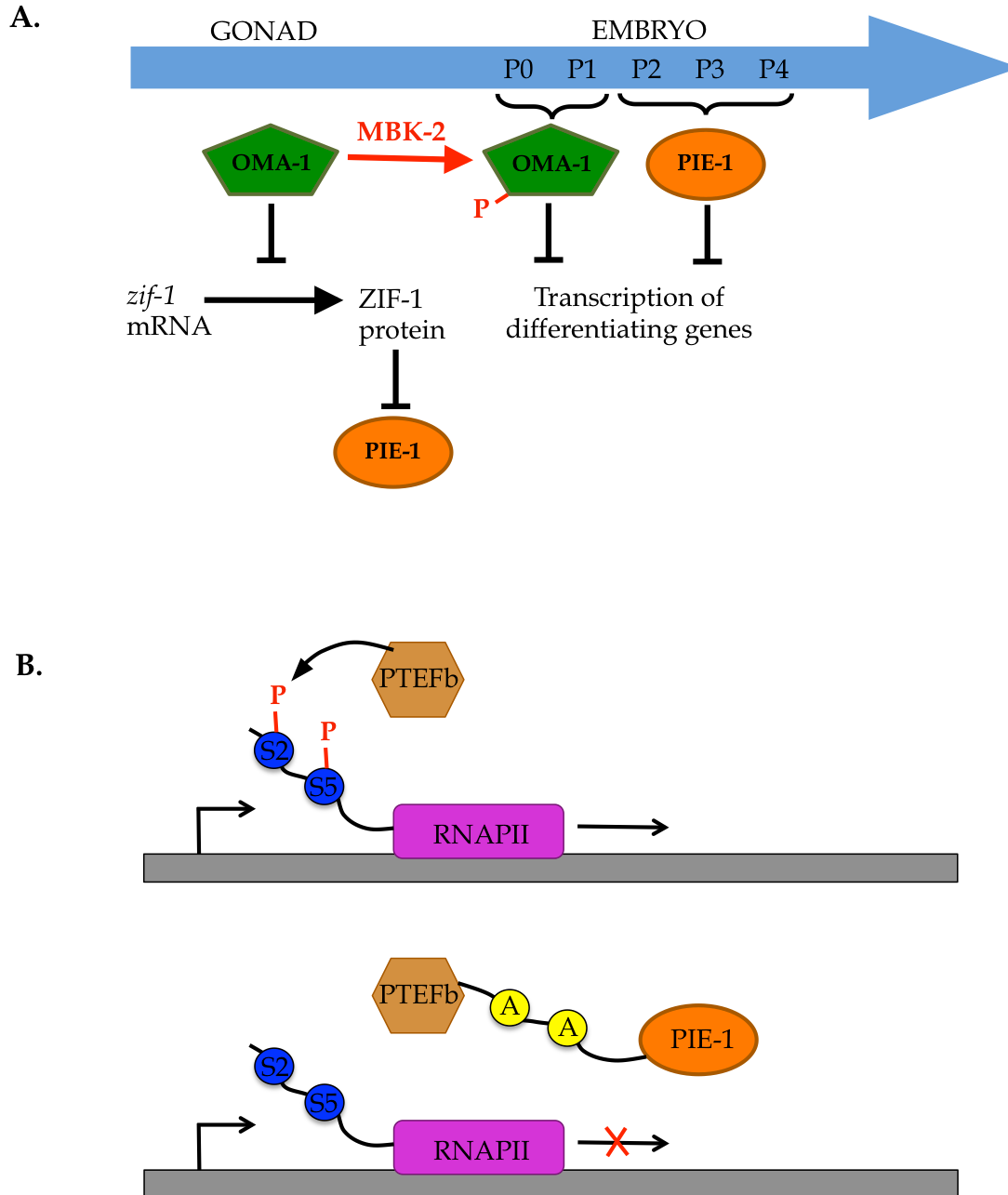
1997; Ghosh and Seydoux 2008). During normal eukaryotic transcription, the transcription elongation factor b (PTEFb) recognizes and binds to the RNA polymerase II (RNAPII) C terminal domain (CTD) thereby phosphorylating serine 2 to drive transcriptional elongation. PIE-1 contains a carboxy-terminal tail sequence similar to that of RNAPII, with an alanine at position 2 instead of a serine, and thereby competes for binding of PTEFb and recruits it away from RNAPII (Figure 1.4)(Batchelder et al. 1999). In addition PIE-1 interferes with formation of the pre-initiation complex by preventing phosphorylation at serine 5 of the RNAPII CTD (Ghosh and Seydoux 2008).

### **1.3 Transcriptional repression in P0-P1 by OMA-1/2**

The earliest germline precursors P0 and P1 are transcriptionally repressed through the action of two functionally redundant CCCH zinc finger-like RBPs, OMA-1 and OMA-2. OMA proteins are expressed at high levels in oocytes and very early embryos and then get rapidly degraded after the first mitotic division (Detwiler et al. 2001; Shimada et al. 2002; Lin 2003). Degradation of OMA occurs through the phosphorylation action of a DYRK2 kinase, MBK-2, at T239 upon fertilization (Figure 1.4) (Nishi and Lin 2005). It has been discovered that OMA's function in the early embryo is to bind to and sequester the TATA-binding protein associated factor 4 (TAF-4). TAF-4 normally interacts with TAF-12 through their histone fold domains (HFD), forming a heterodimer that allows for the complex to be translocated into the nucleus where it will interact with TFIID to initiate transcription. OMA-1 binds to the HFD of TAF-4 through a domain that resembles the HFD of TAF-12 (Guvén-Ozkan et al. 2008).

This interaction prevents TAF-4 from translocating into the nucleus and thus global transcriptional repression is achieved.

The interaction between OMA-1 and TAF-4 only occurs when OMA-1 is phosphorylated at T239 thereby indicating that it only functions to repress transcription in P0 and P1 (after which no OMA-1 can be detected) (Figure 1.4) (Güven-Ozkan et al. 2008). Unphosphorylated OMA-1 in the oocytes functions indirectly to repress transcription by ensuring PIE-1 levels remains high. Unphosphorylated OMA-1 translationally represses *zif-1* by binding to its 3' untranslated region (3' UTR) thereby keeping ZIF-1 from degrading PIE-1 (Figure 1.4)(Güven-Ozkan et al. 2010). Therefore OMA proteins have two separate functions: they act as translational repressors in oocytes to keep PIE-1 levels high, and as transcriptional repressors in 1 and 2-cell embryos to globally repress transcription (Figure 1.4).



**Figure 1.4: Modes of germline blastomere transcriptional repression.**

A) Embryonic stages are indicated by the germline blastomere name (P0-P4). OMA-1 translationally represses *zif-1* in the oocytes thereby ensuring PIE-1 levels remain high. At fertilization OMA-1 gets phosphorylated by MBK-2 turning it into a transcriptional repressor of differentiating genes in P0 and P1. PIE-1 is a transcriptional repressor in P2-P4. B) Cartoon illustrating mode of transcriptional repression by PIE-1. In the absence of PIE-1 PTEFb phosphorylates Serine 2 of the RNAPII CTD thereby leading to transcription initiation and elongation. When PIE-1 is present the CTD of PIE-1 keeps PTEFb from phosphorylating Serine 2 (and Serine 5) of the RNAPII CTD leading to transcriptional repression.

## 2. Germline proliferation

The germline is first established in the early embryo after P4 divides to generate the two PGCs Z2 and Z3 who do not divide until after hatching (Figure 1.3) (Sulston et al. 1983). The PGCs will exponentially begin dividing during the first two larval stages with the first meiotic progression event occurring during the third larval stage. Meiotic progression will continue during the fourth larval stage with oocyte maturation beginning at early adulthood. Although germline proliferation is similar in the two sexes, hermaphrodites produce ~2000 germline stem cells that are separated into the two gonadal arms, whereas males produce ~1000 germline stem cells (Kimble and White 1981).

### 2.1 The distal tip cell and Notch signaling

The somatic gonadal niche in *C. elegans* is comprised of one single cell termed the distal tip cell (DTC). Removal of the DTC leads a lack of mitotic proliferation and subsequent increase in meiotic progression (Kimble and White 1981) whereas duplication or misplacement of the DTC promotes germline stem cell proliferation ectopically (Feng et al. 1999; Kimble and White 1981). It can therefore be determined that the DTC is both necessary and sufficient for germline stem cell proliferation/self-renewal and differentiation and therefore provides a stem cell-like niche for these cells.

Notch signaling is widely conserved across species and has been implicated in many *C. elegans* early embryonic events. Notch interactions are critical for specifying cell fate and in tissue morphogenesis. In addition to its role in embryogenesis, Notch signaling has also been shown to be critical to control germline proliferation during larval

development and to maintain germline stem cells in the adult (Crittenden et al. 2003; Kimble and Simpson 1997; Seydoux and Schedl 2001). The promotion of stem cell proliferation through Notch signaling is also observed in higher organisms (Gaiano and Fishell 2002; Calvi et al. 2003). Removing any of the core components of the Notch signaling pathway, including the ligands APX-1 and LAG-2 and receptor GLP-1, leads to a premature entry of germline stem cells into meiosis. LAG-2 is tethered by a transmembrane domain thereby keeping it localized strictly to the DTC (Henderson et al. 1994), whereas GLP-1 is restricted to the mitotic region where it receives signal to promote mitosis (Figure 1.5) (Austin and Kimble 1987; Crittenden et al. 1994). Another critical germline player, GLD-1, ensures that GLP-1 protein remains restricted to the mitotic region through translational repression of *glp-1* mRNA in the meiotic region of the germline (Figure 1.5).

## 2.2 Entry into meiosis

The decision of transitioning from mitotically dividing germline stem cells into meiotic progression is critical and tightly regulated. It has been shown in many different species that stem cells are controlled through two different strategies. In the most well-known case, stem cells divide asymmetrically to generate one stem cell daughter and one differentiating daughter; however stem cells can also divide symmetrically to generate either more stem cells or only differentiating cells (Watt and Hogan 2000; Marshman et al. 2002). Many key genes regulating the transition from proliferation to differentiation in the germline have been identified. For example, in mammals *Stra8* induces the onset of meiosis once it gets activated by retinoic acid, an active metabolite of vitamin A

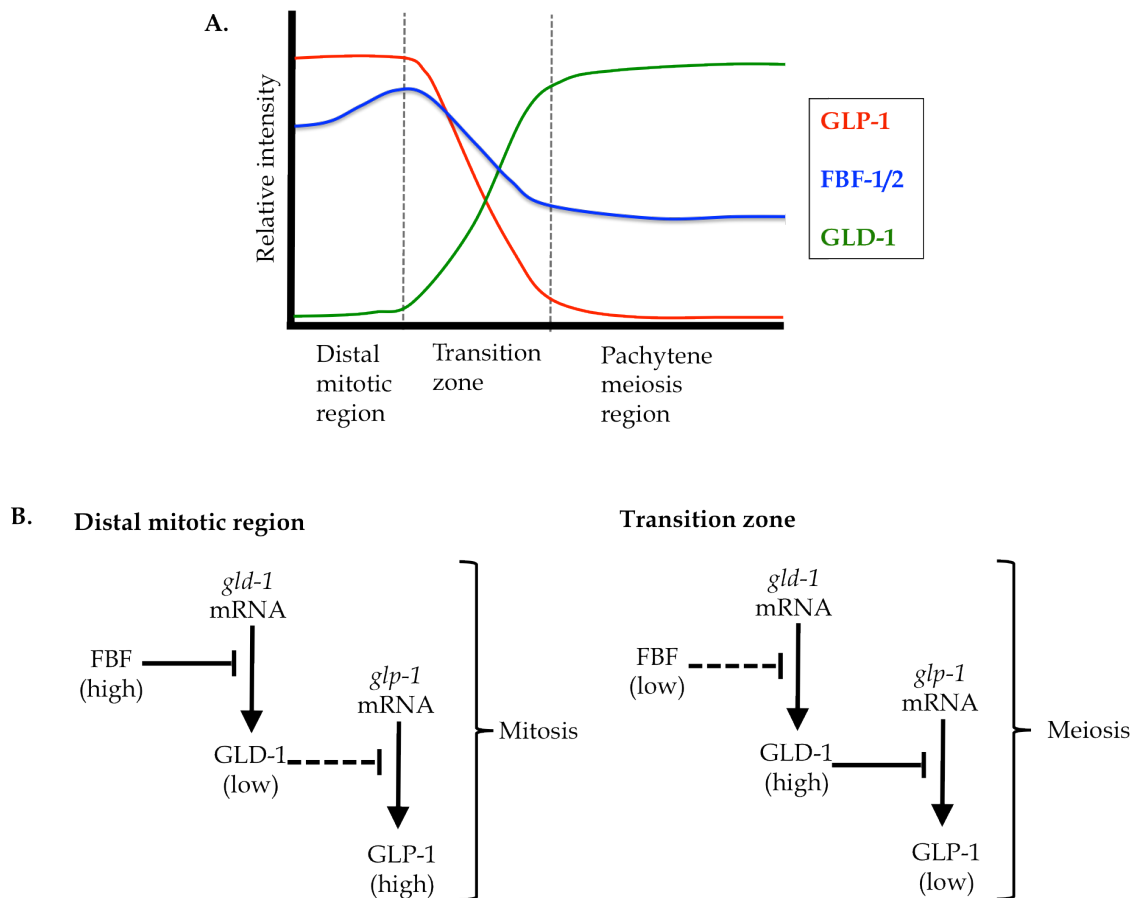


(Western et al. 2008; Bowles et al. 2006; Koubova et al. 2006). In *C. elegans* many regulatory genes have also been identified to control the mitosis to meiosis decision, some of which I will discuss in the next two sections.

### **2.2.a FBF proteins promote mitosis**

FBF-1 and FBF-2 are two almost identical genes that are members of the PUF family of RBPs. They are sequence-specific RNA binding proteins and their binding activity *in vitro* is indistinguishable. FBF proteins were initially identified for their role in sex determination (Zhang et al. 1997) and were later shown to have a function in the maintenance of germline stem cells and entry into meiosis (Crittenden et al. 2002). *C. elegans* PUF proteins are homologs of the well studied Pumilio proteins in *Drosophila*. Pumilio is required for germline stem cell maintenance in female flies through its binding activity to *nanos* mRNA. In addition Pumilio binds to and cooperates with Nanos protein to translationally repress differentiating genes (Lin and Spradling 1997). FBF proteins can repress translation of target mRNAs by binding to their 3' UTR. Many FBF target mRNAs are known, including *gld-1*, *gld-3*, and their own mRNA. *gld-1* and *gld-3* both encode for regulators that promote meiotic cell cycle progression, therefore FBF proteins impede entry into meiosis (Figure 1.5) (Crittenden et al. 2002). Binding to their own mRNA is a form of autoregulation that ensures FBF levels remain relatively low in the germline (Lamont et al. 2004). In addition to FBF regulation on pro-meiotic target mRNA FBF-2 is also a direct downstream target of the notch ligand LAG-1 (Lamont et al. 2004). Though FBF proteins have similar roles in translational regulation they do appear to have distinct effects on the size of the mitotic region. *fbf-1* mutants display a

mitotic region that is significantly smaller than wildtype, whereas *fbf-2* mutants display a mitotic region that is significantly larger (Lamont et al. 2004). The underlying reasons for this observation are yet to be discovered.



**Figure 1.5: The mitosis-to-meiosis decision in *C. elegans***

A) Diagram illustrating the relative levels of GLP-1, FBF-1/2 and GLD-1 in the gonad.

B) Diagrams illustrating the mitosis-to-meiosis decision. Left: In the distal mitotic region, high amounts of FBF protein can translationally repress *gld-1*, which in turn leads to active *glp-1* translation and a mitotic fate. Right: In the transition zone, low amounts of FBF protein leads to active translation of *gld-1* and therefore inhibition of *glp-1* translation leading to a meiotic fate.

### **2.2.b. GLD-1, GLD-2, GLD-3, and NOS-3 promote meiosis**

Entry into meiosis is controlled through two major pathways involving four total RNA regulators, GLD-1, GLD-2, GLD-3, and NOS-3. Removing any of these genes individually does not affect entry into meiosis, whereas removing two from separate pathways simultaneously abolishes meiotic entry and leads to the formation of germline tumors (Kadyk and Kimble 1998; Hansen et al. 2004, Eckmann et al. 2004). GLD-2 and GLD-3 act in the same pathway and encode for the catalytic portion of a poly(A) polymerase and a Bicaudal-C homologue respectively (Kadyk and Kimble 1998, Eckmann et al. 2002). GLD-2 and GLD-3 bind to each other and GLD-3 enhances GLD-2 activity (Eckmann et al. 2004). GLD-1 is homologous to the mouse Quaking and has been extensively shown to be a translational inhibitor through binding to 3' UTRs of target mRNAs (Lee and Schedl 2004). NOS-3 acts in the same pathway as GLD-1 and is related to the *Drosophila* NANOS (Kraemer et al. 1999). GLD-1 levels are low, or absent, in the distal tip before sharply rising in the transition zone where cells enter meiosis (Figure 1.5). When GLP-1 mediated Notch signaling is eliminated, however, GLD-1 levels rise significantly in the distal mitotic region and germline stem cells prematurely enter meiosis, suggesting that low GLD-1 levels at the distal end allows for these cells to remain proliferative (Hansen et al. 2004). GLD-2 and GLD-3 also show a non-uniform expression pattern throughout the germline although the importance of their spatial distribution is unclear. Given the nature of these four regulators, genes downstream of these pathways are most likely regulated at the level of translational control and/or mRNA stability. Though some mRNA targets are known, it is still unclear how these proteins precisely coordinate entry into meiosis. Since GLD-1 and NOS-3 act

as translational inhibitors and GLD-2/GLD-3 function as poly(A) polymerases it seems that the two pathways have opposite functions: GLD-1/NOS-3 likely repress translation of mRNAs that promote mitosis whereas GLD-2/GLD-3 likely promote translation of mRNAs that promote meiosis.

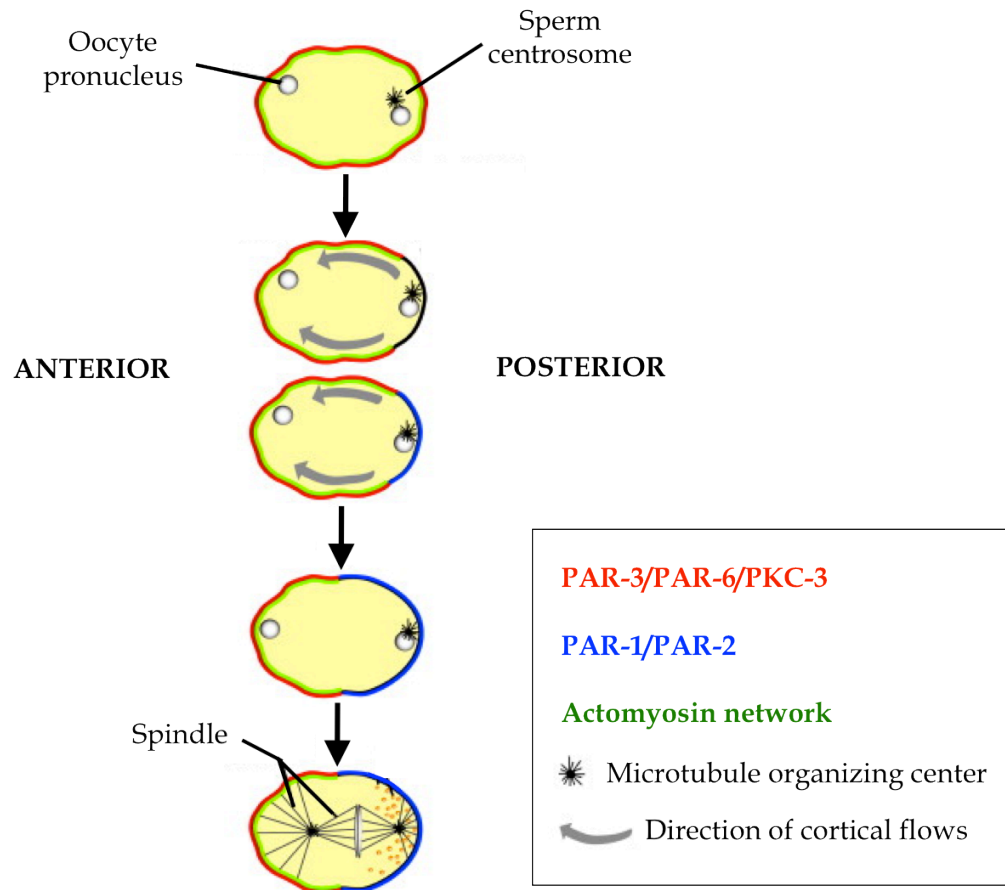
### **III. Regulation of early embryogenesis**

Many events occur simultaneously upon fertilization of an oocyte to generate the zygote. An oocyte, although loaded with a multitude of factors that are critical for embryogenesis, remains quiescent. Upon fertilization the zygote rapidly begins dividing utilizing the maternally deposited factors from the oocyte. Precise regulatory mechanisms are in place to ensure proper segregation and expression of these maternal factors. Although early embryonic events and regulation of maternal factors have been studied in mammalian systems, limitations have prevented a complete understanding of these critical events. *C. elegans* provides an excellent platform due to their rapid cell divisions and easy laboratory manipulation and have therefore provided us with many answers concerning early embryogenesis.

#### **1. A/P polarity determination**

Initial symmetry of an oocyte is broken when it traverses the spermatheca and membrane fusion with sperm occurs. Sperm will enter the oocyte opposite from the side of the oocyte nucleus and establish that side of the zygote as the posterior end (Figure 1.6). The sperm-derived microtubule organizing center initiates polarity in the zygote

leading to an asymmetrical distribution of cell fate determinants in the 1-cell zygote. This cascade of events will lead to two blastomeres with unique developmental potentials after the first mitotic division (Lyczak et al. 2002; Jenkins et al. 2006). The sperm-derived components induce contractions of the cortical actomyosin network thereby driving a kinase complex, PAR-3/PAR-6/PKC-3 away from the site of sperm entry (Figure 1.6)(Cheeks et al. 2004; Munro et al. 2004). This, now anterior localized, kinase complex, via direct phosphorylation, forces the RING finger protein PAR-2 and another kinase PAR-1 to the posterior cortex (Figure 1.6) (Hao et al. 2006). PAR-1 is responsible for restricting the Tis-11 like CCCH zinc finger proteins MEX-5 and MEX-6 to the anterior side of the embryo (Schubert et al. 2000). MEX-5/6 in turn are required to limit many other developmental regulators to the posterior end of the embryo. Two other PAR proteins, PAR-4 and PAR-5 are present in a uniform manner throughout the cytoplasm and cortex. Upon the first mitotic division all the factors that localized to the anterior end of the zygote will get segregated to the somatic blastomere AB, whereas all the posterior factors will segregate to the germline blastomere P1 resulting in two blastomeres with very distinct developmental potentials.



**Figure 1.6: Polarization of the *C. elegans* zygote (figure adapted from Bastock and Johnston 2011).**

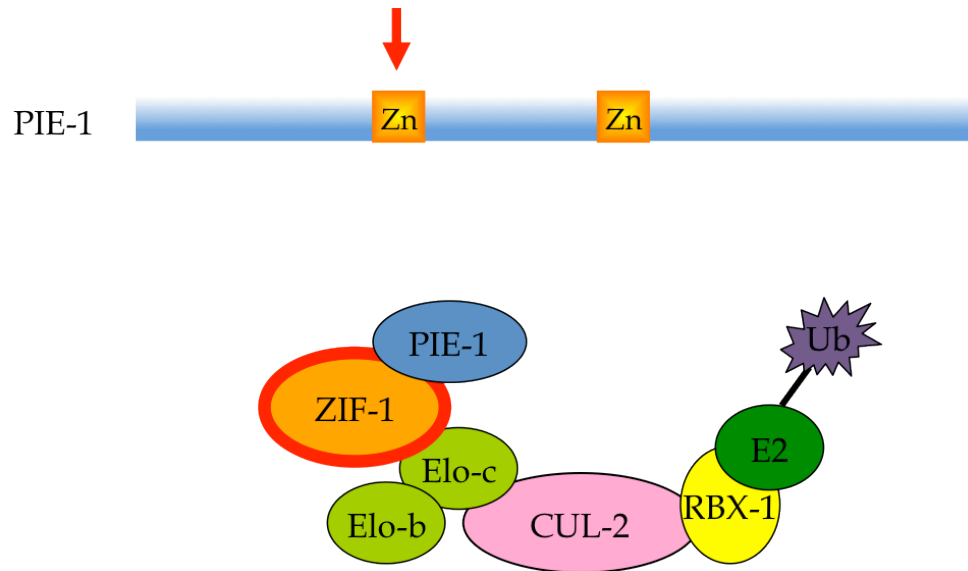
Before polarization, actomyosin (green) and PAR-3/PAR-6/PKC-3 (red) are distributed throughout the cortex. Sperm enters the oocyte opposite of the oocyte nucleus where the sperm centrosome triggers anteriorly directed actomyosin flows (gray arrows). This clears the anterior PAR-3/PAR-6/PKC-3 complex from the posterior to allow the posterior accumulation of PAR-1/2 (blue). This domain expands until the cortex reaches a stable polarized state leading to asymmetric cell division.

## 2. ZIF-1 as a soma-germline determinant

The germline potential in *C. elegans* is segregated after the first mitotic division. After the PAR proteins set up polarity, all the factors present in the 1-cell zygote will segregate either to the germline blastomere or to the somatic blastomere. It is critical that all the germline factors properly segregate to the germline blastomere at the first division

as well as the three subsequent divisions in order to maintain integrity of the germline. In addition to the PAR-mediated processes, it has been shown that ubiquitin/proteasome-mediated pathways play a major role in the soma-germline asymmetry. For example, although the transcriptional repressor and germline determinant PIE-1 gets polarized before each division, the small amount of PIE-1 left in the somatic blastomere after division gets rapidly degraded through a ubiquitin/proteasome mediated pathway (DeRenzo et al. 2003, mello et al. 1996, reese et al. 2000).

The *C. elegans* encodes four major classes of ubiquitin ligases: the HECT-domain proteins, the U-box proteins, the monomeric RING finger proteins, and the multi-subunit RING finger complexes. Within the multi-subunit RING finger class are CUL-1, CUL-2, CUL-3, and CUL-4 based complexes. One particular CUL-2 containing RING finger complex contains a subunit called ZIF-1. ZIF-1 can bind to the first zinc finger of PIE-1 thereby degrading it specifically in somatic blastomeres, but not the germline blastomere, after each division (Figure 1.7) (Reese et al. 2000; DeRenzo et al. 2003). In addition to PIE-1, ZIF-1 also binds to the zinc fingers of many other RBPs, including the germline-enriched factors POS-1, and MEX-1 (DeRenzo et al 2003). Therefore ZIF-1 is thought to be a key factor in generating the soma-germline asymmetry and is critical for embryogenesis. In fact depletion of *zif-1* results in embryonic lethality (DeRenzo et al. 2003). ZIF-1 has to be tightly controlled in a spatial and temporal manner in order to ensure correct degradation of germline-enriched factors in the soma and protection of these factors in the germline. I have carried out a comprehensive study, discussed in chapter two, demonstrating how *zif-1* mRNA gets spatio-temporally regulated through its 3' UTR thereby allowing for soma-restricted expression.



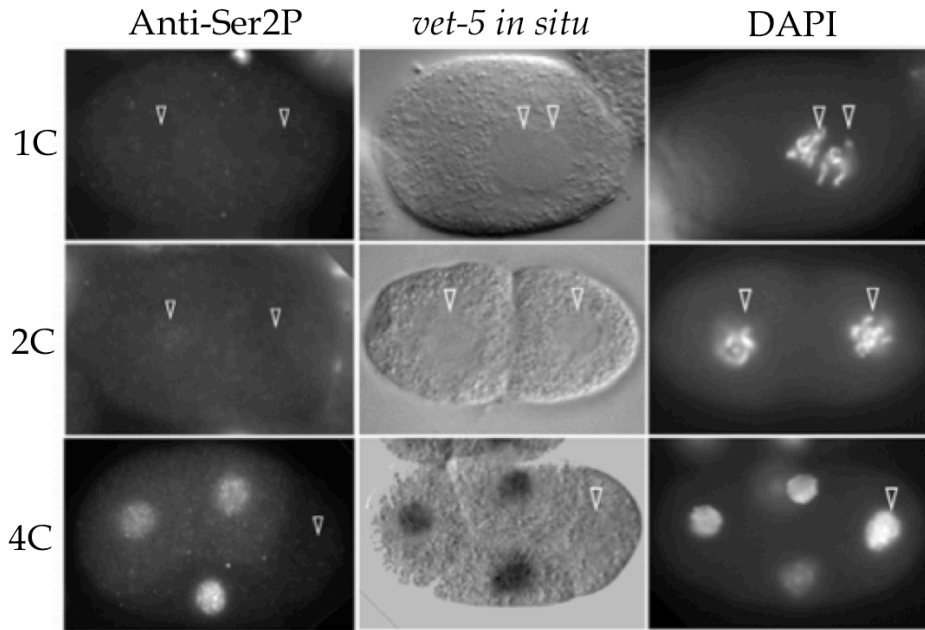
**Figure 1.7: Structure of the PIE-1 degradation complex (figure adapted from deRenzo et al. 2003)**

PIE-1 is degraded by a CUL-2 based RING ubiquitin ligase degradation complex. ZIF-1 is the substrate specific binding partner that interacts with the first zinc finger of PIE-1 (as annotated by the red arrow).

### 3. Importance of maternal factors

No zygotic transcription is detected in *C. elegans* until the 4-cell stage embryo (Figure 1.8); therefore early embryos rely heavily upon maternal mRNA deposited from the oocytes into the zygote. These maternal mRNAs accumulate during oogenesis and allow for the initial establishment of embryonic cell lineages. The importance of maternal contribution has been studied in many systems and it is clear that precise regulation of maternal mRNAs is critical for proper embryogenesis.



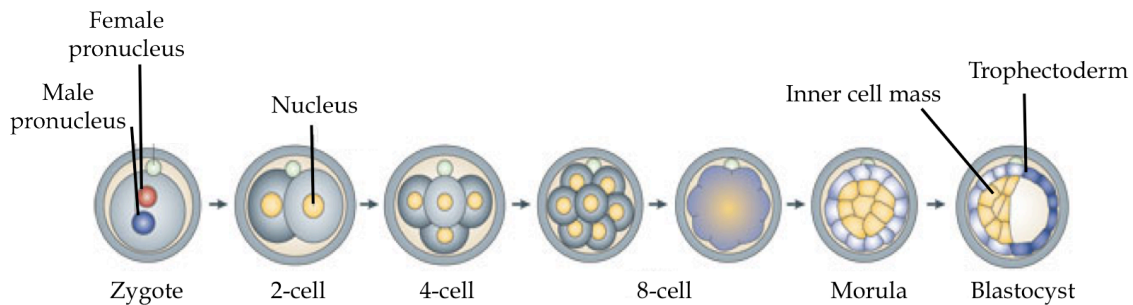


**Figure 1.8: Early zygotic transcription in *C. elegans* (figure adapted from Guven-Ozkan et al. 2008)**

The left column is RNAPII Serine 2 phosphorylation staining to indicate active transcription. The middle column is an in situ hybridization of one of the earliest detectable zygotic transcripts: *very early transcript 5 (vet-5)*. The right column is DAPI staining. No zygotic transcription can be detected until the 4-cell stage embryo and at that time only in somatic blastomeres. Open arrowheads point to pronuclei in 1C, nuclei in 2C, and the germline nucleus in 4C embryos, respectively.

Mouse embryos divide symmetrically until the 8- cell stage at which point the embryo compacts to form the morula. Subsequent asymmetric cell divisions results in a blastocyst with two distinct cell populations: the inner cells that will form the ectoderm/endoderm inner cell mass and the outer cells which will contribute to the trophectoderm (Figure 1.9) (Wroblewska 1967; Johnson and Ziomek 1981). The establishment of these two cell types is dependent on maternally provided E-cadherin (De Vries et al. 2004). Interestingly, it has been shown in mouse embryos that many maternal RNAs and proteins get degraded during meiotic maturation of oocytes (Clegg and Piko 1983; De Leon et al. 1983) most likely through small non-coding RNA mechanisms.

Degradation of maternal RNAs must occur in order to allow for the embryonic developmental programs to get activated. Therefore there are precise selection mechanisms in place that allows critical maternal factors, such as E-cadherin, to remain protected from this degradation process (Alizadeh et al. 2005). It is still unclear how this selection occurs.



**Figure 1.9: Early mouse embryonic divisions (figure adapted from Zernicka-Goetz 2005)**

In the zygote, the female and male pronuclei are labeled red and blue respectively. The embryo undergoes symmetric divisions until the 8-cell stage embryo. At the 8-cell stage the embryo compacts to form the morula. After subsequent asymmetric division the blastocyst stage embryo will consist of two distinct cell populations: the inner cell mass and the outer cells that will contribute to the trophoblast.

The importance of maternal contribution is even more reiterated in species where the egg develops outside of the mother, such as frogs, flies, and worms. During mammalian development the embryo continues to rely on factors produced by the mother that can be provided to the embryo through the placenta. Embryos that develop ex-utero have no connection to their mother and therefore rely heavily on maternal factors during the early stages of development until their own transcriptional machinery takes over. Therefore systems such as *C. elegans* provide excellent platforms for studying the regulation of maternal factors.

### 3.1 The maternal to zygotic transition

At some point during early development, depending on the species, all embryos will pass through a stage during which developmental control is handed over from maternally provided gene products to zygotically transcribed products (Figure 1.10). This maternal-to-zygotic transition (MZT) has been extensively studied and can be classified into two main events: the elimination of a subset of maternal mRNAs and proteins, and zygotic transcription initiation.

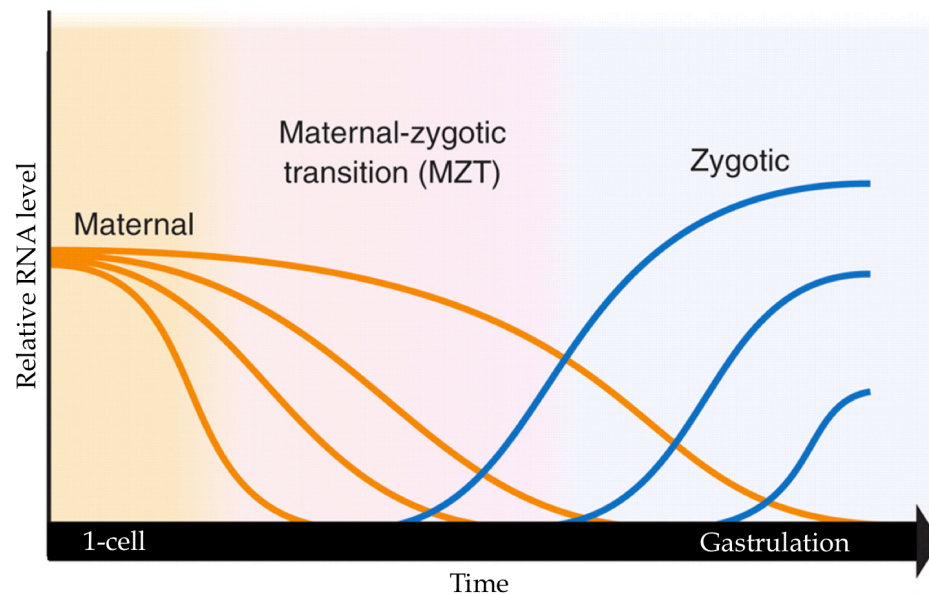
The elimination of maternal products occurs through two types of degradation activities. “Maternal activity” is exclusively maternally encoded and functions upon egg activation without the initiation of zygotic transcription, whereas “zygotic activity” requires the onset of zygotic transcription. In *Drosophila* 20% of maternal transcripts are eliminated maternally and an extra 15% get degraded zygotically (Tadros et al. 2007; De Renzis et al. 2007). In mouse embryos a large fraction of maternally supplied mRNA gets degraded by the 2-cell stage embryo with fertilization triggering at least some of this destabilization (Piko and Clegg 1982; Alizadeh et al. 2005). Approximately 30% of maternal mRNAs are destabilized and degraded in *C. elegans* although it is not known what fraction is owed to maternal degradation and what fraction is owed to zygotic degradation (Baugh et al. 2003). The precise function of maternal mRNA destabilization and degradation is not known. It has been postulated, however, that degradation is required to prevent abnormal mRNA dosage in the early embryo and that elimination of specific maternal mRNAs could be essential for early development (Tadros and Lipshitz 2009). Most maternal mRNAs that are targeted for degradation are eliminated in a spatially regulated manner. For example some are protected from degradation in the

germline blastomeres where they may function to specify certain aspects of germ cell fate and behavior (Seydoux and Fire 1994).

Zygotic transcriptional activation occurs in successive waves (Figure 1.10). In mouse and sea urchin embryos zygotic transcription can be detected as early as the 1-cell stage embryo with additional transcriptional activation occurring during later developmental stages (Aoki et al. 1997). In *Drosophila*, transcription is first detected at cycle 8 with high rates of transcription occurring at cycle 14, just prior to the onset of gastrulation (Edgar and Schubiger 1986). In *C. elegans* earliest zygotic transcription is detected beginning at the 4-cell stage embryo. In early embryos only 11% of total transcripts detected account for zygotic transcripts with the remaining 89% being maternally provided thereby highlighting the importance of maternal mRNA in the early *C. elegans* embryo (Baugh et al. 2003).

Zygotic transcription in *C. elegans* embryos can be blocked by removing the RNAPII component *ama-1*. Knocking down *ama-1* after the completion of oogenesis leads to an impairment of zygotic transcription without affecting maternal mRNA. Embryos lacking *ama-1* fail to initiate gastrulation (around the 28 cell stage) and arrest by approximately the 100-cell stage. Development up until the 28-cell stage occurs with no observable defects (Powell-coffman 1996). Zygotic transcription has been inhibited in the amphipod *Parhyale hawaiiensis* and embryonic development also occurred without observable defects until the onset of gastrulation (Alwes et al. 2011). Maternal-effect *screeching halt* mutants in *zebrafish* arrest due to aberrant zygotic genome activation. In these mutants, the first observable defects are detected at the sphere stage during the mid-blastula transition (right before gastrulation)(Wagner et al. 2004). All of these studies

indicate that the earliest time point in embryonic development that zygotic transcription is critical is for the onset of gastrulation. The early embryo does not appear to require zygotic transcription, but rather can rely on maternally provided factors (Figure 1.10).



**Figure 1.10: The maternal to zygotic transition (figure adapted from Schier 2007)**

Maternal mRNAs are deposited by the mother into the embryo to drive early development. Maternal mRNAs are degraded, and zygotic transcription is activated, in successive waves during embryogenesis. The embryo becomes fully dependent on zygotic transcription for gastrulation.

### 3.2 RNA binding proteins are critical for embryogenesis

Forward genetic screens in *C. elegans* have discovered factors that are important for cell fate specification during early embryogenesis. Interestingly many of the factors identified through these screens contain RNA binding motifs, highlighting the importance of post-transcriptional regulation during embryogenesis. The association of many of these RBPs with early embryonic phenotypes underscores their importance in *C. elegans* development. Though RBPs localize to only a subset of blastomeres in the early embryo,

their mRNA is ubiquitously present throughout the adult germline and early embryos, indicating that RBPs are also under post-transcriptional regulation. Post-transcriptional regulation can occur at several different steps, including splicing, polyadenylation, mRNA stability, mRNA localization, and translation.

RNA regulatory networks are continuously being discovered, such as the repressive role of FBF-1/2 and, possibly, activating role of NOS-3 and GLD-2 on *gld-1* mRNA translation (as discussed in section II) (Crittenden et al. 2002; Hansen et al. 2004). GLD-1 itself has also been shown to contain a KH RNA binding motif and has been implicated in germline development and early embryogenesis through interactions with target mRNAs. One target mRNA of GLD-1, *mex-3*, is important for specifying the identities of the anterior AB somatic blastomeres (Draper et al. 1996). MEX-3 and GLD-1 together, in turn, are spatially non-overlapping repressors of *pal-1* mRNA (Mootz et al. 2004). The majority of RBPs are provided maternally and, although many of them are highly expressed in oocytes, they asymmetrically localize to only a subset of blastomeres in embryos (Figure 1.11). In the next paragraph I will highlight the function of several RBPs that are important in the context of this dissertation.

### **3.2.a Germline enriched RNA binding proteins**

Most RBP get segregated to only a subset of blastomeres after the first mitotic division. Some become enriched specifically in the germline blastomeres whereas other RBPs segregate to a subset of somatic blastomeres. The germline-enriched RBPs that I will be discussing in the next paragraph are PIE-1, POS-1, SPN-4, and MEX-1 (Figure 1.11).

PIE-1, as previously discussed, is a CCCH-type zinc finger RBP that is critical for global transcriptional repression in the germline blastomeres P2-P4. PIE-1 gets segregated to the germline blastomere through a combination of enrichment in the cytoplasm that will become the germline daughter blastomere prior to division, and rapid degradation in the somatic daughter blastomere after division by a CUL-2 containing E3 ligase containing ZIF-1 (Reese et al. 2000; DeRenzo et al. 2003). *pie-1* mutant embryos contain excess pharyngeal and intestinal cells due to a fate transformation of the germline blastomere P2 into its sister blastomere EMS and is therefore critical for maintaining germline integrity (Mello et al. 1992). POS-1 also encodes a CCCH-type zinc finger binding protein and gets segregated to the germline blastomere upon each cell division. POS-1 is critical for proper fate specification of germ cells, intestine, pharynx, and hypodermis (Tabara et al. 1999). Direct binding of POS-1 to *apx-1* and *glp-1* mRNA has been detected leading to positive and negative translational regulation respectively. POS-1 has also been shown to bind directly to SPN-4, which may function to negatively regulate POS-1 activity (Ogura et al. 2003). The germline segregated protein SPN-4 contains an RNP-type RNA binding domain and is critical for rotation of the mitotic spindle in the P1 blastomere (Gomes et al. 2001). In addition, SPN-4 is required for the proper localization of maternal cell-fate determinants such as SKN-1. SPN-4 binds to the 3' UTR of several mRNAs, including *glp-1*, and *pal-1* to positively and negatively regulate their translation respectively (Labbe and Goldstein 2002; huang et al. 2002). MEX-1, is a CCCH-type zinc finger protein that is required for segregation of P granules, germ cell formation, and somatic cell differentiation in the early embryo (Guedes and

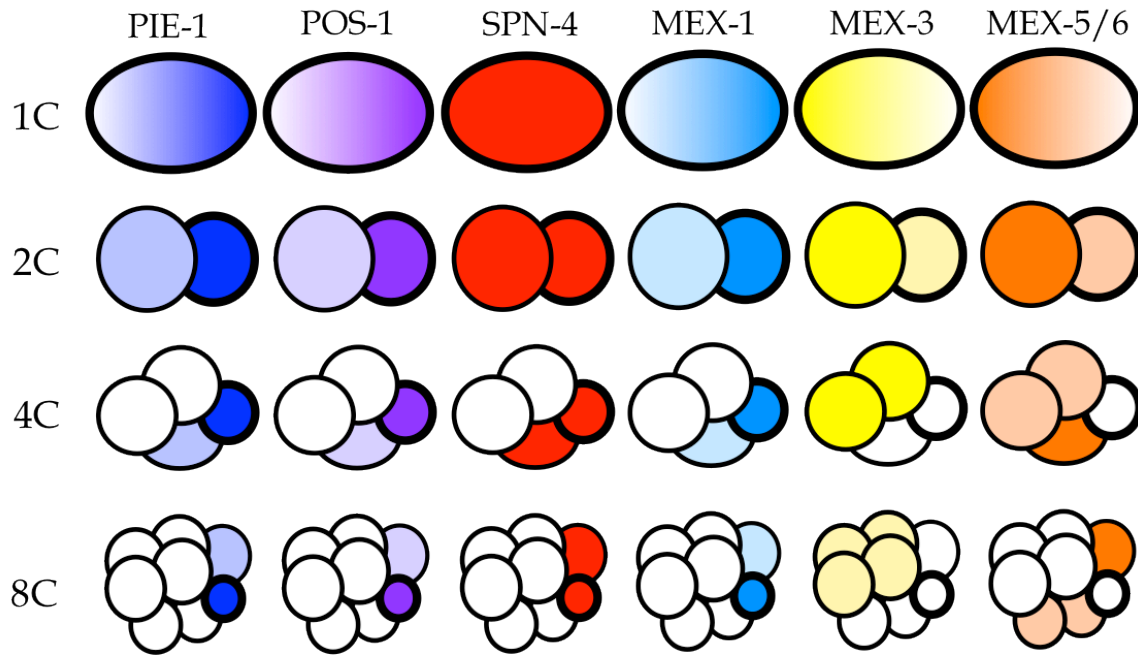
Priess 1997). In addition, MEX-1 has also been implicated in restricting PIE-1 expression and function to germline blastomeres (Tenenhaus et al. 1998).

### **3.2.b Soma-enriched RNA binding proteins**

In addition to the germline-enriched RBPs discussed above three soma-enriched RBPs, MEX-3, MEX-5 and MEX-6, are critical in the context of this dissertation (Figure 1.11). MEX-3 encodes for a KH domain containing protein and is required for specifying the identities of the anterior somatic blastomeres and their descendants. In wildtype embryos, posterior, but not anterior, blastomeres produce muscle cells. This fate specification is compromised in *mex-3* mutants leading to production of muscle cells from anterior blastomeres. MEX-3 protein is detected at high levels in the oocytes and 1-cell embryo and becomes enriched towards the somatic blastomere after the first mitotic division (Figure 1.11)(Draper et al. 1996). MEX-5 and MEX-6 share high sequence similarity, exhibit identical expression patterns, and have partially redundant functions in vivo. They encode for CCCH-type zinc finger proteins and are essential for transducing polarity cues and establishing soma/germline asymmetry in the early embryo (Schubert et al. 2000). Ectopic expression of MEX-5 throughout the early embryo results in reduced expression of germline proteins in germline blastomeres whereas *mex-5/6* mutants show ectopic expression of germline-enriched proteins in the soma (Schubert et al. 2000). MEX-5/6 become enriched towards the somatic blastomere after the first mitotic division and show a very dynamic expression profile after subsequent divisions. In the 4-cell embryo high MEX-5/6 is detected in the newly formed somatic blastomere EMS with lower expression in the two AB somatic blastomeres. In the 8-cell stage embryo low



expression is detected in the two EMS descendent blastomeres and high expression in the newly formed somatic blastomere C (Figure 1.11)(Schubert et al. 2000).



**Figure 1.11: Localization of some maternally provided RNA binding proteins**

The thick outlined blastomere annotates the germline blastomere. Most RNA binding proteins are already asymmetrically localized in the 1-cell embryo as shown by the color gradient.

### 3.3 Regulation of maternal mRNA

The majority of gene regulation has been studied at the transcriptional level. Specifically, gene specific promoters and enhancer elements are critical for driving expression of a particular gene in a spatio-temporal manner. The presence of many maternal factors in the form of mRNA has demonstrated, however, that a unique post-transcriptional mechanism must be utilized during early embryonic development. In fact, the majority of maternal mRNAs in *C. elegans* primarily get regulated post-

transcriptionally through their 3' UTR. Fusing a ubiquitous germline promoter to GFP:H2B and a gene specific 3' UTR is sufficient to drive gene specific GFP expression (Merritt et al. 2008). Moreover, an experiment carried out by the Seydoux lab demonstrated that 3' UTRs predominate over promoters regions. The promoter regions of 12 different genes were fused to GFP:H2B and to a germ cell permissive 3' UTR. Expression patterns conveyed that 11 of the 12 transgenes tested (with the exception being a sperm specific promoter) did not show gene specific expression, but rather were expressed according to the 3' UTR (Merritt et al. 2008). These experiments highlight the importance of 3' UTR mediated post-transcriptional mechanisms during early embryogenesis.

#### **4. Cell-cell interactions in the early embryo**

Early development and cell fate specification in *C. elegans* relies heavily on the integration of many signaling pathways and highly specific cell-cell interactions. As previously described, the notch signaling pathway is critical for establishing a sufficient pool of germline stem cells in early germline development (Crittenden et al. 2003; Kimble and Simpson 1997; Seydoux and Schedl 2001). In addition to its role in early germline development, notch signaling is also critical for embryogenesis. Cell-cell interactions govern when and where signaling in the early embryo will occur. For example in the 4-cell stage embryo the germline blastomere P2 expresses the notch ligand whereas both AB blastomeres, ABa and ABp, express the notch receptor (Evans et al. 1994). However, signaling is only observed from P2 to ABp due to the posterior displacement of ABp that allows it to come into contact with P2. Another Notch

interaction occurs at the 12-cell stage embryo where the daughters of EMS, MS and E, now express the notch ligand and all eight AB daughters express the notch receptor (Hutter and Schnabel 1994; Mango et al. 1994; Lin et al. 1995). The signaling cell MS comes into contact with only two out of the four ABa descendants where it subsequently activates the receptor. These two early notch signaling events are both critical for proper development of pharyngeal cells. Misregulation of ligand and/or receptor at any of these interactions leads to lack of or excess pharyngeal cells (Petcherski and Kimble 2000; Goutte et al. 2002; Hutter and Schnabel 1994; Mango et al. 1994).

Cell-cell interactions have also been shown to be critical in other systems. One example is the hedgehog signaling pathway in *Drosophila*. Hedgehog signaling is initiated by binding of hedgehog ligand to its receptor, Patched (Ptc) (Cohen 2003; Jia and Jiang 2006; Huangfu and Anderson 2006). Ptc in turn is an inhibitor of the 7-transmembrane protein Smoothed (Smo) and downstream of Smo is the hedgehog signaling complex (HSC). In the presence of Hedgehog, Ptc represses Smo thereby preventing the activation of hedgehog signaling (Hooper and Scott 2005; Kogerman 2006). Once Hedgehog is generated in the *Drosophila* embryo, it only activates a thin stripe of cells that it comes into direct contact with. Ectopic activation of Hedgehog leads to a broader band of activated cells and defects in embryonic development (Von Ohlen et al. 1997; Ingham and McMahon 2001).

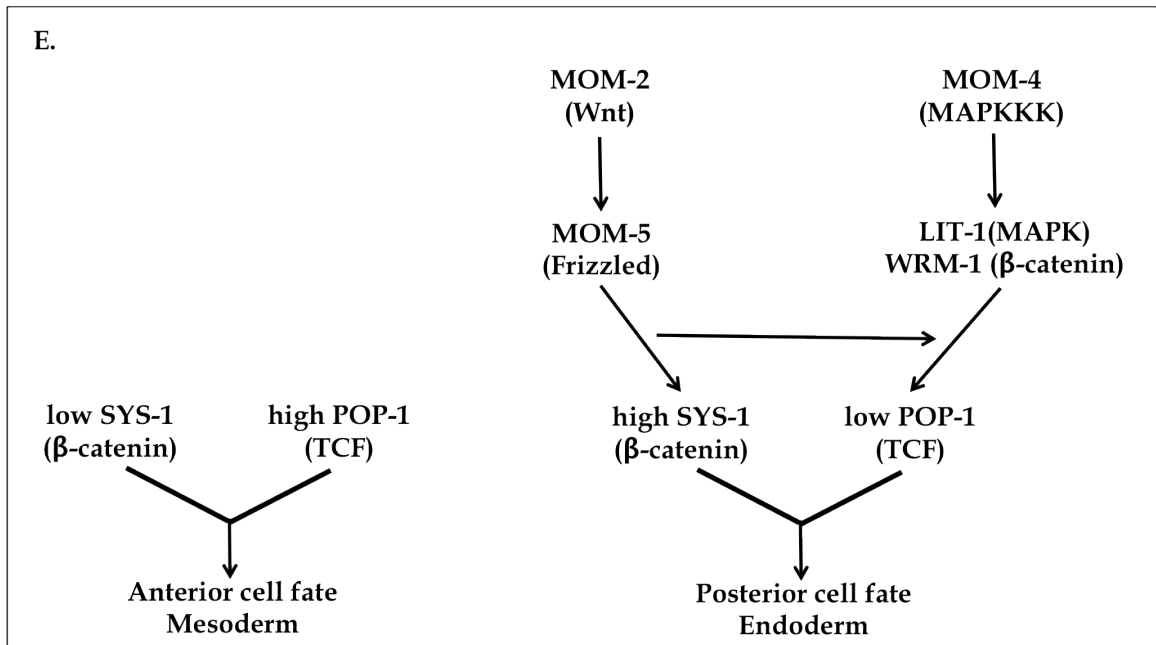
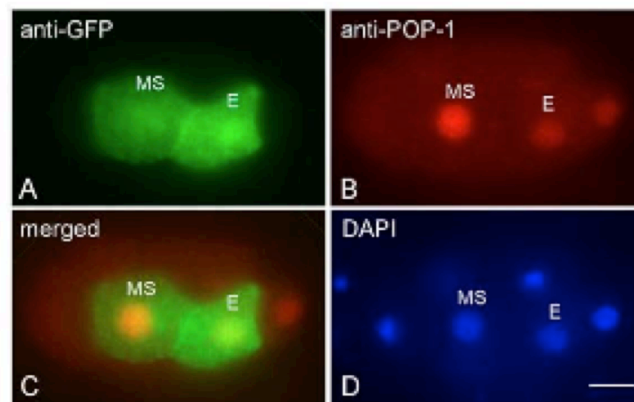
#### **4.1. Wnt signaling**

Wnt signaling is an evolutionarily conserved signal transduction pathway used extensively across different species to control many different aspects of development.

Two distinct Wnt signaling pathways exist: the canonical pathway and the noncanonical pathway. In the canonical pathway, absence of ligand causes  $\beta$ -catenin to interact with APC thereby marking it for GSK-3 dependent phosphorylation. Phosphorylated  $\beta$ -catenin gets ubiquitinated and degraded thereby allowing the TCF transcription factor to repress transcription of its target genes. In the presence of Wnt signal  $\beta$ -catenin is stabilized, translocates to the nucleus and interacts with TCF to prevent transcriptional repression and target genes are activated. The noncanonical Wnt signaling pathway functions in a  $\beta$ -catenin-independent manner.

The earliest Wnt mediated signal in *C. elegans* occurs at the 4-cell stage embryo where the germline blastomere P2 signals to its sister somatic blastomere EMS. This signaling event is required to polarize EMS leading to an anterior MS daughter and a posterior E daughter after division. Blastomere isolation experiments have indicated that the P2-EMS signal is required for proper endoderm production. All the components that are required for P2-EMS signaling have been identified through forward and reverse genetics and include 4 *mom* (more of mesoderm) genes, the kinase *gsk-3*, the  $\beta$ -catenin *wrm-1*, and the TCF *pop-1* (Lin et al. 1995; Rocheleau et al. 1997; Thorpe et al. 1997; Meneghini et al. 1999; Schlesinger et al. 1999; Shin et al. 1999; Walston et al. 2004). The anterior daughter MS, which produces mesoderm, contains high levels of TCF/POP-1, while the E blastomere, which produces endoderm, contains low levels of TCF/POP-1 (Figure 1.12). In addition, the POP-1 co-activator SYS-1 has a reciprocal expression profile (Figure 1.12)(Huang et al. 2007). Active Wnt signaling, through the Wnt ligand MOM-2, from the germline blastomere P2 leads to high SYS-1 levels in E, whereas nuclear POP-1 levels are decreased in E through a MAPK signaling pathway. The precise

ratio of SYS-1 to POP-1 promotes asymmetric cell fates of E and MS and allows for E-derived endoderm production (Figure 1.12)(Huang et al. 2007). In *mom-2* mutants the E blastomere adopts an MS blastomere fate leading to defects in endoderm production (Thorpe et al. 1997; Bowerman 1997). *pop-1* mutants, on the other hand, show excess endoderm production due to the MS blastomere adopting the E blastomere fate (Lin et al. 1995).



**Figure 1.12: E and MS blastomere fates (figure adapted from Huang et al. 2007).** (A-D) Transgenic embryos carrying SYS-1::GFP at 1MS/1E stage stained with (A) anti-GFP antibody, (B) anti-POP-1 antibody and (D) DAPI to visualize nuclei (C) Merged image. SYS-1 signal is high in E whereas POP-1 signal is high in MS. E) Diagram depicting the outcome of E and MS blastomere fate based on SYS-1 and POP-1 levels. High SYS-1 and low POP-1 leads to posterior (endoderm) cell fate whereas low SYS-1 and high POP-1 leads to anterior (mesoderm) cell fate.

Another well studied Wnt signaling event occurs at the 8 cell stage embryo. Four of the *mom* genes, *mom-1*, *mom-2*, *mom-3*, and *mom-5* all cause spindle alignment defects in the ABar blastomere as it divides at the 8-cell stage embryo (Rochelau et al. 1997; Thorpe et al. 1997). Three of the AB granddaughters divide with their spindle orientation parallel to one another, whereas the fourth AB granddaughter, ABar, divides roughly perpendicular to the other three. In any of the *mom* mutants, ABar will orient its spindle parallel to the other AB granddaughters. Correct spindle orientation of ABar requires a MOM-2/Wnt signal from the C blastomere through a direct cell-cell interaction (Walston et al. 2004). After division of the ABar blastomere, its posterior daughter ABarp will remain in contact with C, whereas this contact is lost in wnt-signaling defective mutants (Walston et al. 2004; Park et al. 2003).

#### **4.2. Importance of proper ligand and receptor localization**

Many of the components of the signaling pathways previously described are maternally provided and therefore their mRNAs are ubiquitously localized in the germline and early embryo. Precise post-transcriptional mechanisms are in place to ensure that ligands and/or receptors do not get precociously or ectopically expressed. For embryogenesis to proceed flawlessly, signaling events must occur at precise time points

and in specific blastomeres. Therefore mis-expression of any of the signaling components can lead to detrimental effects during embryogenesis. I have carried out a comprehensive study (discussed in chapter three) demonstrating how one signaling component, the Wnt ligand MOM-2, gets post-transcriptionally regulated through its 3' UTR to regulate its spatio-temporal protein expression.

#### **IV. Degradation of mRNA**

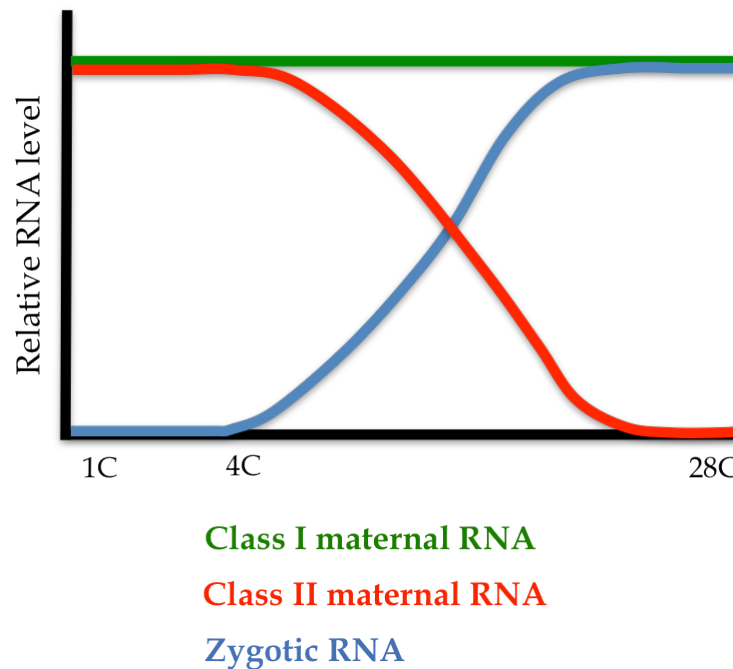
All organisms have mechanisms in place that allows them to turn genes on or off. As previously described, transcriptional regulation and post-transcriptional regulation play a major role in gene regulation, however degradation of RNAs cannot be overlooked. When comparing RNA production with steady-state levels, it is clear that cells transcribe more RNA than what they accumulate. Generally RNA molecules are degraded after they are no longer needed; therefore the dynamics of degradation vary greatly within a cell. For example ribosomal RNA is needed and protected from degradation for most of the cell's life, whereas other RNA species such as short excised introns or spacer fragments are rapidly degraded (Houseley and Tollervey 2009). Dynamics of RNA degradation is also documented for mRNA molecules. Certain mRNAs stay around for the majority of development whereas others get degraded at specific spatio-temporal timepoints. Maternally provided mRNAs, though they are ubiquitously localized at high abundance in the adult germline, have dynamic degradation profiles during early embryonic development. It is therefore clear that RNA degradation

machineries must be very carefully controlled to allow for accurate recognition of target RNAs in specific cells.

### **1. Dynamics of different classes of mRNAs**

Based on their localization patterns in the early embryo, two main classes of maternally provided mRNAs have been identified in *C. elegans* (Seydoux and Fire 1994). Class I maternal RNAs, which tend to be housekeeping genes important for many aspects of development, are maintained in all blastomeres throughout development. Class II maternal RNAs, on the other hand, are specifically degraded in somatic blastomeres, but retained in germline blastomeres. The majority of class II maternal RNAs begin to get degraded at the 4-cell stage embryo. (Figure 1.13) (Seydoux and Fire 1994). The transcriptome remains relatively stable before the 4-cell stage suggesting that all embryonic processes occurring up until then are mostly under maternal control. Beginning at the 4-cell stage embryo, however, a number of mRNAs begin to show dramatic changes in transcript abundance, which continues until just before gastrulation. At this time, the transcriptome stabilizes once again reflecting the onset of embryonic transcriptional control.





**Figure 1.13: Class I and class II maternal RNA in *C. elegans***

Y-axis shows relative mRNA level and the X-axis shows embryonic cell stage as annotated. Class I maternal mRNA levels stay constant throughout development. Class II maternal mRNAs start to degrade at the 4-cell stage coinciding with the onset of zygotic transcription.

## 2. Known RNA degradation machineries

Although specific RNA degradation pathways show immense complexity, there are substantial similarities in the basis of RNA degradation between bacteria, archaea, and eukaryotes. These similarities underline the major and long-standing importance of these processes. There are three major classes of intracellular RNA-degrading enzymes that are found in all domains of life: endonucleases that cut RNA internally, 5' exonucleases that hydrolyze RNA from the 5' end, and 3' exonucleases that hydrolyze RNA from the 3' end. Most organisms contain many RNA degrading enzymes with overlapping activities. Therefore redundancy tends to be a general feature of RNA

degradation and commonly a complete block of degradation cannot be achieved by mutating a single enzyme (Housely and Tollervy 2009). To further complicate RNA degradation machineries, it has been shown that many of the enzymes involved are multi-functional. For example the 5' exonuclease Rat1 and the 3' exonucleases of the exosome complex in yeast are responsible for degrading mRNAs transcribed from polymerase I, II, or III, but yet they can also function to generate mature termini of stable RNA species (Deutscher 2006). These dual functions allow for precise processing of RNAs and underline the complex dynamics of the transcriptome.

ATP-dependent RNA helicases and polymerases participate in almost all pathways of RNA processing and degradation. Polymerases can generate double stranded RNA for further processing or add additional sequence to RNAs to allow for recognition by exonucleases. RNA helicases are capable of rapidly hydrolyzing secondary structures or double stranded RNA by translocating along nucleic acids thereby making room for RNA degradation enzymes to carry out their function. In addition, RNA helicases are believed to signal and recruit the degradation machinery (Cordin et al. 2006; Rajkowitsch et al. 2008). Chaperones represent another class of proteins that are present in most organisms to aid in RNA degradation. For example eukaryotes contain LSM complexes that promote RNA-RNA and RNA-protein interactions to regulate degradation of RNA (Beggs 2005; Mullen and Marzluff 2008). Finally, numerous RBPs also function in various RNA degradation pathways (Glisovic et al. 2008).

A large number of evolutionary conserved small regulatory RNAs are found in essentially all eukaryotes, including the miRNAs, siRNAs, and piRNAs, which can all target mRNAs and other RNA molecules for degradation (Ambros 2004; Baulcombe

2004; Lippman and Martienssen 2004; Mello and Conte 2004). siRNAs and miRNAs are the most broadly distributed and are characterized by the double-stranded nature of their precursors. The precursors of piRNAs are less well understood but appear to be derived from a single-stranded precursor (Malone and Hannon 2009). miRNAs and siRNAs are both initially processed by an enzyme called Dicer. Many organisms, including *C. elegans*, contain a single dicer that is responsible for processing both RNA species. Some organisms, such as *Drosophila*, have two or more dicers with functional specialization (Tomari and Zamore 2005). The PAZ and RNase III domains of Dicer function to cleave double stranded RNA thereby generating small double stranded RNA species with ~2 nucleotide 3' overhangs and a 5' monophosphate on the product ends (Tomari and Zamore 2005). Once double stranded RNAs are processed by dicer, they are loaded into a RISC complex where argonaute proteins act to unwind the two strands of RNA to leave one mature siRNA or miRNA molecule that can target mRNAs for degradation (Parker et al. 2004; Song et al. 2004; Ma et al. 2005). Once mature small RNA molecules are made they generally go through a second round of amplification by utilizing RNA dependent RNA polymerases (RdRPs) thereby strengthening and perpetuating the silencing response (Sijen et al. 2007; Gent et al. 2010). Generally mature siRNAs and miRNAs target mRNAs that show extensive complementarity to their own sequence, however miRNAs can also target mRNAs that show only partial complementarity typically in the 3' UTR region. Target recognition by miRNAs and siRNAs results in decreased translation and increased 5' to 3' degradation (Eulalio et al. 2008; Wu and Belasco 2008).

## 2.1 The exosome pathway

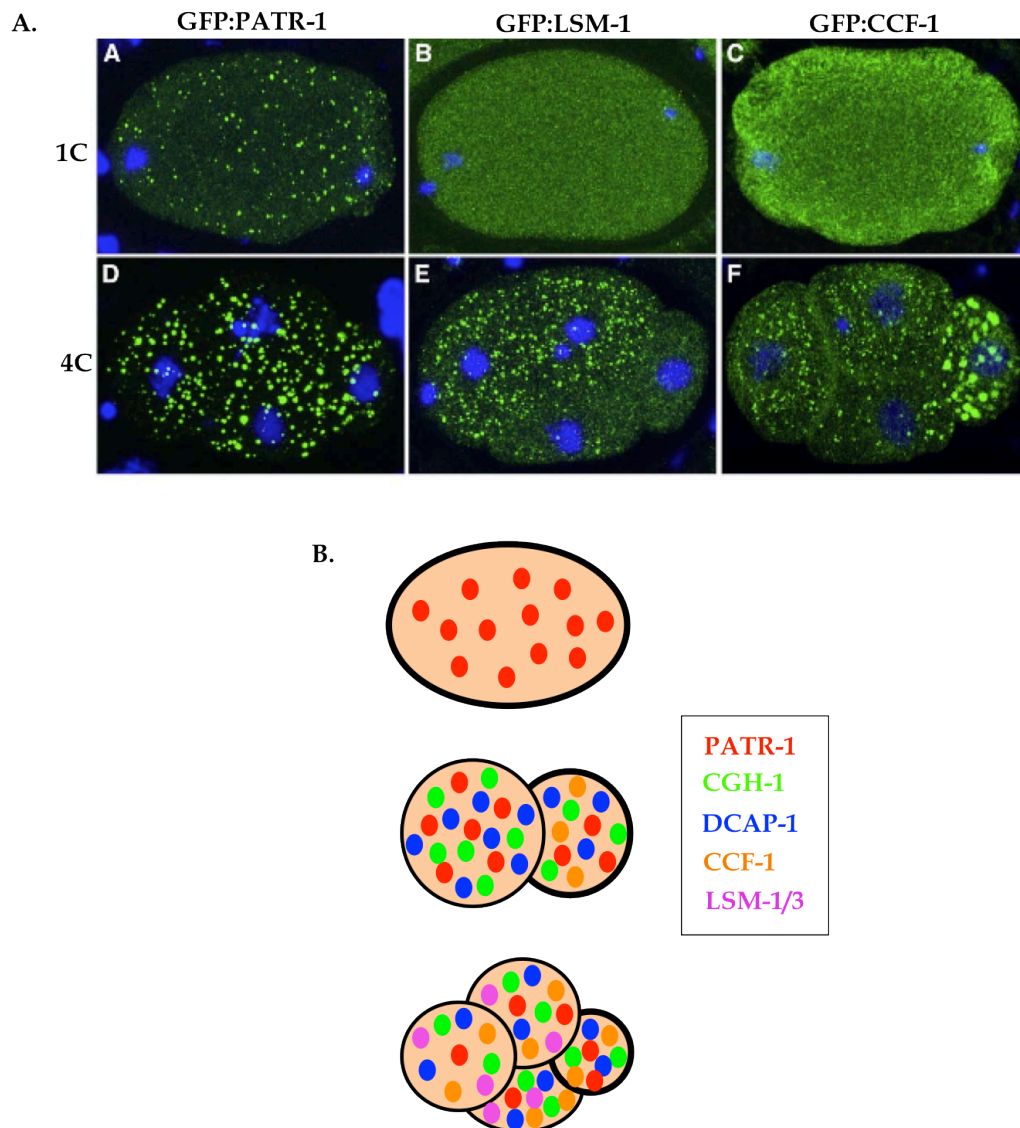
In addition to the miRNA and siRNA pathways, another well conserved mRNA degradation pathway involves the exosome. 5'-3' exonucleases most often are responsible for the degradation of unprotected RNA molecules. mRNAs, however, contain a protective 5' methylguanosine-cap structure and a 3' poly(A) tail thereby preventing degradation through exonucleases. The degradation of mRNAs is often carried out from the 3' end through a tightly controlled exonuclease complex, the RNA exosome (Garneau et al. 2007). The eukaryotic RNA exosome is composed of a central core structure made up of nine subunits with additional subunits and co-factors assembling on top of this basic entity. All of the core subunits are very highly conserved between eukaryotes and are present in cytoplasmic and nuclear exosomes (Allmang et al. 1999). More loosely attached to the core exosome complex are the 3'-5' exonuclease Rrp44 and the RNase D family enzyme Rrp6p (Allmang et al. 1999; Chen et al. 2001). Rrp44 and Rrp6p contain, in addition to their nuclease domains, different conserved motifs that are required to bind regulatory factors or to recognize specific substrates (Midtgaard et al. 2006; Schneider et al. 2007). In two different species of yeast, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, poly(A) polymerase-containing TRAMP complexes have been shown to act as major cofactors of the exosome by tagging defective RNAs with a poly(A) tail (Housely et al. 2006, Buhler et al. 2007). TRAMP complexes have also been found in *C. elegans* and although human TRAMP complexes have yet to be identified, human cells contain homologs of all components of the TRAMP complex and poly(A) tail containing RNA intermediates have been detected (Slomovic et al. 2006; West et al. 2006). The poly(A) tail tagging of RNAs provides a

“landing pad” for exonucleases to initiate degradation. In the cytoplasm, the exosome appears to act only on mRNAs thereby contributing to steady-state mRNA turnover. In the nucleus, the exosome has been shown to play additional functions such as 3' processing of non-coding RNAs (Schilders et al. 2005) and degradation of malformed RNA species (Housely et al. 2006).

## **2.2 P-body pathway in *C. elegans***

Cytoplasmic granule foci are observed in all organisms throughout development. For example, germ granules are cytoplasmic RNA-rich organelles that localize specifically to germ cells. Since germ granules contain mRNAs and many mRNA-binding proteins it is suggested that they play an important function in post-transcriptional gene regulation (Seydoux and Braun 2006). P-bodies are also detected as cytoplasmic foci have been implicated in mRNA degradation and inhibition of mRNA translation (Anderson and Kedersha 2006). P-bodies contain several proteins implicated in translational repression and activation of mRNA decapping. In addition they also contain enzymatic complexes, including decapping and deadenylase complexes and 5'-3' exonucleases (Parker and Sheth 2007). Though P-bodies segregate to both germline blastomeres and somatic blastomeres in *C. elegans*, different components are recruited in the two different cell types (Figure 1.14) (Gallo et al. 2008). In somatic blastomeres, but not germline blastomeres, thereby coinciding with maternal RNA degradation, P-bodies recruit the decapping activators LSM-1 and LSM-3 (Figure 1.14) (Gallo et al. 2008), which have also been implicated in mRNA degradation in yeast and *Drosophila* (Semotok et al. 2005; Tharun et al. 2000). Other components, such as the evolutionarily

conserved P-body components PATR-1, DCAP-1, CGH-1, and CCF-1 localize to germline and somatic blastomeres (Lall et al. 2005; Navarro et al. 2001; Gallo et al. 2008). It is generally believed that PATR-1 containing foci mature in somatic blastomeres into complete P-bodies that contain many components essential for mRNA degradation. Not all somatic P-body foci contain LSM proteins and therefore the recruitment of LSM-1 and LSM-3 may mark specific P-bodies to be degradation competent (Figure 1.14) (Gallo et al. 2008).



**Figure 1.14: Localization of P-body components in *C. elegans* (figure adapted from Gallo et al. 2008)**

A) Confocal images of indicated 1-cell and 4-cell embryos carrying indicated transgenes. B) Localization of P-body components in 1- 2- and 4-cell embryos. Blastomeres outlined in thick are germline blastomeres. PATR-1 is present at high levels in the 1-cell embryos and every blastomere thereafter. CGH-1 and DCAP-1 are present in all blastomeres beginning at the 2-cell stage embryo. CCF-1 is present in the germline blastomere at the 2-cell stage embryo and in all blastomeres of the 4-cell stage embryo. LSM-1/3 are only present in the somatic blastomeres beginning at the 4-cell stage embryo.

### 3. The siRNA pathway

siRNAs are found in all eukaryotes and function in a variety of transcriptional and post-transcriptional gene silencing processes that are collectively referred to as RNA-mediated interference (RNAi). In addition to their role in target mRNA degradation, as previously described, siRNAs were initially characterized to be essential for maintaining genome stability against viral infections (Jamalkandi and Masoudi-Nejad 2011). siRNA pathways have been studied extensively in *C. elegans* and exhibit very high complexity. First of all, though some species contain only a single argonaute protein, the *C. elegans* genome encodes for 27 different argonaute proteins (Yigit et al. 2006; Carthew and Sontheimer 2009). Many of these argonautes have been comprehensively studied and it is becoming evidently clear that each argonaute functions in a specific pathway on only a subset of target genes. For example the argonaute ALG-3/4 is found to function specifically for sperm-specific silencing whereas ERGO-1 functions specifically in the early embryo (Yigit et al. 2006; Han et al. 2009; Vasale et al. 2010; Conine et al. 2010). Secondly, *C. elegans* utilizes two main siRNA-dependent pathways, endogenous and exogenous. Though these two pathways utilize some of the same regulators, for most

parts of the pathway a distinct set of regulators is utilized (Lee et al. 2006, Jamalkandi and Masoudi-Nejad 2011). I will describe the differences and similarities between the exogenous and endogenous RNAi pathways in the next two sections.

### **3.1 Exogenous RNAi in *C. elegans***

Exogenous RNAi was first discovered in *C. elegans* in the 1990's and has since been used extensively in all model systems as a method of gene silencing. Exogenous siRNAs are derived from dsRNA precursors that become further processed to target endogenous mRNAs for degradation and/or silencing. Exogenous RNAi has been dissected extensively in *C. elegans* and most of the regulators involved are now known.

In *C. elegans*, upon introduction of exogenous dsRNA, the dsRNA binding protein RDE-4 binds to the RNA through one of its dsRNA binding motifs thereby recruiting Dicer (DCR-1) to initiate the silencing process (Figure 1.15) (Parker et al. 2006; Blanchard et al. 2011). Once DCR-1 is bound to the dsRNA, the argonaute protein RDE-1 and the Dicer related helicase (DRH-1) are recruited and bind tightly to the dsRNA (Yigit et al. 2006). DCR-1 then cleaves the dsRNA to produce primary double-stranded siRNAs bound by RDE-1 (Parker et al. 2006). RDE-1 cleaves off the passenger strand through its DDH catalytic motif. Although many argonaute proteins used in the passenger cleavage step are also used for degradation of target mRNA, RDE-1 seems to act specifically in passenger strand cleavage (Steiner et al. 2009). Primary siRNAs are targeted for secondary amplification before they are capable of target degradation (Pak et al. 2012; Aoki et al. 2007; Yigit et al. 2006). The RdRPs RRF-1 and EGO-1 stimulate production of secondary siRNAs by using selected mRNAs as templates (Sijen et al.



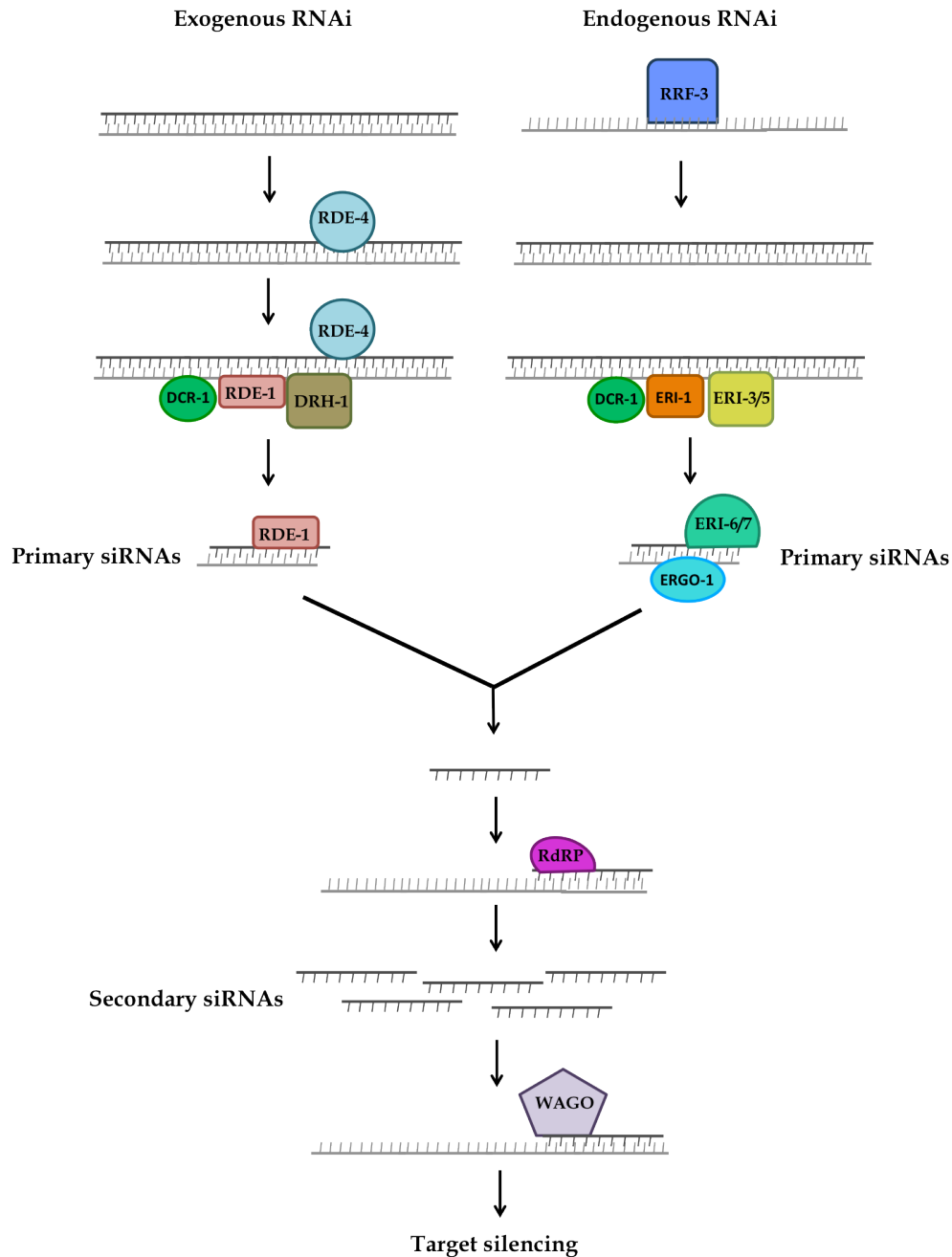
2001; Smardon et al. 2000). Specific WAGO argonautes then bind to the secondary siRNAs to target mRNAs for silencing through the use of additional co-factors including RDE-10 and RDE-11 (Figure 1.15) (Yigit et al. 2006; Gu et al. 2009; Yang et al. 2012; Zhang et al. 2012). The precise silencing mechanisms by exogenous siRNAs are still poorly understood, however some insights have recently been discovered. It was found that the argonaute NRDE-3 binds secondary siRNAs thereby translocating them to the nucleus where a subsequent decrease in pre-mRNA synthesis and an increase in histone H3 lysine 9 methylation is observed (Burkhart et al. 2011; Guang et al. 2010).

### **3.2 Endogenous siRNAs in *C. elegans***

Initial studies of RNAi responses in *C. elegans* suggested that siRNAs were strictly used for anti-viral roles whereas miRNAs appeared to be important as endogenous regulators of gene expression. This view changed with the discovery of an enhanced exogenous RNAi response in worms mutant for the argonaute RRF-3, suggesting a possible endogenous role by this enzyme and a competition between the exogenous and endogenous RNAi pathways (Simmer et al. 2002). Sequencing experiments have since revealed numerous endogenous siRNAs that read anti-sense to protein-coding genes and have a prevalence of a guanosine in the 5' position (a feature known to be unique to primary siRNAs)(Ambros et al. 2003; Lee et al. 2006).

Since the discovery of endogenous siRNAs, many enhanced RNAi, *eri*, genes have been identified, demonstrating a clear competition between the exogenous and endogenous RNAi pathways. In the ERI-dependent endogenous RNAi pathway, specific messages are selected as templates for the RdRP RRF-3 to generate dsRNA (Figure

1.15). The precise selection of these templates is currently still unknown, but appear to include recognition of duplicated genes. dsRNA produced by RRF-3 is cleaved by DCR-1 (the same dicer that is used for exogenous RNAi) to produce 26G-duplex siRNAs with a passenger strand of 19 nucleotides (Gent et al. 2010; Han et al. 2009; Vasale et al. 2010). Several co-factors interact with DCR-1 to guide RNA cleavage, including the RNase ERI-1, the tudor domain protein ERI-5, and ERI-3 (Duchaine et al. 2006; Gabel and Ruvkun 2008). Different argonautes, ALG-3/4 for sperm-specific targets and ERGO-1 for embryonic targets, can now bind the 26G siRNAs to cleave the passenger strand (Yigit et al. 2006; Vasale et al. 2010). Recently the helicase proteins ERI-6 and ERI-7 have been characterized to aid in ERGO-1-dependent production of 26G siRNAs, most likely through unwinding of the 26G dsRNA (Fischer et al. 2008; Fischer et al. 2011). The mRNAs that are targeted by these endogenous siRNAs are themselves used as templates for secondary amplification through the use of RdRPs to produce 22G secondary siRNAs. Finally the 22G secondary siRNAs are bound by specific WAGO argonautes or CSR-1 to initiate gene silencing (Figure 1.15) (Claycomb et al. 2009; Gu et al. 2009). Most CSR-1 associated siRNAs are anti-sense to protein coding genes whereas the majority of WAGO associated siRNAs seem to target transposons, pseudogenes and cryptic loci (Gu et al. 2009). The exact silencing mechanisms of endogenous siRNAs are yet to be discovered.



**Figure 1.15: The exogenous and ERGO-1 endogenous siRNA pathways in *C. elegans***

In the exogenous pathway double stranded RNA gets bound by RDE-4 leading to the recruitment of DCR-1, RDE-1 and DRH-1 and slicing of the double stranded RNA into primary siRNAs. RDE-1 then cleaves off the passenger strand and the resulting siRNA goes through a second round of amplification utilizing RdRPs. Finally secondary siRNAs initiate gene silencing through WAGO argonautes. In the endogenous pathway RNA gets polymerized into double stranded RNA through RRF-3. The double stranded RNA then gets bound by DCR-1, ERI-1, ERI-3, and ERI-5 to slice it primary siRNAs. ERGO-1, along with ERI-6/7, cleave off the passenger strand and the resulting siRNA goes through a second round of amplification as in the exogenous pathway.

## CHAPTER 2: POST-TRANSCRIPTIONAL REGULATION OF MATERNAL *ZIF-1* IN THE EARLY EMBRYO

### Summary

Transcriptional repression in germline blastomeres in *C. elegans* requires PIE-1 protein. Germline blastomere-specific localization of PIE-1 depends, in part, upon regulated degradation of PIE-1 in somatic cells. Our lab and others have shown that the temporal and spatial regulation of PIE-1 degradation is controlled by translation of the substrate-binding subunit, ZIF-1, of an E3 ligase. It is therefore critical that ZIF-1 is exclusively expressed in somatic blastomeres. In this chapter I show that ZIF-1 expression in embryos is regulated by five maternally-supplied RBPs. POS-1, MEX-3, and SPN-4 function as repressors of ZIF-1 expression, whereas MEX-5 and MEX-6 antagonize this repression. All five proteins bind directly to the *zif-1* 3' UTR in vitro. I have shown that, in vivo, POS-1 and MEX-5/6 have antagonistic roles in ZIF-1 expression. In vitro, they bind to a common region of the *zif-1* 3' UTR, with MEX-5 binding impeding that by POS-1. The region of the *zif-1* 3' UTR bound by MEX-5/6 also partially overlaps with that bound by MEX-3, consistent with their antagonistic functions on ZIF-1 expression in vivo. Whereas both MEX-3 and SPN-4 repress ZIF-1 expression, neither protein alone appears to be sufficient, suggesting that they function together in ZIF-1 repression. MEX-3 and SPN-4 repress ZIF-1 expression exclusively in 1- and 2-cell embryos, the only period during embryogenesis when these two proteins co-localize. As the embryo divides, ZIF-1 continues to be repressed in germline blastomeres by POS-1, a germline blastomere-specific protein. MEX-5/6 antagonize repression by POS-1 and

MEX-3, enabling ZIF-1 expression in somatic blastomeres. The spatio-temporal expression of ZIF-1 results from a net summation of complex positive and negative translational regulation by 3' UTR-binding proteins, with expression in a specific blastomere dependent upon the precise combination of these proteins in that cell.

## Introduction

The single founder blastomere, P4, for the entire germline in *C. elegans* is specified through a series of four asymmetric cell divisions (see Figure 1.3)(Strome 2005). Each of these divisions generates a smaller germline precursor (P1 through P4, termed the P lineage) and a larger somatic sister cell. P4 divides symmetrically to generate Z2 and Z3, which go on to generate the entire germline post-embryonically. All P lineage germline blastomeres are transcriptionally repressed whereas their somatic sisters undergo rapid transcriptional activation and lineage-specific differentiation (Nakamura and Seydoux 2008 and Seydoux and Fire 1994).

As discussed in the introduction of this dissertation, transcriptional repression in the *C. elegans* germline precursors is achieved through the action of two classes of maternally-supplied proteins. OMA-1 and OMA-2 globally repress transcription initiation in P0 and P1 (Guvén-Ozkan et al. 2008) whereas PIE-1 globally represses transcription initiation and elongation in P2-P4 (see Figure 1.4) (Batchelder et al. 1999; Ghosh and Seydoux 2008; Seydoux and Dunn 1997; Zhang et al. 2003). OMA-1, OMA-2, and PIE-1 proteins are all expressed from maternally-supplied mRNAs and are first detected in developing oocytes (Detwiler et al. 2001; Mello et al. 1996). OMA-1 and OMA-2 are degraded soon after the first mitotic division and are not detected in

subsequent P lineage blastomeres (Detwiler et al. 2001; Lin 2003). PIE-1 is segregated preferentially to the germline blastomere at each P lineage division (Mello et al. 1996). Chromatin in germline blastomeres must remain primed for transcriptional activation to allow for rapid differentiation-driven transcription in their sister blastomeres after division. Therefore global transcriptional repression by both OMA and PIE-1 is a robust but readily reversible way to transcriptionally silence the germline precursors, while maintaining the chromatin primed for transcriptional activation in the somatic sisters (Schaner et al. 2003).

The germline blastomere specific localization of PIE-1 is the result of selective enrichment towards the presumptive germline blastomere prior to division, coupled with selective degradation of any PIE-1 remaining in the somatic blastomeres following division (Reese et al. 2000). Degradation of PIE-1 in somatic cells is initiated by a CUL-2 containing E3 ligase (DeRenzo et al. 2003). The substrate-binding subunit of this E3 ligase, ZIF-1, binds PIE-1 via the first of the two CCCH zinc fingers found in PIE-1 (see Figure 1.7) (DeRenzo et al. 2003). GFP fused to the first zinc finger of PIE-1 (GFP::PIE-1 ZF1) functions as a reporter for PIE-1 degradation and undergoes ZIF-1-dependent degradation specifically in somatic blastomeres (Reese et al. 2000). Our lab has shown that the spatial and temporal regulation of this E3 ligase activity is controlled at the level of *zif-1* translation (Güven-Ozkan et al. 2010). In *C. elegans*, correct spatio-temporal expression of almost all maternally-supplied transcripts is regulated post-transcriptionally via the 3' UTR (Merritt et al. 2008). In this chapter I show that, using a translational reporter containing the *zif-1* 3' UTR, *zif-1* is translated in somatic cells, where PIE-1 is degraded, but not in oocytes or germline blastomeres, where PIE-1 levels are normally

maintained.

Our lab recently showed that OMA-1 and OMA-2 bind to the *zif-1* 3' UTR and, in an IFET-1 dependent manner, repress translation of *zif-1* in oocytes (Güven-Ozkan et al. 2010; Li et al 2009). This repression is relieved by phosphorylation of OMA-1 and OMA-2 by MBK-2 (Güven-Ozkan et al. 2010). OMA proteins are phosphorylated by MBK-2 soon after completion of meiosis II, and therefore it is unlikely that they are responsible for the continuing repression of *zif-1* translation in 1-cell embryos. It was not known how *zif-1* translation remains repressed in 1- and 2-cell embryos, and is asymmetrically activated only in somatic blastomeres at the 4-cell stage. As discussed earlier, translational control through maternally provided RBPs is critical during early embryogenesis. Most of these RBPs are asymmetrically localized to one or a few blastomeres. For example, POS-1, MEX-1, and SPN-4 are localized primarily to germline blastomeres (Guedes and Priess 1997, Ogura et al. 2003 and Tabara et al. 1999), whereas MEX-3, MEX-5, and MEX-6 are localized primarily to somatic blastomeres (see Figure 1.11) (Draper et al. 1996 and Schubert et al. 2000). POS-1, MEX-1, MEX-5, and MEX-6, like PIE-1, all contain tandem CCCH zinc-finger RNA-binding motifs, and all are targets of the ZIF-1 containing E3 ligase (DeRenzo et al. 2003; Guedes and Priess 1997, Mello et al. 1996; Reese et al. 2000; Schubert et al. 2000; Tabara et al. 1999). ZIF-1-dependent degradation in somatic blastomeres contributes to the restricted localization pattern of these proteins. MEX-5/6 are the only ZIF-1 substrates with an expression pattern that coincides both temporally and spatially with ZIF-1 activity. Despite being ZIF-1 substrates, MEX-5/6 have been shown to be required for ZIF-1-dependent degradation through an unknown mechanism (DeRenzo et al. 2003). OMA-1 and OMA-

2, although containing tandem CCCH zinc fingers, are not degraded via a ZIF-1-dependent mechanism (DeRenzo et al. 2003; Detwiler et al. 2001).

In this chapter I will discuss the spatial and temporal control of ZIF-1 expression in the early embryo. The work presented in this chapter has been published *developmental biology* in 2012. I show that the somatic cell-specific translation pattern of *zif-1* is a result of both net positive regulation in soma and repression in germline blastomeres. POS-1, MEX-3, and SPN-4 negatively regulate, whereas MEX-5 and MEX-6 positively regulate the expression of *zif-1*. All five proteins can bind to the *zif-1* 3' UTR in vitro, suggesting direct regulation. POS-1, MEX-3, MEX-5, and MEX-6 share partially overlapping binding sites on the *zif-1* 3' UTR and have antagonistic roles in *zif-1* expression. The precise combinatorial expression of these five RNA-binding proteins determines whether the *zif-1* transcript is translated in a particular blastomere.

## **Materials and Methods**

### Strains

N2 was used as the wildtype strain. Genetic markers are: LGII, *mex-6* (pk440); LGIII, *unc-119*(ed3); LGIV, *oma-1*(zu405), *oma-1*(zu405,te33), *mex-5* (zu199); and LGV, *oma-2*(te51). Plasmids used, strain names and transgenes are as follows: TX1246 (teIs113 [Ppie-1gfp::h2b::UTRzif-1,771bp]), TX1251 (teIs604 [Ppie-1gfp::h2b::UTRzif-1,771bp,Δ4-63]), TX1288 (*oma-1*(zu405; te33)/nT1; *oma-2*(te51)/nT1; teIs114 [Ppie-1gfp::h2b:: UTRzif-1,771bp]), TX1298 (teEx607 [Ppie-1gfp::h2b::UTRzif-1,771bp, Δ184-243]), TX1311 (teEx610 [Ppie-1gfp::h2b::UTRzif-1,771bp, 64–183]), TX1481 (*mex-6*(pk440); *unc-30*(e191)*mex-5*(zu199)/nT1; teIs113 [Ppie-1gfp::h2b:: UTRzif-



1,771bp]), TX1513 (mex-6(pk440); unc-30(e191)mex-5(zu199)/nT1; axEx1120 [Ppie-1gfp::pie-1 zf1::UTRppe-1, pRF4]), TX1533 (teIs140 [Ppie-1gfp::h2b::UTRzif-1,771bp,  $\Delta$ 64-123]), TX1541 (teIs143 [Ppie-1gfp::h2b::UTRzif-1,771bp,  $\Delta$ 114-183]), TX1543 (teIs145 [Ppie-1gfp::h2b::UTRzif-1,771bp,  $\Delta$ 64-183]), TX1567 (teEx719 [Pmed-1gfp::mex-3]), TX1570 (teIs113 [Ppie-1gfp::h2b::UTRzif-1,771bp]; teEx719 [Pmed-1gfp::mex-3]), JH1436 (axEx1120 [Ppie-1gfp::pie-1 zf1::UTRppe-1, pRF4]).

### Plasmid construction

Most plasmids were constructed with the Gateway cloning technology. The *in vivo* 3' UTR functional assays used the 771 nt genomic sequence downstream of the *zif-1* stop codon, which was cloned downstream of *pie-1* promoter-driven GFP::H2B in the germline expression vector pID3.01B (Reese et al., 2000). All deletion constructs were derived from the 771 nucleotide sequence (Guvén-Ozkan et al., 2010). The *mex-3* cDNA was cloned downstream of the *med-1* promoter-driven GFP (Maduro et al., 2001).

### *C. elegans* transformation

All integrated lines were generated by microparticle bombardment (Praitis et al., 2001) whereas other transgenic lines were generated by complex array injection (Kelly et al., 1997). The GFP::MEX-3 expressing line was generated by micro-injection. For each construct, expression was analyzed and found to be consistent in at least two independent lines.

### RNA interference

Feeding RNAi was performed by transforming IPTG-inducible plasmids containing the cDNA sequence of the gene of interest into HT115 bacteria. After bacteria was grown up at 37°C to the mid-log phase it was seeded on NGM plates containing 1 mM IPTG. L1 larvae were then fed for 2 days at 25 °C.

### Immunofluorescence

Immunofluorescence for *C. elegans* embryos was carried out as described previously: for anti-SPN-4 (1/10,000, rabbit) (Ogura et al., 2003). Secondary antibodies used were Alexa568 conjugated goat anti-rabbit (Invitrogen, 1/250).

### Protein purification and RNA binding assay

MBP-tagged proteins were prepared by cloning individual cDNAs into pDEST-MAL (Invitrogen). These expression clones were transformed into Rosetta cells, grown to log phase at 37 degrees and induced with 1 mM IPTG for 4 h at room temperature in the presence of 0.2% glucose and 0.1 mM zinc. After bacterial cultures were spun down they were resuspended in 10 mM TRIS, 200 mM NaCl, 1 mM EDTA, and 10 mM  $\beta$ -mercaptoethanol and protease inhibitor. Bacteria was lysed by sonication, 0.1% Triton was added and protein was allowed to bind to an amylose resin (NEB) for two hours at 4 degrees. Protein was eluted using 50 mM maltose. Biotinylation of RNA and pull-downs were performed as previously described (Guvén-Ozkan et al., 2010 and Lee and Schedl,

2001) except for the following modifications. The optimal amount of purified protein and the *zif-1* 3' UTR were empirically determined by titration series. Each binding reaction contained 150 ng of the purified MBP-tagged protein and 100 ng/60 nucleotides of biotinylated RNA. For the competition binding assay shown in Fig. 2D, 1 µg of each MBP-tagged protein and 30 ng/60 nt RNA were used.

#### Analysis of embryos, imaging, and quantification

All images except those shown in Figure 2.8A were obtained with an Axioplan microscope (Zeiss) equipped with a MicroMax-512EBFT CCD camera (Princeton Instruments) controlled by the Metamorph acquisition software (Molecular Devices) (Güven-Ozkan et al., 2008). Imaging for Figure 2.8A was performed with a LSM 510 Meta confocal microscope (Zeiss) and quantified with ImageJ software (Güven-Ozkan et al., 2008). Nuclear GFP intensity was quantified for cells in 28-cell embryos. Embryos were imaged at multiple time points to evaluate the expression of GFP::MEX-3 at 12–16-cell stages and the levels of GFP at 20–50-cell stages. The ratios of nuclear GFP intensities in EMS descendants to that in AB descendants for individual embryos were plotted in Figure 2.8A.

## **Results**

### **I. Nucleotides 64–123 of the *zif-1* 3' UTR are critical for *zif-1* regulation**

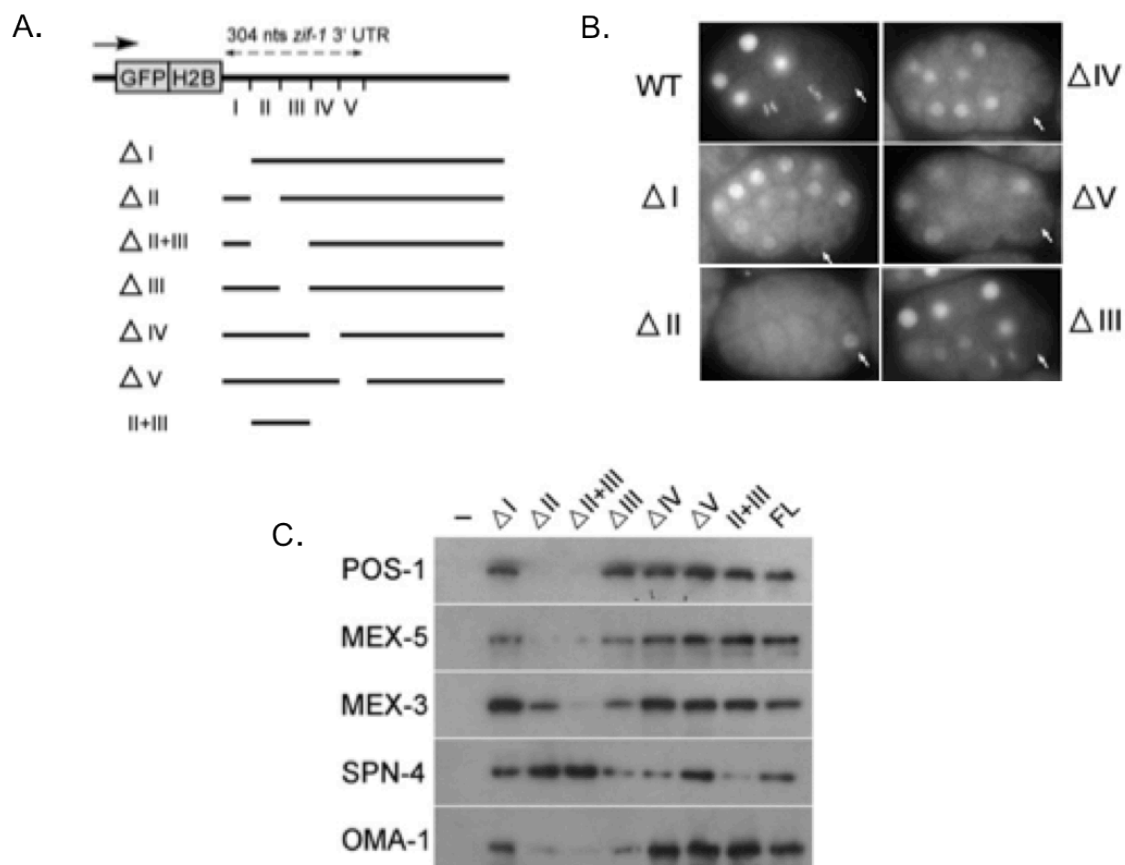
To investigate the mechanism by which the temporal and spatial expression pattern of *zif-1* is regulated in embryos, I performed deletion analyses of the *zif-1* 3' UTR

in vivo. The *zif-1* 3' UTR (304 nucleotides) was arbitrarily divided into five approximately 60 nucleotide regions (I–V, Figure 2.1A). GFP::H2B reporters driven by the *zif-1* 3' UTR with each of these five regions individually deleted were generated, and the expression pattern in transgenic embryos was analyzed. No change in GFP::H2B expression was detected with any of the *zif-1* 3' UTR single region deletion reporters, with the exception of Region II deletion ( $\Delta$ II), which lacks nucleotides 64–123 (Figure 2.1B,  $n > 100$  for each transgene). This GFP reporter will be referred to as GFP::H2B<sup>*zif-1* $\Delta$ II</sup>. GFP::H2B<sup>*zif-1* $\Delta$ II</sup> exhibited nuclear GFP in germline blastomeres P3 and P4, and no GFP in somatic blastomeres, a highly noteworthy expression pattern as it is reciprocal to what is observed in wildtype embryos (Figure 2.1B,  $n > 200$ ). This result suggests (1) that somatic blastomere-specific expression of GFP::H2B<sup>*zif-1*</sup> is due to both negative regulation in germline blastomeres and positive regulation in somatic blastomeres, and (2) that Region II of the *zif-1* 3' UTR mediates binding by both negative and positive regulators of ZIF-1 expression.

## II. POS-1 and MEX-5/MEX-6 bind to nucleotide 64-123 of the *zif-1* 3' UTR

To identify RNA-binding protein(s) that bind to *zif-1* 3' UTR Region II and regulate spatial and temporal expression of ZIF-1, I directly tested RNA-binding proteins known to play key functional roles in early embryos, including POS-1, SPN-4, MEX-3, MEX-5, PIE-1, and MEX-1 (Draper et al. 1996; Guedes and Priess 1997; Mello et al. 1992; Ogura et al. 2003; Schubert et al. 2000; Tabara et al. 1999). Biotinylated single-stranded *zif-1* 3' UTR RNA was incubated with bacterially-expressed MBP-fused test proteins. RNA, along with associated proteins, was pulled down using streptavidin-

conjugated magnetic beads. MBP-fused protein pulled down with the RNA was analyzed by western blots using an anti-MBP antibody. I found that POS-1, SPN-4, MEX-3, and MEX-5 all bound to the full-length *zif-1* 3' UTR (Figure 2.1C). No specific binding was detected with PIE-1 or MEX-1 (data not shown). I also performed RNA pull-downs with RNAs corresponding to the *zif-1* 3' UTR with the five arbitrarily-defined regions deleted, either individually or in combination (Figure 2.1C). I observed that binding by both POS-1 and MEX-5 was dramatically reduced if RNA with Region II deleted was used in the pull down. Individual deletion of any other region had minor or no effect upon POS-1 or MEX-5 binding (Figure 2.1C).



**Figure 2.1: In vitro and in vivo analyses of *zif-1* 3' UTR regions.**

A) Schematic of the *zif-1* 3' UTR, the five ~ 60 nt subregions, and the deletion constructs utilized. B) Representative fluorescence micrographs of embryos expressing GFP::H2B reporters under the control of the indicated *zif-1* 3' UTR. C) In vitro RNA pulldowns using the indicated purified MBP-tagged protein and various forms of the *zif-1* 3' UTR RNAs as shown in (B). After pulldown of RNA, protein bound to the RNA was assayed by Western blot using anti-MBP antibodies. FL: full length (304 nt). – : no RNA. OMA-1 was used as a positive control as it was previously shown to bind to regions II and III of the *zif-1* 3' UTR (Guvén-Ozkan et al 2010).

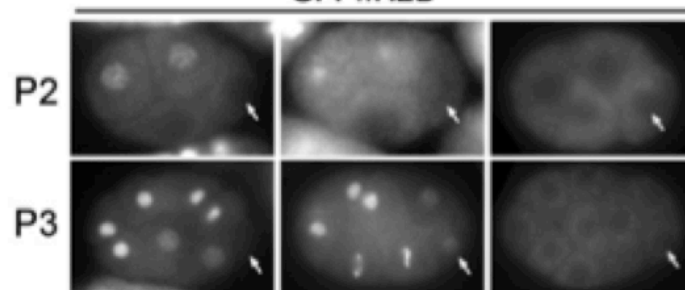
## 1. POS-1 represses and MEX-5/6 promote expression of GFP::H2B<sup>zif-1</sup>

To determine whether POS-1 and/or MEX-5 regulate expression of ZIF-1, I depleted *pos-1*, or *mex-5* and *mex-6*, by RNAi in the transgenic strain expressing GFP::H2B<sup>zif-1</sup> (Figure 2.2A). Depletion of *pos-1* by RNAi resulted in derepression of GFP::H2B<sup>zif-1</sup> in germline blastomeres, which can be detected, albeit weakly, as early as P2 (20% embryos scored positive, n = 40), and clearly in P3 (100% embryos scored positive, n = 55). MEX-5 and MEX-6 share high sequence similarity, exhibit identical expression patterns, and have partially redundant functions in vivo, which suggests that they are likely to bind to the same RNA targets. Simultaneous depletion of *mex-5* and *mex-6*, either by RNAi or genetic mutation [*mex-5(zu199);mex-6(pk440)*], resulted in a complete loss of GFP::H2B<sup>zif-1</sup> in embryos (100%, n = 120 and 300, respectively). I also observed abnormal persistence of GFP::PIE-1 ZF1 in *mex-5/6*(RNAi) embryos (100%, n = 90), and precocious degradation in *pos-1*(RNAi) embryos (100% embryos with a reduced GFP in P4, n = 166) (Figure 2.2A), phenotypes previously reported for *mex-5/6*(–) and *pos-1*(–) mutant embryos (DeRenzo et al. 2003; Tabara et al. 1999). The phenotypes generated by either *pos-1*(RNAi) or *mex-5/6*(RNAi), with respect to GFP::H2B<sup>zif-1</sup> expression, are dependent on Region II of the *zif-1* 3' UTR, as depletion of *pos-1* or *mex-5/6* resulted in no change in the expression of GFP::H2B<sup>zif-1ΔII</sup> (n > 50 for

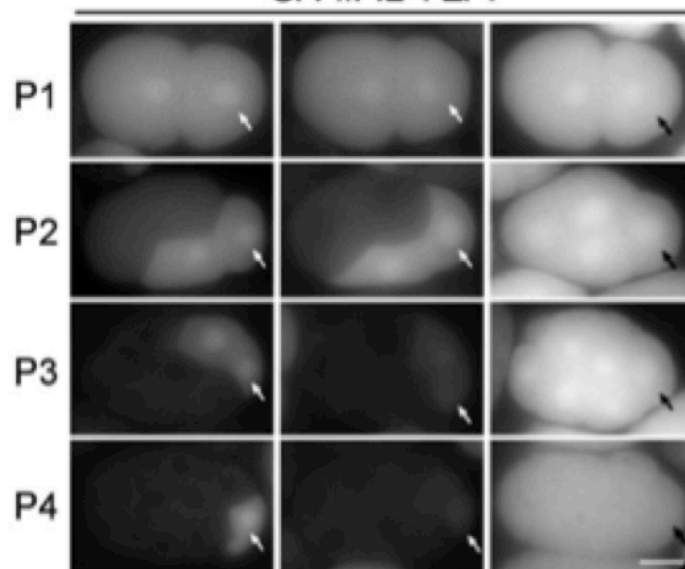
each RNAi, (Figure 2.2B). Taken together, these results support a model whereby POS-1 negatively regulates, and MEX-5/6 positively regulate, the expression of ZIF-1, both via direct binding to Region II of the *zif-1* 3' UTR.

Depletion of *pos-1* or *mex-5/6* results in embryos with altered polarity, which could indirectly affect the expression of GFP::H2B<sup>zif-1</sup>. Ideally, one would generate reporters with the *zif-1* 3' UTR mutated for POS-1 binding sites or MEX-5 binding sites and assay their expression in embryos. The preferred binding sequences for the zinc fingers of POS-1 and MEX-5 in vitro have been investigated using electrophoretic mobility shift (EMSA) and fluorescence polarization (FP) assays (Farley et al. 2008; Pagano et al. 2007). Examination of the *zif-1* 3' UTR identified four likely binding sites for POS-1 in Region II. However, three overlap with putative binding sites for MEX-5 (Figure 2.2C), precluding conclusive mutational analyses.

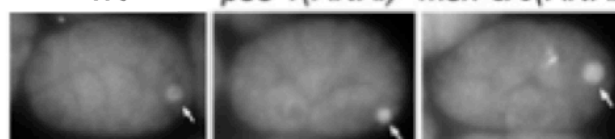
A. wildtype *pos-1(RNAi)* *mex-5/6(RNAi)*  
GFP::*H2B<sup>zif1</sup>*



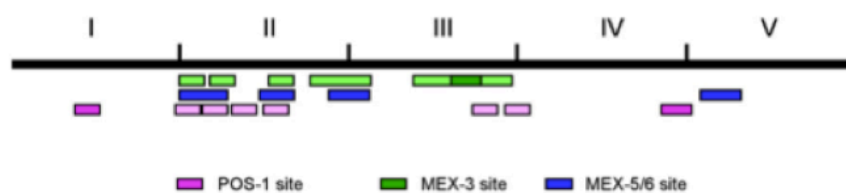
GFP::*PIE-1 ZF1*



B. GFP::*H2B<sup>zif1</sup>ΔII*  
wt *pos-1(RNAi)* *mex-5/6(RNAi)*



C.

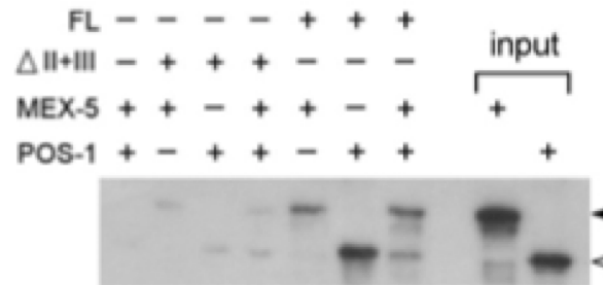




**Figure 2.2: POS-1, MEX-3, and SPN-4 repress whereas MEX-5/6 promote ZIF-1 expression in vivo.** A) Fluorescence micrographs of staged embryos, indicated on the left by the germline blastomere (arrows), expressing GFP::H2B<sup>*zif-1*</sup> (top panels) or GFP::PIE-1 ZF1 (bottom panels) in wildtype, *pos-1*(RNAi), and *mex-5/6*(RNAi) backgrounds. Bar is 10  $\mu$ m B) Embryos expressing GFP::H2B<sup>*zif-1 $\Delta$ III*</sup> in *pos-1*(RNAi) and *mex-5/6*(RNAi) backgrounds. C) Schematic of putative binding sites for POS-1 (pink, 5' UAU2–3RDN1–3G 3'), MEX-3 (green, 5' (A/G/U)(G/U)AGN(0–8)U(U/A/C)UA 3') and MEX-5/6 (blue, minimum 6 U per 8- nucleotide stretch) on the five regions of the *zif-1* 3' UTR (Farley et al., 2008; Pagano et al., 2009; Pagano et al., 2007). Sequences that deviate by one nucleotide from the published optimal POS-1- and MEX-3-binding sites are indicated in lighter pink and lighter green, respectively.

## 2. MEX-5 antagonizes POS-1 binding to the *zif-1* 3' UTR

The overlapping putative POS-1 and MEX-5/6 binding sites within Region II of the *zif-1* 3' UTR suggested that these proteins might have antagonistic functions in *zif-1* expression in cells where POS-1 and MEX-5/6 co-localize. I therefore tested whether POS-1 and MEX-5/6 binding to the *zif-1* 3' UTR in vitro is antagonistic. The biotinylated *zif-1* 3' UTR RNA efficiently pulled down POS-1 or MEX-5 in the in vitro binding assay when either protein was presented alone. However, when both MEX-5 and POS-1 were presented simultaneously, MEX-5 was preferentially pulled down at the expense of POS-1 (Figure 2.3). This result argues that MEX-5 binding to the *zif-1* 3' UTR is preferred over POS-1 binding, and that MEX-5 binding impedes POS-1 binding. All together, these results support an antagonistic function between POS-1 and MEX-5 in the expression of ZIF-1 in vivo. In addition, they provide an explanation for how expression of MEX-5/6 could overcome the repressive function of POS-1 in somatic blastomeres newly separated from germline blastomeres.



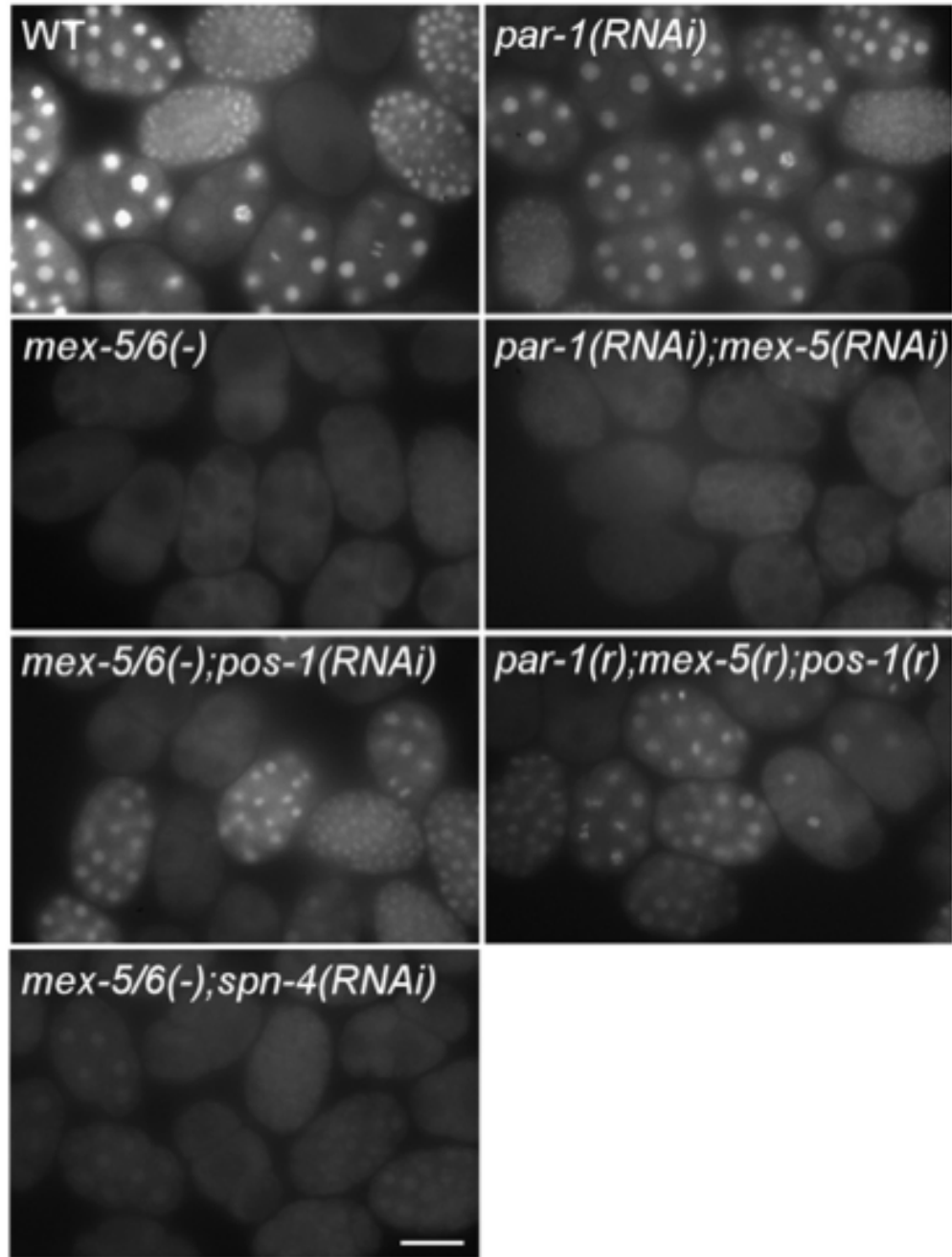
**Figure 2.3: Competition binding between POS-1 and MEX-5**

POS-1 and MEX-5/6 binding to the *zif-1* 3' UTR in vitro is antagonistic. In vitro RNA pulldowns using MEX-5 (arrow head), POS-1 (open arrowhead), or both, and indicated forms of the *zif-1* 3' UTR RNAs were performed as in figure 2.1C. FL = full length

### 3. Two distinct functions for MEX-5/6

In *mex-5(zu199);mex-6(pk440)* mutant embryos, many proteins that are normally localized to the germline blastomeres, such as POS-1, have been shown to be uniformly distributed throughout the embryo (Schubert et al. 2000). Uniform distribution of POS-1, a repressor for *zif-1* expression, could account for, or contribute to, the loss of GFP::H2B<sup>zif-1</sup> expression in *mex-5(zu199);mex-6(pk440)* embryos. Indeed, depletion of *pos-1* by RNAi in *mex-5(zu199);mex-6(pk440)* embryos results in the expression of GFP::H2B<sup>zif-1</sup> in all cells in embryos at the 4-cell stage and older (100%, n > 200; Figure 2.4). This result suggests that ectopic POS-1 accounts for most, if not all, of the loss of *zif-1* expression in *mex-5/6(-)* embryos. In addition, it suggests that MEX-5/6 are not absolutely required for ZIF-1 expression when POS-1 is absent. In wildtype embryos, MEX-5 and MEX-6 levels are low in germline blastomeres due to the germline-blastomere-specific serine/threonine kinase, PAR-1. In *par-1*(RNAi) embryos, MEX-5 and MEX-6 are present at high levels in all early blastomeres (Schubert et al. 2000), and ZIF-1-dependent degradation of CCCH finger-containing proteins is detected in all cells

(DeRenzo et al. 2003). I observed uniform distribution of GFP::H2B<sup>zif-1</sup> in *par-1*(RNAi) embryos that is dependent on MEX-5/6 activity. GFP::H2B<sup>zif-1</sup> is not expressed in *par-1*(RNAi);*mex-5*(RNAi) embryos (0%, n > 200), it is expressed when *pos-1* is also depleted (100% embryos 4-cell and older, n > 200; Figure 2.4). Taken together, these results demonstrate two distinct functions for MEX-5 and MEX-6 as positive regulators of *zif-1* expression. First, MEX-5 and MEX-6 restrict the repressor POS-1 to germline blastomeres, releasing repression in somatic blastomeres. Second, in cells where MEX-5, MEX-6, and POS-1 co-localize, MEX-5/6 compete with POS-1 for overlapping binding sites on the *zif-1* 3' UTR, antagonizing a POS-1 repressive effect.



**Figure 2.4: MEX-5/6 positively regulate *zif-1* expression in embryos by antagonizing POS-1 mediated repression.** Fluorescence micrographs of groups of embryos expressing GFP::H2B<sup>*zif-1*</sup> in the indicated genetic backgrounds. Depletion of *pos-1* suppresses the GFP::H2B<sup>*zif-1*</sup> defect in *mex-5(zu199);mex-6(pk440)* [*mex-5/6(-)*] and *par-1(RNAi);mex-5(RNAi)* embryos. In the bottom right hand panel, (RNAi) was abbreviated to (r) due to space limitation. Bar: 30  $\mu$ M.

### III. MEX-3 and SPN-4 repress translation of *zif-1*

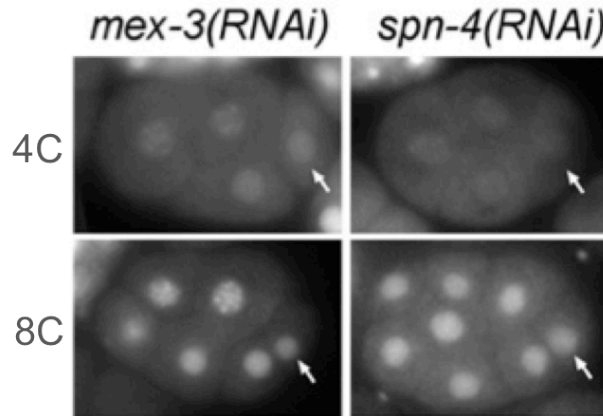
The above results demonstrate that MEX-5/6 are not absolutely required for the expression of *zif-1* in somatic blastomeres. However, the GFP reporter regulated by the *zif-1* 3' UTR lacking Region II (GFP::*H2B<sup>zif-1ΔII</sup>*), and therefore independent of POS-1 and MEX-5/6 regulation (Figures 2.1B and 2.2B), is nonetheless repressed in somatic blastomeres. This observation suggests one or more additional RNA-binding proteins that repress expression of *zif-1* in somatic blastomeres through regions outside of Region II in the 3' UTR. Because POS-1 accounts for most, if not all repression in *mex-5(zu199);mex-6(pk440)* embryos, this hypothetical RBP(s) is expected to be absent or inactive in *mex-5(zu199);mex-6(pk440)* embryos.

#### 1. MEX-3 and SPN-4 bind directly to the *zif-1* 3' UTR

Indeed, through RNA pulldown experiments I have identified two other proteins that exhibit specific binding to the *zif-1* 3' UTR: MEX-3 and SPN-4 (Figure 2.1C). MEX-3 binds to Regions II and III of the *zif-1* 3' UTR. Deleting Region II or III alone results in a modest reduction whereas deleting both Region II and III results in a dramatic reduction in MEX-3 binding to the *zif-1* 3' UTR. It has been shown previously that MEX-3 expression is abolished in *mex-5(zu199);mex-6(pk440)* mutant embryos (Schubert et al. 2000), making it a likely candidate for this predicted RNA-binding protein. SPN-4, on the other hand, although demonstrating *zif-1* 3' UTR sense strand specificity, exhibited less sequence-specificity in its binding. SPN-4 binding was reduced, but not abolished, after pulldown of RNAs corresponding to the *zif-1* 3' UTR deleted of either regions III or IV.

Most dramatically, depletion of either *mex-3* or *spn-4* resulted in uniform

expression of GFP::H2B<sup>*zif-1*ΔII</sup> in all cells as early as in the 4-cell stage (100% embryos 4-cell and older,  $n > 100$  for each RNAi; Figure 2.5). These results together support the model that MEX-3 and SPN-4 repress expression of ZIF-1, also by direct binding to the *zif-1* 3' UTR.



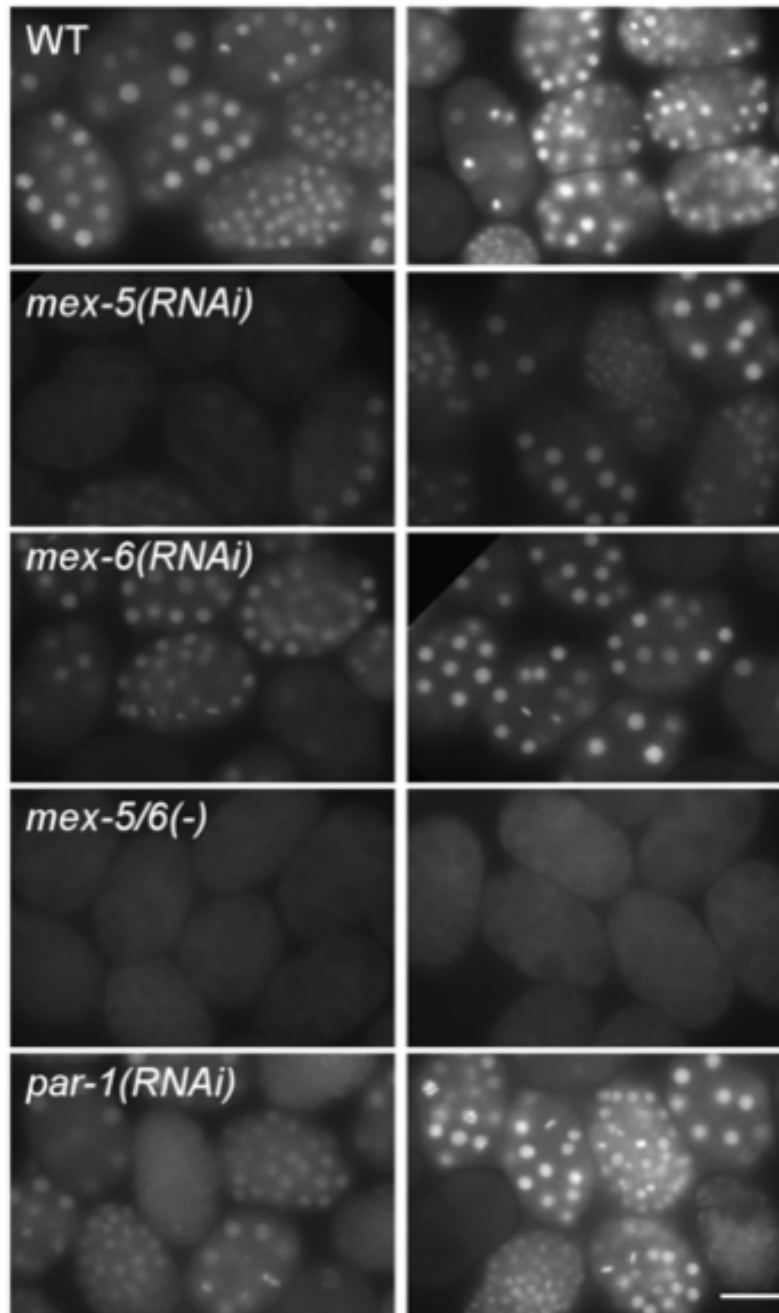
**Figure 2.5: MEX-3 and SPN-4 repress translation of *zif-1***

Fluorescence micrographs of embryos expressing GFP::H2B<sup>*zif-1*ΔII</sup> in the indicated genetic backgrounds. Arrows point to the germline blastomere. Removing *mex-3* or *spn-4* leads to derepression as early as the 4-cell embryo.

## 2. MEX-3 and MEX-5/6 function antagonistically to regulate *zif-1*

The preferred RNA sequence in vitro for the MEX-3 RNA-binding domain has also been determined (Pagano et al. 2009). Analysis of the *zif-1* 3' UTR revealed many possible MEX-3 binding sites, all within Regions II and III, that also overlap putative binding sites for POS-1, MEX-5, or both (Figure 2.2C). Consistent with their partially overlapping binding sites, I could show that MEX-3 and MEX-5/6 function antagonistically in regulating *zif-1* expression in vivo. Embryos depleted of *mex-5* alone exhibit a reduction in GFP::H2B<sup>*zif-1*</sup> expression (Figure 2.6). This reduction can be suppressed when *mex-3* is simultaneously depleted. With *mex-6(RNAi)*, which resulted in

a mild reduction in GFP::H2B<sup>zif-1</sup> expression, a similar suppression was observed. In fact, depletion of *mex-3* in wildtype or *par-1*(RNAi) embryos resulted in a detectable increase in overall levels of GFP::H2B<sup>zif-1</sup>. Depletion of *mex-3* did not suppress *mex-5(zu199);mex-6(pk440)* embryos where MEX-3 is already low or absent (Figure 2.6).



**Figure 2.6: MEX-3 represses *zif-1* expression.**

Fluorescence micrographs of GFP::H2B<sup>*zif-1*</sup> expression in groups of embryos depleted of various proteins (indicated in left column). Each row compares GFP::H2B<sup>*zif-1*</sup> intensities in the indicated genetic backgrounds between *mex-3* depletion (right) or nondepletion (left). Depletion of *mex-3* increases GFP signal except in the *mex-5(zu199);mex-6(pk440)* [*mex-5/6(-)*] background which expresses little or no MEX-3 protein. Bar: 30  $\mu$ M.

**IV. MEX-3 and SPN-4 function together to repress *zif-1***

The result that depletion of either *mex-3* or *spn-4* resulted in a uniform expression of GFP::H2B<sup>*zif-1* $\Delta$ II</sup> in all cells as early as the 4-cell stage (Figure 2.5) was somewhat unexpected, as SPN-4 has always been considered germline blastomere-enriched, and MEX-3 somatic blastomere-enriched (Draper et al. 1996; Ogura et al. 2003). Both SPN-4 and MEX-3 proteins are present at high levels in 1-cell embryos. At each germline blastomere division, SPN-4 protein is initially equal between the two daughters, but is then degraded only in the somatic daughters (see Figure 1.11). MEX-3, on the other hand, is enriched in AB after the first mitotic division. Low levels of MEX-3 remain in germline blastomeres where it, like many RNA-binding proteins identified in germline blastomeres, associates with germline specific P-granules (see Figure 1.11). The uniform expression of GFP::H2B<sup>*zif-1* $\Delta$ II</sup> in *spn-4*(RNAi) and *mex-3*(RNAi) embryos, combined with asymmetric localization of both SPN-4 and MEX-3 after the 2-cell stage, suggests a repressive function for both SPN-4 and MEX-3 in 1- or 2-cell stage embryos.

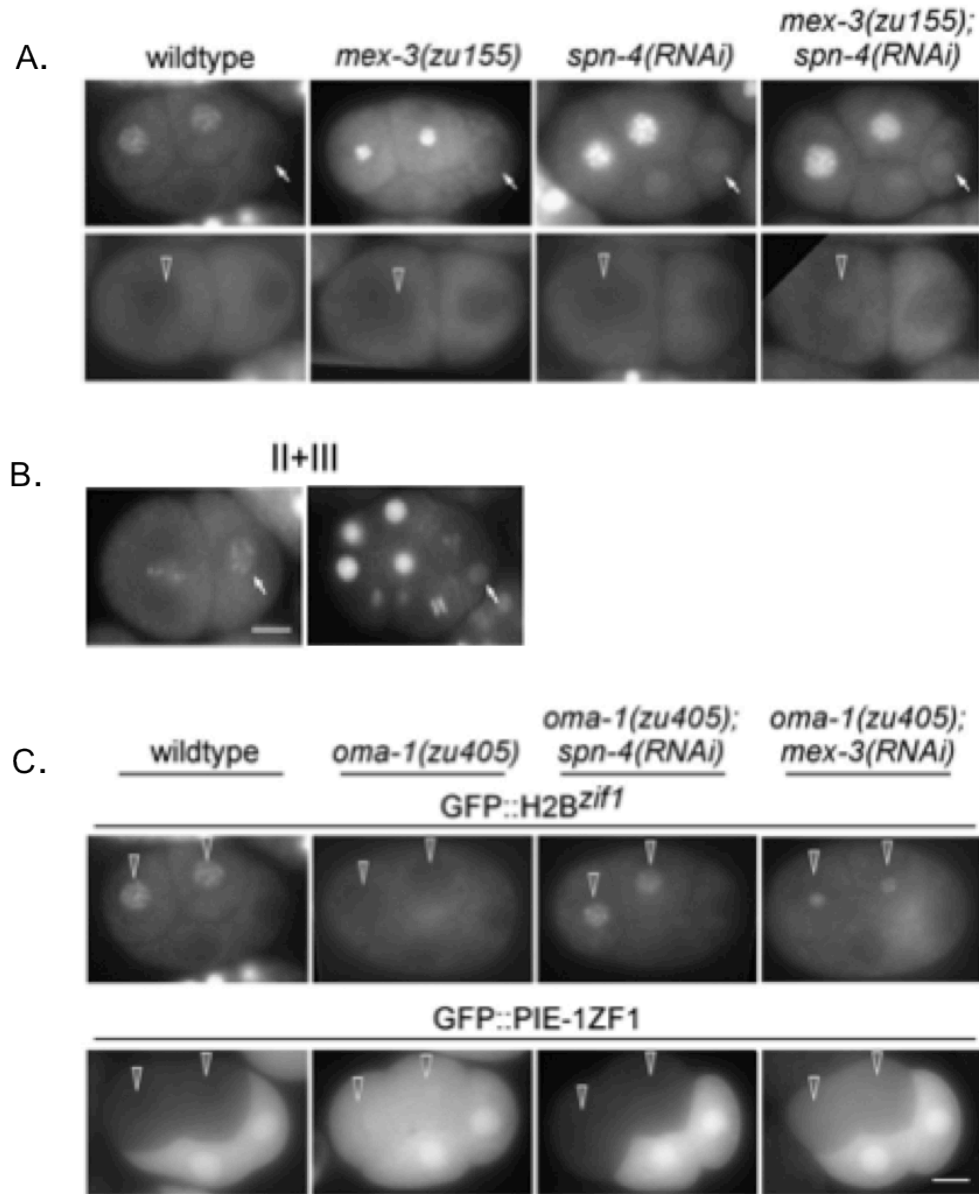
Consistent with the above possibility, depletion of *spn-4* in wildtype embryos by RNAi resulted in the detection of GFP::H2B<sup>*zif-1*</sup> in P2 and EMS of very young 4-cell embryos (100%, n = 120, Figure 2.7A). GFP::H2B<sup>*zif-1*</sup> can also be detected in both P2 and EMS of early 4-cell embryos derived from *mex-3(zu155)* mutant worms (100%, n = 40). For *mex-3(zu155)* worms that are also depleted of *spn-4* by RNAi, GFP::H2B<sup>*zif-1*</sup> signal



can be detected, albeit weakly, as early as 2-cell embryos (10%,  $n = 50$ ). Further supporting the early repressive function of SPN-4, I showed that a GFP::H2B reporter regulated by only Regions II and III of the *zif-1* 3' UTR (GFP::H2B<sup>*zif-III+III*</sup>), a region not bound by SPN-4 in our in vitro binding assay (Figure 2.1C), is properly repressed in oocytes, where repression is OMA-1/2-dependent (Güven-Ozkan et al. 2010), but uniformly expressed in embryos as early as the 2-cell stage (Figure 2.7B).

MEX-3 and SPN-4 have been shown to bind each other in a yeast 2-hybrid assay (Huang et al. 2002). Two lines of evidence support a model that these two proteins function together in regulating *zif-1* expression. First, neither SPN-4 nor MEX-3 appears to be sufficient on its own for the translational repression of *zif-1*, as shown by the following three examples. (1) Depletion of *pos-1* results in derepression of *zif-1* in germline blastomeres, despite a high level of SPN-4 (and low MEX-3) (Fig. 2A). (2) SPN-4, like POS-1, is uniformly distributed in *mex-5(zu199);mex-6(pk440)* embryos (100%,  $n = 40$ ), a genetic background where MEX-3 protein is absent (Schubert et al., 2000). However, POS-1, but not SPN-4, is responsible for the *zif-1* repression in *mex-5(zu199);mex-6(pk440)* embryos. Depletion of *pos-1*, but not *spn-4*, restored GFP::H2B<sup>*zif-1*</sup> expression in *mex-5(zu199);mex-6(pk440)* embryos (Figure 2.4). (3) Similarly, I observed no ectopic repression of GFP::H2B<sup>*zif-1*</sup> in *spn-4(RNAi)* embryos (data not shown), which have been shown to maintain high MEX-3 levels in all blastomeres until approximately the 28-cell stage (Huang et al. 2002). The second line of evidence supporting SPN-4 and MEX-3 acting together comes from analysis in the *oma-1(zu405)* genetic background. Our lab showed previously that the *oma-1(zu405)* missense mutation results in OMA-1 protein persisting past the 1-cell stage and repressing translation of *zif-*

*I* in somatic blastomeres (Güven-Ozkan et al. 2010; Nishi and Lin 2005). The ectopic *zif-1* repression in *oma-1(zu405)* embryos is most apparent in 4-cell embryos, and progressively lessens in older embryos (Güven-Ozkan et al., 2010). Degradation of many maternally-supplied proteins is also defective in *oma-1(zu405)* embryos, including MEX-5/6, POS-1, MEX-3, and SPN-4 (Lin 2003). I find that depletion of *mex-3* or *spn-4*, but not *pos-1* or *mex-5/6*, partially suppresses the ectopic repression of *zif-1* [40%, n = 20 for *mex-3*(RNAi) and 70%, n = 20 for *spn-4*(RNAi)] and degradation of GFP::PIE-1 ZF1 in *oma-1(zu405)* embryos [(50%, n = 50 for *mex-3*(RNAi) and 85%, n = 50 for *spn-4*(RNAi); Figure 2.7C).



**Figure 2.7: MEX-3 and SPN-4 function together to repress *zif-1* expression.**

A) Fluorescence micrographs of GFP::H2B<sup>zif-1</sup> expression in 2-cell and 4-cell wildtype, *mex-3(zu155)*, *spn-4(RNAi)*, or *mex-3(zu155); spn-4(RNAi)* embryos. GFP::H2B<sup>zif-1</sup> signal can be detected in all blastomeres in early 4-cell embryos upon depletion of *mex-3*, *spn-4*, or both. GFP was detected in some 2-cell embryos depleted of both genes. B) Fluorescent micrographs of embryos expressing GFP::H2B<sup>zif-1 II+III</sup>. GFP::H2B is derepressed in all blastomeres when only regions II and III are presented. C) Fluorescence micrographs of 4-cell embryos expressing GFP::H2B<sup>zif-1</sup> or GFP::PIE-1 ZF1 in the indicated genetic backgrounds. GFP::H2B<sup>zif-1</sup> expression in somatic blastomeres (arrows) is ectopically repressed in *oma-1(zu405)* embryos. The GFP::H2B<sup>zif-1</sup> defect in *oma-1(zu405)* is suppressed when *mex-3* or *spn-4* is depleted. A reciprocal pattern is seen in embryos carrying GFP::PIE-1 ZF1. Bar: 10  $\mu$ M.

## V. Expression of *zif-1* in newly formed somatic cells

One interesting question that remains is how *zif-1* expression is turned on in somatic daughters (EMS, C, and D) newly divided from a germline blastomere where *zif-1* is actively repressed. Although POS-1 and SPN-4 are germline-enriched proteins, their levels are initially equal and high between newly formed somatic cells and their sister germline blastomeres (Ogura et al. 2003; Tabara et al. 1999). Therefore, initiation of *zif-1* expression cannot be explained by the asymmetric localization of its repressors, POS-1 and SPN-4. The levels of MEX-5/6, the positive regulators of *zif-1* expression, are coincidentally increased in all newly formed somatic blastomeres (Schubert et al. 2000). Because MEX-5/6 antagonizes repression by POS-1, it is possible that an increase in MEX-5/6 levels is sufficient to allow *zif-1* expression in these newly-formed somatic blastomeres. In addition, I considered whether two other factors contribute to the initiation of *zif-1* expression in newly formed somatic cells. First, levels of MEX-3 protein, which negatively regulates *zif-1* expression and is a likely cofactor for SPN-4, are low in newly formed somatic blastomeres (Draper et al. 1996), and this might be important to relieve repression. Second, degradation of POS-1, which is both a substrate of ZIF-1 as well as a repressor of *zif-1* expression, could initiate a positive feedback loop that facilitates *zif-1* expression to high levels. SPN-4 is subsequently degraded by a yet unknown mechanism.

### 1. Expression of *zif-1* is decreased when MEX-3 is ectopically expressed

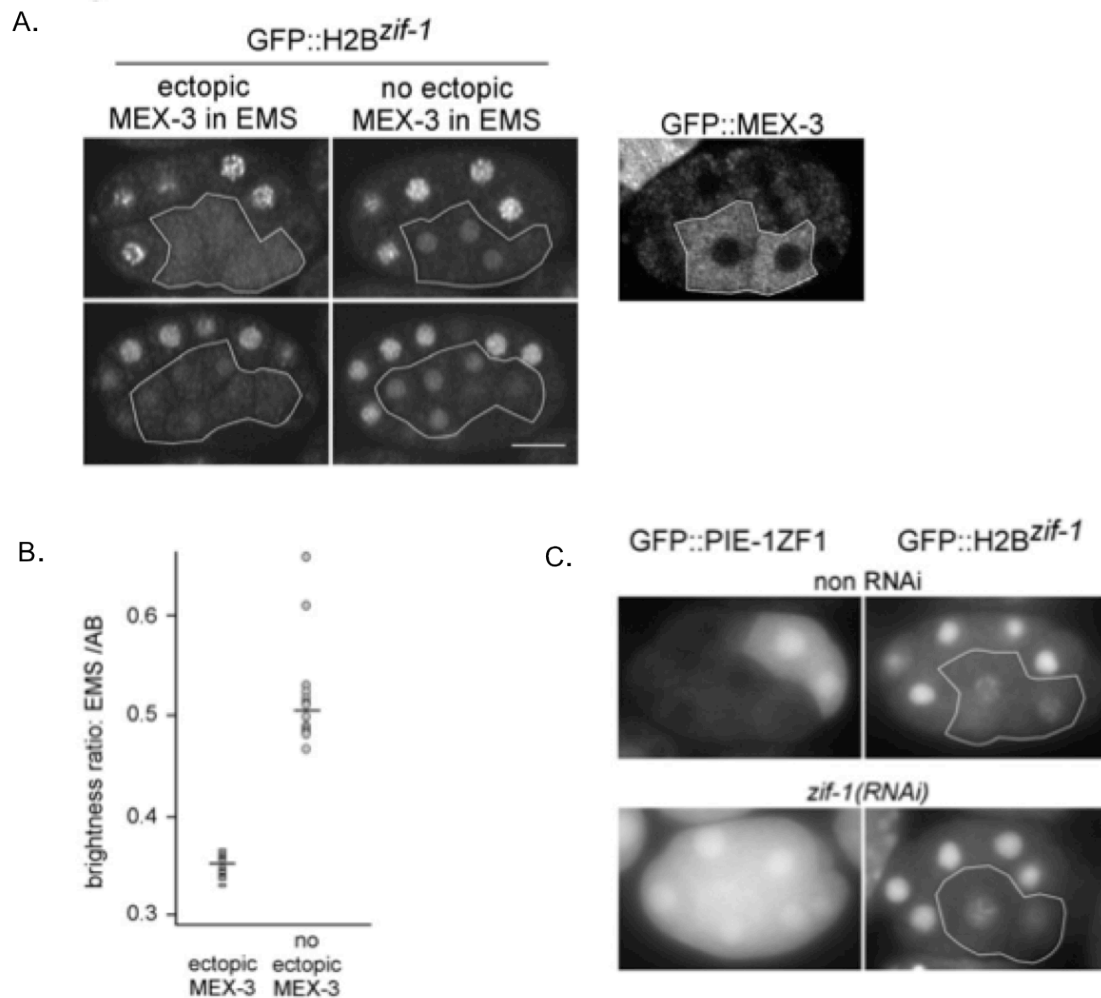
If a low level of MEX-3 protein is critical for the onset of translation of *zif-1*, then in embryos in which MEX-3 is ectopically expressed in the EMS lineage, GFP:: $\text{H2B}^{\text{zif-1}}$

expression should be decreased or abolished. The *med-1* promoter has been shown to drive GFP-fused transgenic proteins specifically in the EMS lineage (Maduro et al. 2001). I was unable to use two different fluorescent tags for this experiment for technical reasons: H2B<sup>*zif-1*</sup> tagged with *mCherry* exhibited slower maturation and degradation than GFP and I was unable to recover *mCherry::MEX-3* expressing lines for unknown reasons. However, because MEX-3 is expressed exclusively in the cytoplasm (Figure 2.8A) and GFP::H2B<sup>*zif-1*</sup> is primarily nuclear, I was able to assay the effect on GFP::H2B<sup>*zif-1*</sup> expression by confocal microscopy of embryos also expressing cytoplasmic GFP::MEX-3. In addition, cytoplasmic GFP::MEX-3 is degraded very rapidly and is mostly undetectable in 28-cell stage embryos, the stage at which I analyzed the effect on nuclear GFP::H2B<sup>*zif-1*</sup> expression (Figure 2.8A). At the 28-cell stage, there are six blastomeres derived from the EMS blastomere, four from MS and two from E blastomeres. I measured nuclear GFP signal in the EMS descendants and AB descendants on the same focal planes. Nuclear GFP signal in the four MS descendants and two E descendants is presented as a ratio to the nuclear GFP signal in the AB blastomeres of the same embryo. Nuclear GFP in EMS descendants is significantly lower in embryos expressing ectopic GFP::MEX-3 than embryos without ectopic GFP::MEX-3 (Figure 2.8A and B). This supports the model that a low level of MEX-3 protein in newly formed somatic blastomeres is important to relieve *zif-1* translational repression.

## **2. Degradation of POS-1 is not important for translation of *zif-1***

To test whether degradation of POS-1 in EMS is required for translation of *zif-1* in EMS, I depleted *zif-1* by RNAi and assayed the onset of GFP::H2B<sup>*zif-1*</sup> signal in EMS

compared to that in nonRNAi embryos. I also depleted *zif-1* in the strain expressing GFP::PIE-1 ZF1 in a parallel experiment as a control to monitor the effect of *zif-1*(RNAi) on the degradation of ZIF-1 targets. *zif-1*(RNAi) resulted in a defect in the degradation of, and therefore uniform distribution of, GFP::PIE-1 ZF1 in embryos up to at least the 20-cell stage (100%,  $n > 100$ ). Despite successful inhibition of GFP::PIE-1 ZF1 degradation, I observed no difference in the timing of onset of expression for GFP::H2B<sup>*zif-1*</sup> in the EMS lineage (0%,  $n = 30$ ; Figure 2.8C). This result suggests that degradation of POS-1 is not important for translation of *zif-1* in EMS.



**Figure 2.8: Low MEX-3 levels are important for *zif-1* expression in newly-formed somatic cells.** A) Top two rows: confocal microscopy images of embryos expressing GFP::H2B<sup>*zif-1*</sup> with (left column) or without (right column) ectopic GFP::MEX-3 in the EMS lineage descendants (outlined). Top row: 16-cell stage. Second row: the same embryos at 28-cell stage. The bottom panel shows the exclusive cytoplasmic signal from GFP::MEX-3 in a 12-cell embryo without GFP::H2B<sup>*zif-1*</sup>. B) Quantification of GFP::H2B<sup>*zif-1*</sup> signal in embryos with or without GFP::MEX-3 in the EMS lineage of 28-cell embryos. Y axis represents the ratio of average nuclear GFP intensity in the EMS lineage to that in the AB lineage in individual embryos. Thirteen embryos of each genotype were scored. —=mean. C) Fluorescence micrographs of embryos expressing GFP::PIE-1 ZF1 (left column) or GFP::H2B<sup>*zif-1*</sup> (right column) in wildtype and *zif-1*(RNAi) backgrounds. The onset of GFP::H2B<sup>*zif-1*</sup> expression remains unchanged upon *zif-1*(RNAi). Bar: 10  $\mu$ M.

## Discussion

Spatial and temporal restriction of ZIF-1 expression exclusively to somatic blastomeres is critical to achieve localization of PIE-1 specifically to germline blastomeres. I showed in this chapter that the combination of both repression in germline blastomeres and activation in somatic blastomeres results in somatic cell-specific expression of *zif-1*. Multiple RNA-binding proteins, which individually can either activate or repress *zif-1* expression via direct binding to the *zif-1* 3' UTR, function in a combinatorial fashion to effect either net repression or net activation of *zif-1* translation. Many of these RNA-binding proteins physically interact with one another as well as compete for the same or overlapping binding sites (Figure 2.9). As *zif-1* RNA is maternally supplied and uniformly distributed in early embryos, I believe that the effect on ZIF-1 expression by these RNA-binding proteins is primarily through translational regulation.

I have shown that POS-1, SPN-4, and MEX-3 all repress expression of the *zif-1* reporter (Figure 2.9). MEX-3 and SPN-4 have been shown to physically interact (Huang

et al. 2002). The results that both MEX-3 and SPN-4 are required for complete repression of ZIF-1 expression support a model that they function together to effect *zif-1* repression. However, I cannot rule out the possibility that MEX-3 and SPN-4 function in parallel pathways. It is possible that physical binding between these two proteins stabilizes their binding to target RNA(s). Further biochemical analyses will be needed to address this possibility. I propose that wherever MEX-3 and SPN-4 co-localize, such as P0, they repress translation of *zif-1*. After the first division, both MEX-3 and SPN-4 are initially present at high levels in both daughters (Draper et al. 1996; Ogura et al. 2003). Therefore, *zif-1* continues to be repressed in both blastomeres. Translational repression of *zif-1* in AB is lifted following degradation of SPN-4 later in the cell cycle through an unidentified mechanism, which results in ready detection of *zif-1* translational reporters in ABa and ABp. Meanwhile, MEX-3 decreases in level in P1, and becomes primarily localized to the germline P-granules. I do not believe that MEX-3 in P-granules functions in conjunction with SPN-4 to repress ZIF-1 expression, as depletion of POS-1 resulted in the derepression of the *zif-1* transgene in later germline blastomeres where SPN-4 and P-granule-bound MEX-3 are present (Draper et al. 1996; Ogura et al. 2003). It is often observed that the detection of reporter GFP is delayed by one cell cycle compared to the onset of expression of the endogenous protein. Therefore I suggest that continued repression of *zif-1* in the P lineage is maintained by the function of POS-1, most likely beginning with the P2 blastomere. My finding that POS-1 and MEX-3 repress translation of *zif-1* is consistent with previous findings that reduced levels of PIE-1, and therefore derepression of zygotic transcription, were detected in P4 blastomeres of both *pos-1(-)* and *mex-3(-)* mutant embryos (Tabara et al. 1999; Tenenhaus et al. 1998).



In EMS, the somatic daughter of the germline blastomere P1, levels of the translational repressors POS-1 and SPN-4 are initially high but *zif-1* is nonetheless translated. I find that translation of *zif-1* results from the combination of low levels of MEX-3, without which SPN-4 does not repress, along with a high level of activators MEX-5 and MEX-6, which antagonize repression by POS-1. Ectopic expression of MEX-3 in EMS, where SPN-4 levels are high, is sufficient to prevent or delay *zif-1* translation.

Translation of *zif-1* in somatic blastomeres requires MEX-5 and MEX-6. I show that MEX-5/6 have dual roles in promoting translation of *zif-1* in somatic blastomeres. First, they restrict POS-1, along with several other proteins, to germline blastomeres through their function in maintaining embryonic polarity (Schubert et al. 2000). Second, they bind to the *zif-1* 3' UTR, enabling its translation. However, binding of MEX-5/6 to the *zif-1* 3' UTR is not absolutely required for ZIF-1 translation, as evidenced by ZIF-1 expression in *pos-1;mex-5;mex-6* embryos. Instead, MEX-5/6 binding to the *zif-1* 3' UTR enables ZIF-1 expression by preventing binding of repressors to the same region. This model is supported by the extensive overlap of MEX-5/6 and POS-1 binding sites in Region II, and that MEX-5 binding impedes POS-1 binding to the *zif-1* 3' UTR in our in vitro assay. The antagonistic functions between MEX-5/6 and POS-1 are consistent with previous genetic analyses showing that a reduced dosage of *pos-1* suppresses the *mex-5* mutant phenotype (Tenlen et al. 2006). The results from this chapter present a molecular mechanism for the requirement for MEX-5/6 in ZIF-1-mediated target protein degradation (DeRenzo et al. 2003). Furthermore, these results explain how MEX-5/6 can be expressed at a high level in cells where ZIF-1 degrading activity is also high, and how

MEX-5/6 promote ZIF-1 degrading activity while simultaneously being substrates of ZIF-1 themselves (DeRenzo et al. 2003).

The region of the *zif-1* 3' UTR bound by MEX-5/6 also partially overlaps with the binding sites for MEX-3 and OMA-1/2, three other key repressors of *zif-1* translation (Figure 2.9B) (Guven-Ozkan et al. 2010; Pagano et al. 2009). I find that MEX-5/6 also antagonize repression of *zif-1* by MEX-3 and OMA-1/2. I did not, however, observe a clear competitive advantage for MEX-5 binding over MEX-3 binding in vitro pull down assays. It is possible that the mechanism by which MEX-5/6 antagonize repression by MEX-3 is different from that utilized against POS-1. MEX-5/6 bind to MEX-3 in a yeast 2-hybrid assay (Huang et al. 2002). Therefore, MEX-5/6 could antagonize translational repression by MEX-3 through a protein–protein interaction, preventing MEX-3 from binding to the *zif-1* 3' UTR. In cells where activators MEX-5/6 and their competing repressors, POS-1, MEX-3, or OMA-1/2 co-localize, the outcome regarding *zif-1* translation will likely be determined by the relative abundance of MEX-5/6 and their competing repressors.

POS-1 is both a repressor of *zif-1* translation as well as a target of ZIF-1-mediated degradation. This suggests that a positive feedback loop, whereby degradation of some POS-1 leads to further relief from POS-1-mediated repression, could accelerate the relief from translational repression of *zif-1* by POS-1 in newly formed somatic sisters of P lineage blastomeres. However, I detected no difference in the expression of GFP::H2B<sup>*zif-1*</sup> when *zif-1* was depleted by RNAi, suggesting that such a positive feedback loop does not play a major role in the onset of *zif-1* translation in EMS. It is likely that the level of MEX-5/6 in EMS is already sufficiently higher than that of POS-1 such that any further

reduction of POS-1 levels would be inconsequential for the onset of *zif-1* translation. MEX-5/6 are themselves also targets of ZIF-1 degradation (DeRenzo et al. 2003), suggesting a possible negative feedback loop for *zif-1* translation which would limit the duration of ZIF-1 translation in somatic blastomeres. The effect of *zif-1* RNAi on the onset of GFP::H2B<sup>zif-1</sup> expression might therefore be compounded by the simultaneous inhibition of both positive and negative feedback loops.

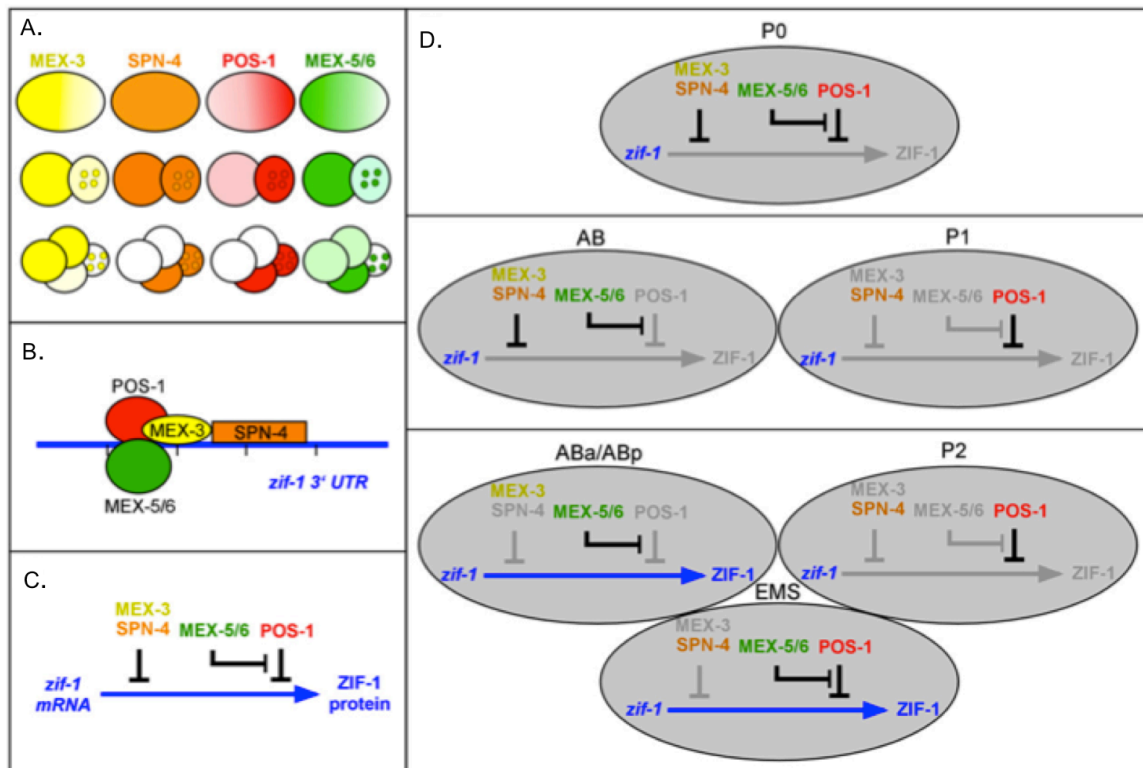
Transcriptional repression is critical for maintaining both the stability and the totipotency of the germline. Proper development of the germline is, therefore, one of the most critical processes to occur during *C. elegans* early embryogenesis. However, the mechanism that has evolved to segregate PIE-1 to the germline precursors is not 100% effective, and some PIE-1 remains in the somatic sisters following division (Reese et al. 2000). Cell cycles last only 10–15 min in early *C. elegans* embryos (Seydoux and Fire 1994), therefore any residual PIE-1 in somatic cells would interfere with the rapid onset of zygotic transcription that is crucial for somatic development. Asymmetric translation of ZIF-1 in somatic blastomere ensures rapid degradation of PIE-1 in somatic blastomeres.

To have so many RNA-binding factors, acting both positively and negatively, regulating the translation of *zif-1* simply to establish a somatic cell-specific translation pattern might seem to be overly complicated. Particularly counterintuitive is the discovery that *zif-1* expression is repressed by MEX-3, a protein enriched in somatic cells where *zif-1* is ultimately expressed. I suggest that *C. elegans* embryos are faced with a particularly unique challenge due to two aspects of their developmental program: (1) the way in which their germ cell precursors are specified (Strome and Lehmann 2007), and

(2) the fact that many maternally-supplied proteins, including almost all key RNA-binding proteins, are already translated prior to fertilization. It makes good sense for worms to utilize the germline blastomere-specific protein, POS-1, to repress the translation of *zif-1* in germline blastomeres, and somatic blastomere-specific proteins, MEX-5 and MEX-6, to compete with POS-1 and promote translation in newly formed somatic blastomeres. However, POS-1 and MEX-5/6 are all maternally-supplied proteins and are all present at a high level in the 1-cell embryo, P0, before being asymmetrically segregated to germline and somatic blastomeres, respectively. P0, like the other P lineage blastomeres, is a precursor for both somatic and germline blastomeres. High levels of POS-1, MEX-5, and MEX-6 in P0 would be predicted to promote the translation of *zif-1*, leading to precocious degradation of PIE-1 and embryonic lethality. *C. elegans* circumvents this dilemma by utilizing two additional RNA-binding proteins, SPN-4 and MEX-3, whose expression patterns only overlap in the 1-cell and 2-cell embryos, to function together to repress *zif-1* translation in 1- and 2-cell embryos. After the 2-cell stage, repression by SPN-4 and MEX-3 is relieved, and germline versus somatic regulation of ZIF-1 expression initiates. Repression in germline blastomeres is continued by the germline protein POS-1, and embryos only need to control *zif-1* translation in newly-formed somatic blastomeres.

I propose that multiple RNA-binding proteins, expressed in individual dynamic temporal and spatial patterns, function in a combinatorial manner to prevent the precocious translation of *zif-1*, localizing ZIF-1 activity strictly to the somatic blastomeres. Such regulation is essential for the stability of PIE-1 in germline blastomeres, global transcriptional repression in the germline precursors, and ultimately

the maintenance of germline integrity and totipotency.



**Figure 2.9: Model of *zif-1* regulation.**

Schematic representation showing (A) endogenous expression patterns of MEX-3 (yellow), SPN-4 (orange), POS-1 (red), and MEX-5/6 (green) in 1-, 2- and 4-cell embryos. B) The deduced binding sites on the *zif-1* 3' UTR for MEX-3, SPN-4, POS-1, and MEX-5/6 based on the RNA-binding assays. C) The proposed effects of these RNA-binding factors on *zif-1* translation. D) Proposed regulation of *zif-1* translation in each early blastomere, as a result of the combinatorial effects of MEX-3, SPN-4, POS-1, and MEX-5/6, resulting from the dynamic temporal and spatial localization of each protein. Gray arrow: no ZIF-1 expression; blue arrow: ZIF-1 expression. See text for details.

## CHAPTER 3: REGULATION OF MATERNAL WNT mRNA IN *C. ELEGANS* EMBRYOS

### Summary

In this chapter I characterized the translational regulation of maternally-supplied *mom-2* mRNA, which encodes a Wnt ligand essential for two separate cell-cell interactions in early embryos. A GFP reporter that includes only the *mom-2* 3'UTR is translationally repressed properly in oocytes and early embryos, and then correctly translated only in the known Wnt signaling cells. I show that the spatiotemporal translation pattern of this reporter is regulated combinatorially by a set of nine maternally-supplied RBPs. These nine proteins all directly bind the *mom-2* 3'UTR in vitro and function as positive or negative regulators of *mom-2* translation in vivo. The net translational readout for the *mom-2* 3'UTR reporter is determined by competitive binding between positive- and negative-acting RBPs for the 3'UTR, along with the distinct spatiotemporal localization patterns of these regulators. The 3'UTR of maternal mRNAs therefore appears to contain a combinatorial code that determines the topography of associated RBPs, integrating positive and negative translational inputs.

### Introduction

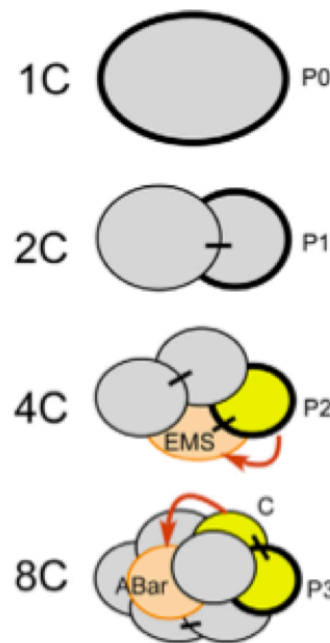
Precise patterns of gene expression during development are regulated predominantly at the level of transcription. In transcription-driven gene regulation, transcriptional activators bind to enhancer elements and orchestrate the co-localization of proteins at the gene promoter to activate transcription. The transcriptional status of a

particular gene in a cell or developing tissue is, therefore, determined primarily by the precise combination of transcription factors that can bind to the enhancer sequence in that specific cell. However, my work presented in chapter 2 shows that there are clear exceptions to the transcription-driven gene regulation.

Correct spatiotemporal translation of the majority of germline mRNAs in *C. elegans* is controlled via the 3'UTR (Merritt et al. 2008) as I discussed in chapter 2 for *zif-1* maternal mRNA (Güven-Ozkan et al. 2010). I showed that correct spatiotemporal translation of maternal *zif-1* mRNA requires seven maternally-supplied RBPs - OMA-1, OMA-2, POS-1, SPN-4, MEX-3, MEX-5, and MEX-6. Translation of *zif-1* mRNA is repressed by OMA-1 and OMA-2 in oocytes, by MEX-3 and SPN-4 in 1- and early 2-cell embryos, and by POS-1 in germline blastomeres P2-P4. In somatic blastomeres, MEX-5 and MEX-6 relieve translational repression by outcompeting POS-1 for binding to the *zif-1* 3'UTR.

In this chapter, I characterized the translational regulation of maternally-supplied *mom-2* mRNA using a reporter carrying the *mom-2* 3'UTR. *mom-2* encodes a Wnt ligand essential for two Wnt-mediated cell-cell interactions during *C. elegans* early embryogenesis (Rocheleau et al. 1997; Thorpe et al. 1997; Park and Priess 2003; Walston et al. 2004). The first MOM-2/Wnt signal occurs at the 4-cell stage when P2 signals EMS, whereas the second signal occurs at the 8-cell stage when C, the somatic daughter of P2, signals ABar (Figure 3.1). The P2-to-EMS Wnt signal specifies the fate of E, the EMS posterior daughter, as the sole intestinal precursor (Rocheleau et al. 1997; Thorpe et al. 1997; Goldstein 1992). In embryos deficient in this interaction, E adopts the fate of its sister, MS, which produces no intestinal cells. The Wnt signal from C orients the

division axis of ABar toward C (Park and Priess 2003; Walston et al. 2004). P2 and both P2 daughters, C and P3, display MOM-2/Wnt signaling activity, whereas no P2 precursor cells or any other cells at the 4- or 8-cell stage do (Park and priess 2003). However, the mechanism by which MOM-2 activity is spatially and temporally restricted within the early embryo remained unknown.



**Figure 3.1: Known Wnt signaling events**

Illustration of two known MOM-2-dependent interactions (red arrows) in early embryos. Stages of embryos shown in all figures are indicated by the number of blastomeres. Blastomeres exhibiting MOM-2-dependent signaling capability are in yellow. Signal-receiving cells are in orange. Short bars connect sister blastomeres. Germline blastomeres are marked with a thick outline

In this chapter I will present work to show that maternally-supplied MOM-2/Wnt activity is regulated post-transcriptionally through the *mom-2* 3'UTR. The results discussed in this chapter were published in *Development* in 2013. Nine maternal RBPs, many shown previously to also regulate maternal *zif-1* expression, bind the *mom-2*



3'UTR in vitro and combinatorially regulate proper spatiotemporal translation of a *mom-2* 3'UTR reporter in vivo. OMA-1 and OMA-2 repress translation in oocytes whereas MEX-3 and MEX-5/6 repress translation in the early embryo. PIE-1, POS-1, and MEX-1 are required to promote translation in the embryo. Some of the positive and negative regulators bind to the same region and show competitive binding to the *mom-2* 3' UTR thereby suggesting a similar regulatory mechanism as was shown for *zif-1* regulation. The combinatorial codes within the 3' UTR, however, are found to be distinct for the expression of the *mom-2* and *zif-1* reporters.

## Materials and Methods

### Strains

LGI, *spe-9(hc88ts)*, LGII, *mex-1(zu121)*, *teIs127(P<sub>pie-1</sub>-gfp::h2b-UTR<sup>mom-2</sup>)*; LGIII, *unc-119(ed3)*, *pie-1(zu154)*; LGIV, *oma-1(zu405d)*, *oma-1(zu405d te33)*; LGV, *oma-2(te51)*, *pos-1(zu148)*, *tnIs17[unc-119(+), pCS410 (oma-1:s:tev:gfp)]*. Strains used are: TX1377 *teIs127(P<sub>pie-1</sub>-gfp::h2b-UTR<sup>mom-2</sup>)*, DG2460 *spe-9(hc88ts); oma-1(zu405d te33)*, DG2581 *spe-9(hc88ts); oma-1(zu405d te33); tnIs17*, DG2620 *unc-119(ed3); oma-1(zu405d te33); oma-2(te51); tnIs17*. *tnIs17* rescues *oma-1(zu405d te33); oma-2(te51)* sterility. All integrated lines were generated by bombardment (Praitis et al. 2001).

### Plasmid construction

Most plasmids were constructed using Gateway technology (Guven-Ozkan et al. 2010). A 557 bp genomic sequence beginning 100 bp upstream of the *mom-2* stop codon was cloned downstream of *pie-1* promoter-driven GFP::H2B in the vector (Guven-Ozkan

et al., 2010; Reese et al., 2000). pCS410 was created by modifying plasmid pRL475 (Lin 2003). DNA encoding a codon-optimized S tag and tobacco etch virus (TEV) protease cleavage site was created using gene synthesis (Hoover and Lubkowski 2002) and ligated into a *NheI* site created between OMA-1 and GFP.

### RNA interference and imaging

Feeding RNAi was performed by feeding L1 larvae for 2 days at 25°C as described in chapter two. All images were acquired, processed, and quantified as described in chapter two. Spindle orientation was assayed using sequential DIC Z-stacks.

### Single molecule mRNA FISH

Probes were purchased from Biosearch Technologies and hybridization was performed as described (Raj and Tyagi, 2010) with few modifications below. Embryos were collected by cutting adult hermaphrodites on poly-lysine coated slides, squashed with a coverslip, incubated in 3.7% formaldehyde for 15 minutes, and frozen on dry ice. Upon removal of the coverslip, embryos were treated with 95 % ethanol/ 10 minutes/ -20°C, 95% ethanol/ 5 minutes/ 20°C, 70% ethanol/ 3 hours/ 4°C, 400 ml wash buffer/ 10 minutes, 100 ml probe/ overnight/ 37°C, three 20-minute 37°C washes, and then mounted. All probes were coupled to CAL590 (1:2.5 dilution). Wash buffer consists of 10% formamide and 2X SCC buffer, hybridization buffer is the same as wash buffer plus 1g/ml dextran sulfate.

### Recombinant proteins and RNA binding assays

MBP-tagged proteins were purified from *E. coli* and Flag-PIE-1 was purified from HeLa cells (Li et al. 2009 and methods in chapter 2). HIS-IFET-1 was purified from *E. coli* as follows. Bacterial cultures were grown at 20 degrees and induced overnight with 0.1 mM IPTG at 16 degrees. After bacterial cultures were spun down, pellets were resuspended in 10mM TRIS, 150mM NaCl, 5mM imidazole, 100 ug/ml lysozyme, protease inhibitor and 1.5% sarkosyl. Bacteria was lysed and bound to talon resin as described in chapter 2. Protein was eluted with 1M imidazole. Biotinylation of RNA and pull-downs were performed as described (Lee and Schedl 2001; Guven-Ozkan et al. 2010). The optimal amounts of purified protein and *mom-2* 3'UTR RNA were empirically determined by titration. A typical binding reaction contained 150 ng of the purified protein (34.5, 30.3, 34.1, 31.5, 41.5, 36.7, and 76.3 nM, respectively, for MBP-tagged OMA-1, MEX-1, MEX-3, MEX-5, POS-1, SPN-4, and Flag-PIE-1) and 400 ng of biotinylated RNA. For the competition binding assays, RNA was kept limiting compared to proteins used (1 mg of each MBP-tagged protein and 130 ng RNA, except for the SPN-4/PIE-1 competition, where 750 ng MBP-SPN-4 and 400 ng FLAG-PIE-1 were used).

#### Purification of OMA-1 and associated RNAs from adult worms

OMA-1:S:TEV:GFP was purified as described (Cheeseman et al. 2004; Cheeseman and Desai 2005) with modifications. Young adults were sonicated in 50 mM Hepes (pH7.5), 100 mM KCl, 1mM MgCl<sub>2</sub>, 10% glycerol, 0.05% NP-40, and protease inhibitors (complete EDTA-free, Roche). OMA-1:S:TEV:GFP was purified from 1 ml of medium-speed supernatant (18,000g/2x10 min, supplemented to 300 mM KCl and 200

units of RNAsin (Promega)) with 20 mg of a-GFP antibody (NB600-308, Novus Biologicals) cross-linked to 150 ml Dynabeads protein A (Invitrogen) at 4°C for 1 hour. Dynabeads were washed seven times with IP wash buffer (sonication buffer supplemented to 300 mM KCl, 20 units/ml RNAsin, 5 mM 2-mercaptoethanol, 5 mM sodium citrate, 10 mM ZnCl<sub>2</sub>) before incubation with 0.25 units/ml AcTEV protease (Invitrogen) overnight at 4°C to release immunoprecipitated OMA-1:S protein. RNAs were purified from this supernatant (IP RNA) or 50 ml of crude lysate (Input RNA) using Trizol (Invitrogen) and precipitated with linear acrylamide (Sigma).

### RT-qPCR

RT-qPCR was performed on IP and Input RNA samples from the same DG2581 lysate and an IP RNA sample from a similarly prepared DG2460 lysate, which lacks the OMA-1:S:TEV:GFP fusion protein. Reverse transcription was performed with IP RNA or input RNA using SuperScript II reverse transcriptase (Invitrogen) and random primers according to manufacturer instructions (oligo-dT primers performed similarly). qPCR was performed using FastStart SYBR Green Master Mix (Roche) on a Mastercycler ep *realplex* instrument (Invitrogen) in triplicate with appropriate controls. Fold-enrichment relative to the mock IP and estimated percent of input RNA were calculated as  $=2^{-(Ct(IP)-Ct(mock\ IP))}$  or  $=100(2^{-(Ct(IP)-Ct(input, corrected))})$ , respectively. In the latter calculation, the raw Ct value of the input sample was corrected to account for the fact that 125-fold less input sample was used for each RT-PCR reaction. Only fold-enrichment relative to input RNA is shown in Figure 1.

## Results

### I. OMA-1 interacts with the *mom-2* 3' UTR

OMA-1 and OMA-2 proteins are expressed only in oocytes and 1-cell embryos and are degraded soon thereafter. The *oma-1(zu405d)* mutant results in OMA-1 protein persisting past the 1-cell stage, and this OMA-1 persistence underlies its embryonic lethality (Detwiler et al. 2001; Lin 2003). Approximately 30% of *oma-1(zu405d)* embryos lack intestinal cells, similar to embryos defective in the Wnt pathway (Lin 2003). This shared phenotype suggests that ectopic OMA-1 in *zu405d* embryos may interfere with the function or expression of one or more components of the Wnt signaling pathway.

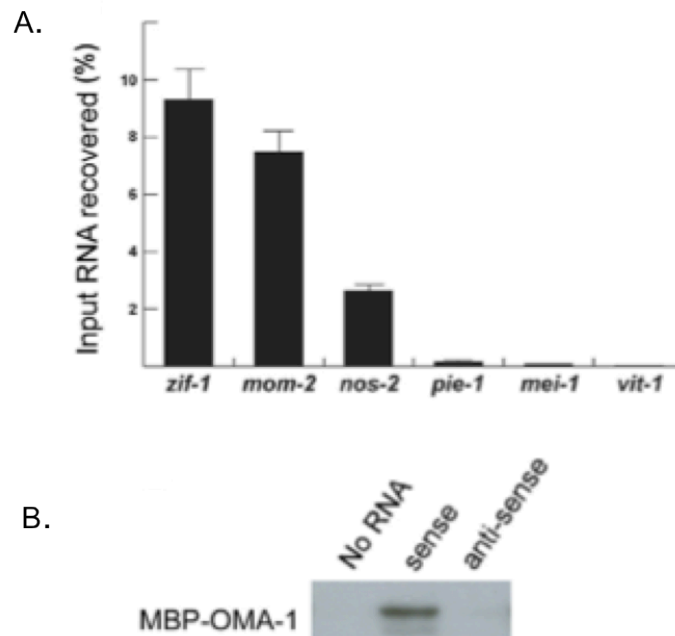
#### 1. *mom-2* is enriched in RNP particles containing OMA-1

In collaboration with the David Greenstein lab, I found *mom-2* mRNA to be highly enriched in RNP particles that contain OMA-1 protein. In that study a rescuing OMA-1::GFP transgene (*tnIs17*) was introduced into the null *oma-1(zu405d te33)* mutant, followed by crossing in the *spe-9(hc88ts)* mutation, which has a temperature-sensitive defect in sperm production. The resulting strain, DG2581, produces normal oocytes but no embryos at 25°C. OMA-1::GFP was pulled down from DG2581 worm lysates using anti-GFP antibody and RNAs co-immunoprecipitated were analyzed using *C. elegans* Gene Chip Arrays (Affymetrix). *mom-2* mRNA was enriched >4-fold in the IP fraction compared to the input RNAs. No RNAs corresponding to other genes known to function in the P2-to-EMS signal, including *mom-1*, *mom-3*, *mom-4*, *mom-5*, *gsk-3*, *apr-1*, *src-1*, *wrm-1*, and *lit-1*, were enriched.

The relative abundance of selected transcripts in the OMA-1 IP was also analyzed by RT-qPCR. *mom-2* transcripts along with *zif-1* and *nos-2* transcripts were assayed, two known targets of OMA-1 (Jadhav et al. 2008; Guven-Ozkan et al. 2010), *pie-1* and *vit-1*, transcripts unlikely to be OMA-1 targets, and *mei-1*, an mRNA that is regulated by OMA-1 but only in embryos and not in oocytes (Li et al. 2009). The established targets of OMA translational repression during oogenesis (*zif-1* and *nos-2*) as well as *mom-2* were efficiently recovered compared to negative controls (*pie-1* and *vit-1*) and *mei-1* (Figure 3.2A).

## **2. Purified OMA-1 interacts directly with the *mom-2* 3' UTR**

To determine whether the co-IP of *mom-2* mRNA with OMA-1 was due to a direct interaction between the *mom-2* 3'UTR and OMA-1 protein, I performed an in vitro RNA binding assay using purified MBP-OMA-1 protein. Following biotinylation, RNA corresponding to the *mom-2* 3'UTR was incubated with MBP-OMA-1 and RNA pulled down with streptavidin-conjugated magnetic beads. The amounts of MBP-OMA-1 pulled down with the RNA were analyzed by western blot using an anti-MBP antibody. MBP-OMA-1 was selectively pulled down by RNA corresponding to the sense, but not the anti-sense strand of the *mom-2* 3'UTR (Figure 3.2B). I therefore conclude that OMA-1 can bind directly to the *mom-2* 3'UTR, and may regulate expression of MOM-2 protein in vivo.



**Figure 3.2: OMA-1 interact with the *mom-2* 3' UTR**

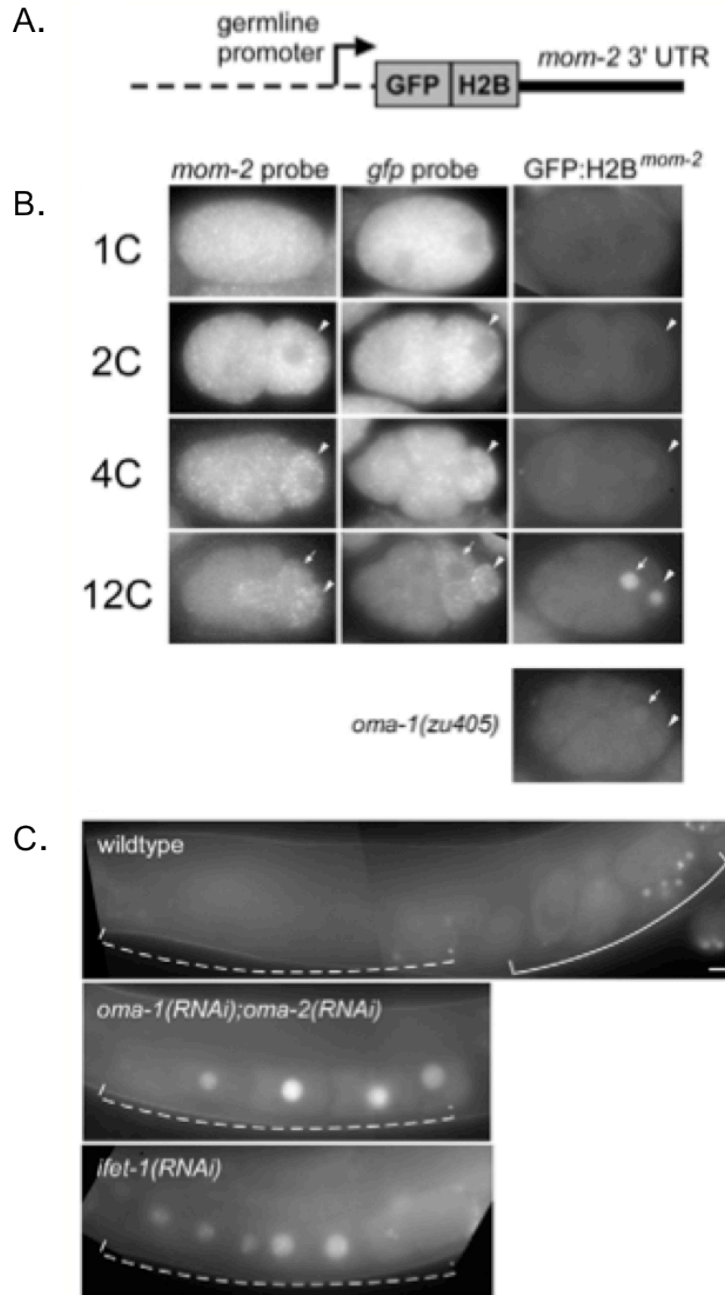
A) Recovery of the indicated mRNAs from the input lysate by OMA-1 immunoprecipitation as measured by RT-qPCR. Error bars indicate s.d. B) *In vitro* RNA pulldown using MBP-OMA-1 and *mom-2* 3'UTR RNA was blotted with anti-MBP antibody. OMA-1 binds directly to the *mom-2* 3' UTR in sense strand specific manner.

## II. The *mom-2* 3' UTR determines localization of its mRNA and protein

The mechanism by which MOM-2 activity is restricted in early embryos is not known. *mom-2* mRNA was detected in oocytes and early embryos by single molecule Fluorescence In Situ Hybridization (smFISH) (Harterink et al. 2011), which I confirmed (Figure 3.3B). I detected uniform *mom-2* mRNA levels in oocytes, 1-, 2-, and early 4-cell embryos. The abundance of *mom-2* mRNA decreases in somatic blastomeres beginning at the late 4-cell stage. By the 12-cell embryo, *mom-2* mRNA was detected at variably decreased levels in different blastomeres but remains relatively unchanged in P3 and C. This preferential degradation in somatic blastomeres has been observed for many, but not all, maternally-supplied mRNAs (Seydoux and Fire 1994).

I generated a reporter construct that expresses nuclear GFP::H2B under the control of the germline-specific *pie-1* promoter and the *mom-2* 3'UTR ( $P_{pie-1}\text{-}gfp\text{:}h2b\text{-}UTR^{mom-2}$ ) (Figure 3.3A). I will refer to the mRNA and GFP::H2B expressed from  $P_{pie-1}\text{-}gfp\text{:}h2b\text{-}UTR^{mom-2}$  as  $gfp\text{:}h2b^{mom-2}$  and GFP::H2B<sup>*mom-2*</sup>, respectively. Using smFISH,  $gfp\text{:}h2b^{mom-2}$  was detected in oocytes and early embryos in a pattern similar to endogenous *mom-2* mRNA (Figure 3.3B). Despite  $gfp\text{:}h2b^{mom-2}$  being detected at a high level in oocytes and early embryos, GFP::H2B<sup>*mom-2*</sup> is only first detected at the 4-cell stage, initially at a very low level in the P2 blastomere (Figure 3.3B and C). The GFP signal is further elevated in P3 and C, the daughters of P2, and remains high in all their subsequent descendants. The strong correlation between onset of GFP::H2B<sup>*mom-2*</sup> signal and the blastomeres known to exhibit MOM-2 activity argues that the GFP::H2B<sup>*mom-2*</sup> expression pattern parallels closely that of the endogenous maternal MOM-2 protein. My analysis also suggests that the *mom-2* 3'UTR is sufficient to confer correct localization of both its mRNA and protein in early embryos. Due to the lack of a good MOM-2 antibody, I characterize how the expression pattern of GFP::H2B<sup>*mom-2*</sup> is regulated in embryos.





**Figure 3.3: The *mom-2* 3'UTR is sufficient to determine the localization pattern of *gfp::h2b<sup>mom-2</sup>* RNA and GFP::H2B<sup>*mom-2*</sup> protein.**

A) Schematic of the reporter construct used. (B) Localization of endogenous *mom-2* (left column) and *gfp::h2b<sup>mom-2</sup>* (middle column) mRNAs assayed using smFISH. Right column shows fluorescence micrographs of live embryos expressing GFP::H2B<sup>*mom-2*</sup>. A 12-cell *oma-1(zu405d)* embryo is also shown. Arrowheads indicate germline blastomeres; arrows indicate the C blastomere. (C) Fluorescence micrographs of GFP::H2B<sup>*mom-2*</sup> in wild-type, *oma-1/2(RNAi)* and *ifet-1(RNAi)* adult hermaphrodites. Dashed lines indicate oocytes and solid line denotes embryos in the uterus. *oma-1/2(RNAi)* and *ifet-1(RNAi)* animals do not produce embryos. Scale bar: 10  $\mu$ m.

### III. OMA proteins repress GFP::H2B<sup>*mom-2*</sup> expression in oocytes

Repression of GFP::H2B<sup>*mom-2*</sup> expression in oocytes depends on OMA-1/2 proteins. High levels of GFP::H2B<sup>*mom-2*</sup> were observed in oocytes following simultaneous depletion of *oma-1* and *oma-2* by RNAi (Figure 3.3C, 100% animals examined, n=50). Furthermore, ectopic OMA-1 appears to be sufficient to repress GFP::H2B<sup>*mom-2*</sup> expression in embryos. In *oma-1(zu405d)* embryos, I observed reduced GFP::H2B<sup>*mom-2*</sup> expression in P3 and C (55% reduction in P3, n=13, SD=3.6%) (Figure 3.3B). Translational repression of maternal *mom-2* mRNA by persisting OMA-1 can explain the Wnt phenotype seen in *oma-1(zu405d)* embryos.

Our lab has shown previously that repression of *zif-1* translation by OMA proteins requires the eIF4E-binding protein, IFET-1 (Güven-Ozkan et al. 2010). IFET-1 binds to both OMA-1 and to the 5' cap-binding protein, eIF4E, presumably creating an inhibitory loop that prevents translation initiation (Li et al. 2009). GFP::H2B<sup>*mom-2*</sup> was repressed in oocytes of 100% *ifet-1(RNAi)* animals (n=25; Figure 3.3C), suggesting that OMA proteins also repress translation of *gfp::h2b<sup>mom-2</sup>* through an IFET-1-dependent mechanism. Together, my in vitro and in vivo results indicate that OMA-1/2 repress the translation of *gfp::h2b<sup>mom-2</sup>*, and presumably the *mom-2* mRNA, in oocytes via direct binding to the *mom-2* 3'UTR.

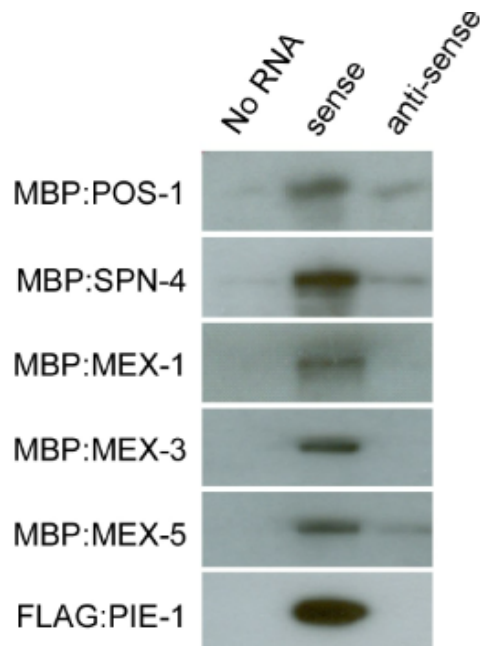
### IV. Expression of GFP::H2B<sup>*mom-2*</sup> requires MEX-1, PIE-1 and POS-1

OMA proteins are degraded soon after the first mitotic division (Lin 2003). Therefore, repression by OMA proteins can not account for the spatiotemporal expression

of GFP::H2B<sup>*mom-2*</sup> in embryos. To identify regulators of MOM-2 expression in embryos, I analyzed GFP::H2B<sup>*mom-2*</sup> in embryos depleted individually of specific RBPs.

### 1. Six RNA binding proteins bind directly to the *mom-2* 3' UTR

All RBPs tested here are maternally-supplied and were initially identified genetically to have essential functions in fate-specification of early blastomeres, which could complicate these analyses. In order to exclude RBPs whose effect on GFP::H2B<sup>*mom-2*</sup> expression could be indirect, possibly via a change in cell fate specification, I first assayed the ability of candidate RBPs to bind to the *mom-2* 3'UTR. I performed a series of in vitro RNA binding assays using biotinylated *mom-2* 3'UTR RNA and candidate RBPs. I found that the germline-enriched RBPs MEX-1, PIE-1, and POS-1, and SPN-4 and the soma-enriched RBPs MEX-3 and MEX-5 are all capable of binding to the *mom-2* 3'UTR in a sense-strand specific manner (Figure 3.4).

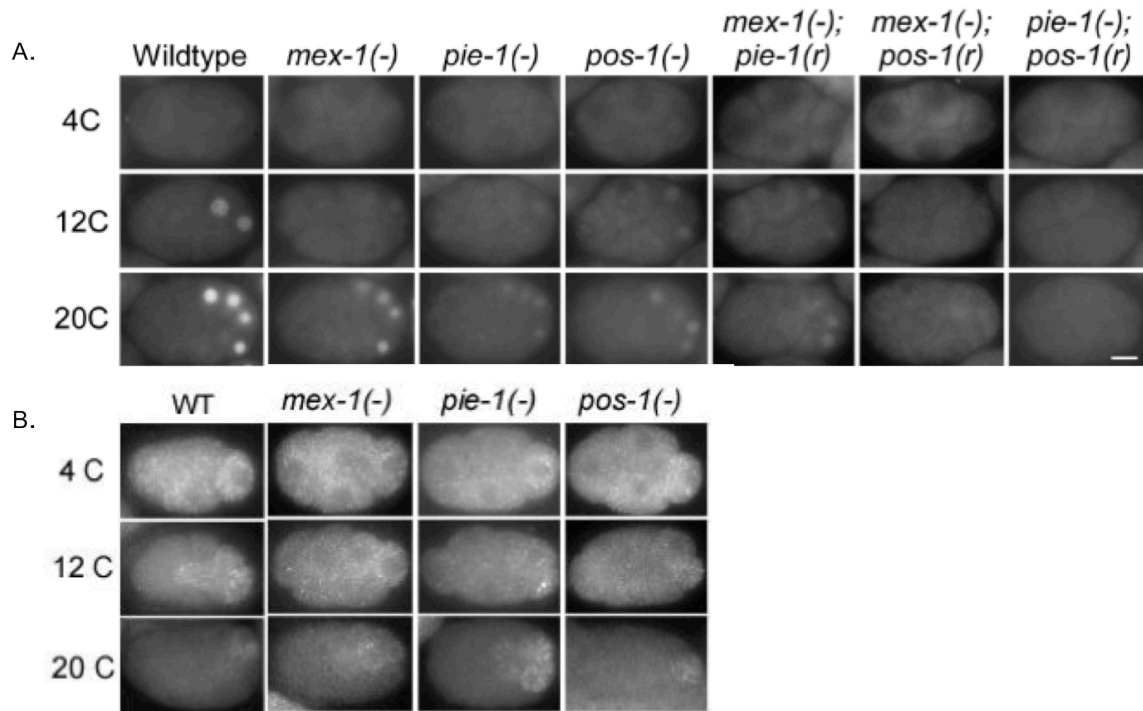


**Figure 3.4: Several RBPs bind directly to the *mom-2* 3' UTR**

*In vitro* RNA pulldown using various MBP tagged proteins and FLAG tagged PIE-1 with the *mom-2* 3'UTR RNA. Westerns were blotted with anti-MBP and anti-FLAG antibodies. All proteins tested bind directly to the *mom-2* 3' UTR in a sense strand specific manner.

**2. MEX-1, PIE-1 and POS-1 are required for GFP::H2B<sup>*mom-2*</sup> expression**

I next analyzed GFP::H2B<sup>*mom-2*</sup> levels in embryos individually depleted of each of these RBPs and found that depleting *mex-1*, *pie-1*, or *pos-1* leads to a reduction, but not abolishment of expression (Figure 3.5A) [*mex-1(zu121)* (50% reduction in P3, n=13, SD=3.2%), *pie-1(zu154)* (54% reduction in P3, n=13, SD=3.6%), *pos-1(zu148)* (33.1% reduction in P3, n=12, SD=3.8%)]. *pos-1(RNAi)* in either the *pie-1(zu154)* or *mex-1(zu121)* strain resulted in no detectable GFP::H2B<sup>*mom-2*</sup> in embryos (n=73 and 59, respectively). On the other hand, combined depletion of *pie-1* and *mex-1* [*pie-1(RNAi);mex-1(zu121)*, n=12, SD=3.2%, or *pie-1(zu154);mex-1(RNAi)*, n=12, SD=3.1%] did not result in a more severe defect in GFP::H2B<sup>*mom-2*</sup> expression than either single mutant (Figure 3.5A). smFISH with probes to either *mom-2* or *gfp* mRNA revealed no gross changes in levels in embryos mutant for any of the three genes (Figure 3.5B). I therefore conclude that PIE-1, MEX-1, and POS-1 function together to promote the translation of *gfp::h2b<sup>mom-2</sup>* in germline blastomeres.



**Figure 3.5: MEX-1, PIE-1, and POS-1 are required for *mom-2* expression**

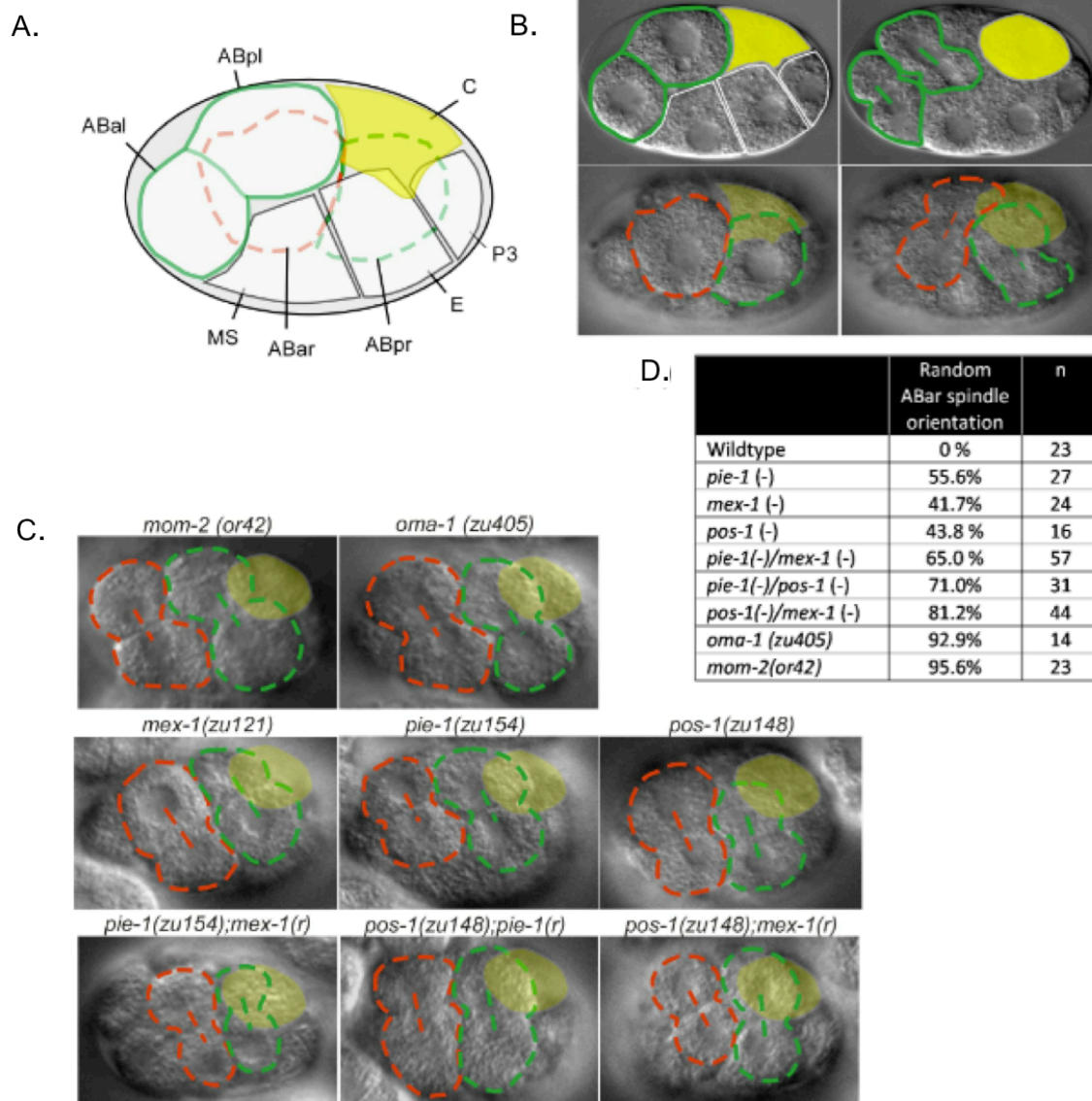
A) Fluorescence micrographs of live embryos expressing GFP::H2B<sup>*mom-2*</sup> in mutant (-) or RNAi (r) backgrounds. Scale bar: 10  $\mu$ m. B) smFISH imaged of embryos in various mutant (-) backgrounds using the endogenous *mom-2* probe.

### 3. *pie-1*, *mex-1*, and *pos-1* mutant embryos have a wnt signaling defective phenotype

Reduced expression of GFP::H2B<sup>*mom-2*</sup> could indicate a defect in Wnt-mediated processes in *mex-1*, *pie-1*, or *pos-1* mutant embryos. Both *pos-1(zul48)* and *mex-1(zul21)* mutants were previously reported to have endoderm defects (Mello et al. 1992; Tabara et al. 1999). All *pos-1(zul48)* embryos produce no endoderm, whereas *mex-1(zul21)* mutant embryos have a cold-sensitive, incompletely penetrant defect in endoderm production. However, the E blastomere in *pos-1(zul48)* or *mex-1(zul21)* mutant embryos does not undergo a homeotic fate change to the MS blastomere, as is the case for E in *mom-2(or42)* embryos. Specification of MS and differentiation of MS-

derived tissue types requires zygotic transcription (Mango et al. 1994; Broitman-Maduro et al. 2006), which might well be defective in these mutant embryos for reasons unrelated to the translation of maternal *mom-2*. Therefore, I examined ABar division axis realignment, a Wnt-dependent event independent of zygotic transcription (Powell-Coffman et al. 1996).

In wildtype embryos, a Wnt signal from the C blastomere aligns the division axis of ABar toward C, perpendicular to the other three AB-derived blastomeres (Park and Pries 2003; Walston et al. 2004) (Figure 3.6A and B). This alignment was shown to be defective in nearly all *mom-2* mutant embryos (Rocheleau et al. 1997; Thorpe et al. 1997), a result I reproduced (96%, n=23) (Figure 3.6C and D). In addition, I observed a similar defect in ABar division axis in 93% (n=23) of *oma-1(zu405d)*, 56% (n=27) of *pie-1(zu154)*, 42% (n=24) of *mex-1(zu121)*, and 44% (n=16) of *pos-1(zu148)* embryos (Figure 3.6C and D). This defect was enhanced when one of the other two positive regulators was also depleted by RNAi in *mex-1(zu121)* or *pos-1(zu148)* embryos. There was only modest or no enhancement when RNAi was combined with *pie-1(zu154)* embryos (Figure 3.6C and D). Although these results suggest that defective Wnt/MOM-2 translation underlies the spindle orientation defect in *pos-1*, *mex-1*, and *pie-1* mutant embryos, I cannot rule out the possibility of an indirect effect. For example, P2 (and its descendants) is not properly specified in the *pie-1* or *pos-1* mutant. Furthermore, in the *mex-1* mutant the AB lineage develops with irregular timing (Schnabel et al. 1996) and the ABar fate is changed (Mello et al. 1992; Tabara et al. 1999).



**Figure 3.6: Embryos depleted of *pie-1*, *mex-1* or *pos-1* exhibit a Wnt signaling-defective phenotype.** A) Schematic of a wild-type 8-cell embryo. Solid and dashed outlines indicate blastomeres on two different focal planes. ABar is outlined in red, with all other AB descendants outlined in green. Yellow, C blastomere. (B) (Left column) DIC images of two different focal planes of a wild-type 8-cell embryo. (Right column) The four AB descendants in the same embryos undergoing division. (C) DIC micrographs showing division axes of ABar and ABpr in embryos with the indicated mutations or RNAi (r) treatment. Scale bar: 10  $\mu$ m. D) Percentages of embryos with a random ABar spindle orientation in indicated mutants.

## V. MEX-3/5/6 and SPN-4 repress GFP::H2B<sup>*mom-2*</sup> expression

In vivo analyses showed that the RBPs MEX-3, SPN-4, MEX-5, and MEX-6 have repressive roles in the expression of GFP::H2B<sup>*mom-2*</sup>. These four proteins are all present at a high level in oocytes and 1-cell embryos, but are asymmetrically localized to different subsets of blastomeres afterward (Draper et al. 1996; Schubert et al. 2000; Ogura et al. 2003). Depletion of *spn-4* or *mex-3* resulted in GFP::H2B<sup>*mom-2*</sup> expression as early as the 1-cell stage, and in all subsequent blastomeres (Figure 3.7A). No GFP::H2B<sup>*mom-2*</sup> was detected in oocytes of *mex-3(RNAi)* or *spn-4(RNAi)* animals (not shown). These results suggest that SPN-4 and MEX-3 are both required for the translational repression of *gfp::h2b<sup>mom-2</sup>* in the 1-cell embryo.

In *mex-5(RNAi);mex-6(RNAi)* embryos, I observed uniform expression of GFP::H2B<sup>*mom-2*</sup> in all blastomeres as early as the 4-cell stage (Figure 3.7A). The spatial localization of MEX-5/6, the derepression of GFP::H2B<sup>*mom-2*</sup> in *mex-5/6(RNAi)* embryos, and the in vitro binding of the *mom-2* 3'UTR by MEX-5, all suggest that MEX-5/6 directly repress translation of *gfp::h2b<sup>mom-2</sup>* mRNA in somatic blastomeres. MEX-5/6 activity is not required for *gfp::h2b<sup>mom-2</sup>* translational repression until the 4-cell stage.

### 1. SPN-4 and MEX-5/6 affect *mom-2* mRNA localization

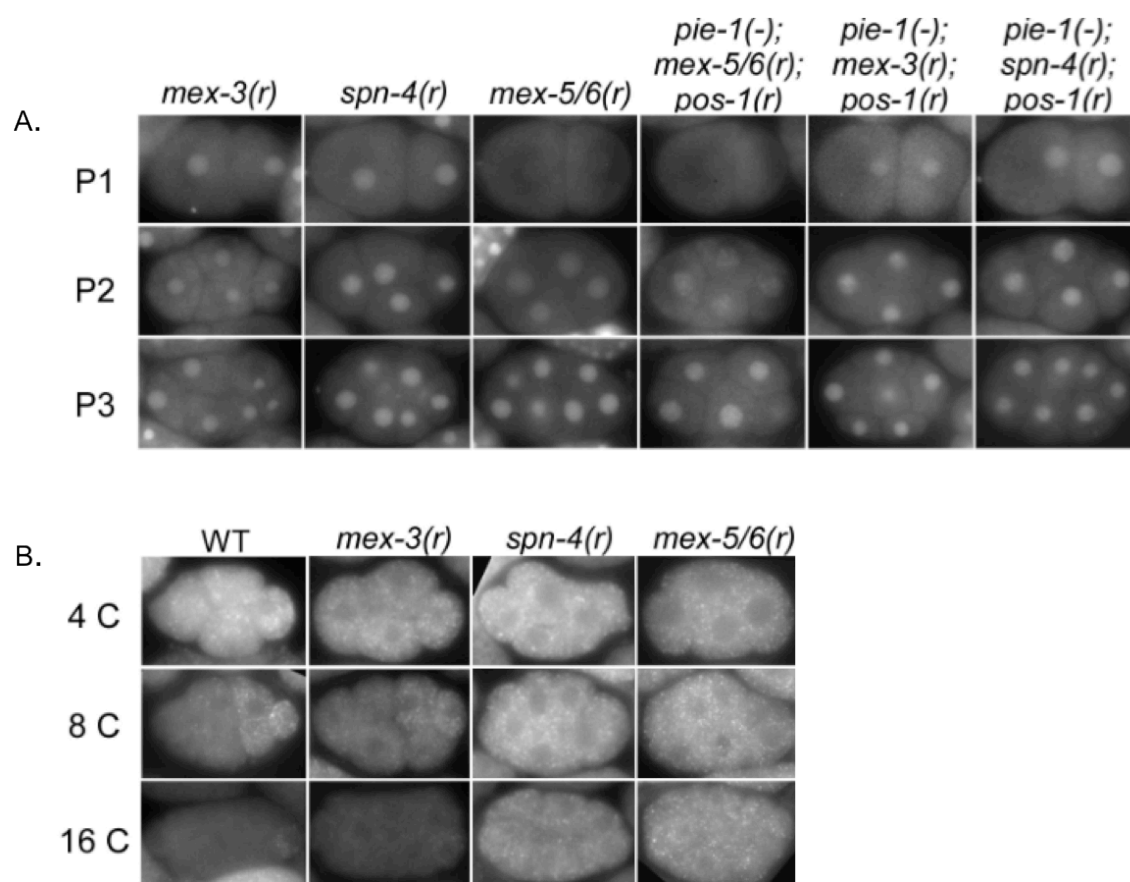
Depletion of either *spn-4* or *mex-5/6* resulted in *mom-2* and *gfp::h2b<sup>mom-2</sup>* mRNAs being uniformly distributed in embryos beyond the 4-cell stage (Figure 3.7B), likely a result of defective degradation of these mRNAs in somatic blastomeres. Whereas ectopic *gfp::h2b<sup>mom-2</sup>* mRNA may contribute to ectopic expression of GFP::H2B<sup>*mom-2*</sup> in 4-cell and



older embryos, it can not account for ectopic expression of GFP::H2B<sup>mom-2</sup> in 1- or 2-cell embryos, where *gfp:h2b<sup>mom-2</sup>* is already high in wildtype embryos.

## **2. Uniform localization of positive regulators in *mex-5/6* embryos is not responsible for uniform GFP::H2B<sup>mom-2</sup> expression**

In *mex-5(RNAi);mex-6(RNAi)* embryos, PIE-1, MEX-1, and POS-1 are all uniformly localized (Schubert et al. 2000). However, uniform localization of all three positive regulators is not the cause for the uniform expression of GFP::H2B<sup>mom-2</sup> observed in *mex-5(RNAi);mex-6(RNAi)* embryos. Simultaneous depletion of *pie-1* and *pos-1* [*(pie-1(zu154);pos-1(RNAi))*], or *mex-1* and *pos-1* [*(mex-1(zu121);pos-1(RNAi))*] in *mex-5(RNAi);mex-6(RNAi)* embryos resulted in no change to the uniform expression of GFP::H2B<sup>mom-2</sup> (Figure 3.7A). Similarly, PIE-1, MEX-1, and POS-1 are not required for the GFP::H2B<sup>mom-2</sup> derepression observed in *mex-3(RNAi)* or *spn-4(RNAi)* embryos (Figure 3.7A). The observation that the positive regulators PIE-1, MEX-1, and POS-1, are not required for GFP::H2B<sup>mom-2</sup> expression when negative regulators are depleted suggests that these positive regulators promote expression of GFP::H2B<sup>mom-2</sup> by antagonizing the negative regulators.



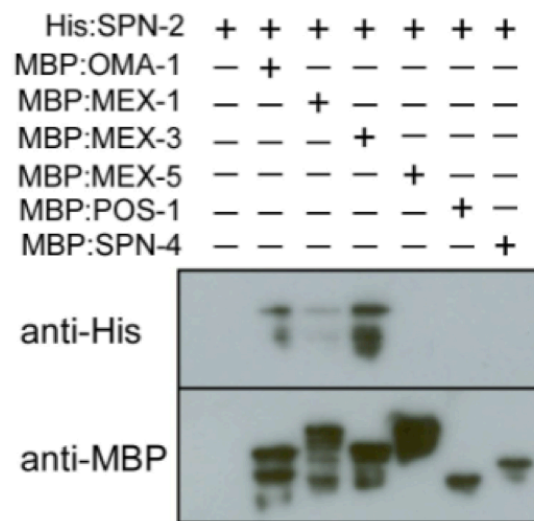
**Figure 3.7: MEX-3, SPN-4 and MEX-5/6 repress *mom-2* expression**

A) Fluorescence micrographs of live embryos expressing GFP::H2B<sup>*mom-2*</sup> in mutant (-) or RNAi (r) backgrounds. Scale bar: 10  $\mu$ m. B) smFISH imaged of embryos in various mutant (-) backgrounds using the endogenous *mom-2* probe.

### 3. MEX-3 binds to IFET-1

The GFP::H2B<sup>*mom-2*</sup> expressed in oocytes of *ifet-1(RNAi)* animals persists into embryos, precluding the determination whether IFET-1 activity is also required for the repression of *mom-2* in embryos. I did, however, investigate whether any of the RBPs functioning in the embryo bind to IFET-1 in vitro. Like MBP-OMA-1, MBP-MEX-3 efficiently pulled down His-IFET-1 in an in vitro pull-down assay (Figure 3.8). Low, but reproducible, amounts of His-IFET-1 were also pulled down by MBP-MEX-1. No

detectable IFET-1 was pulled down with any of the other proteins tested. This result suggests that repression of *gfp::h2b<sup>mom-2</sup>* translation in embryos by MEX-3 is through a mechanism involving IFET-1, similar to the translational repression by OMA-1/2 in oocytes.



**Figure 3.8: MEX-3 binds to IFET-1**

His-IFET-1 was mixed with individual MBP-tagged proteins and pulled down with amylose beads. Proteins pulled down were assayed using the indicated antibodies

## VI. Competitive binding of RNA binding proteins to the *mom-2* 3' UTR determines

### GFPH2B<sup>mom-2</sup> expression

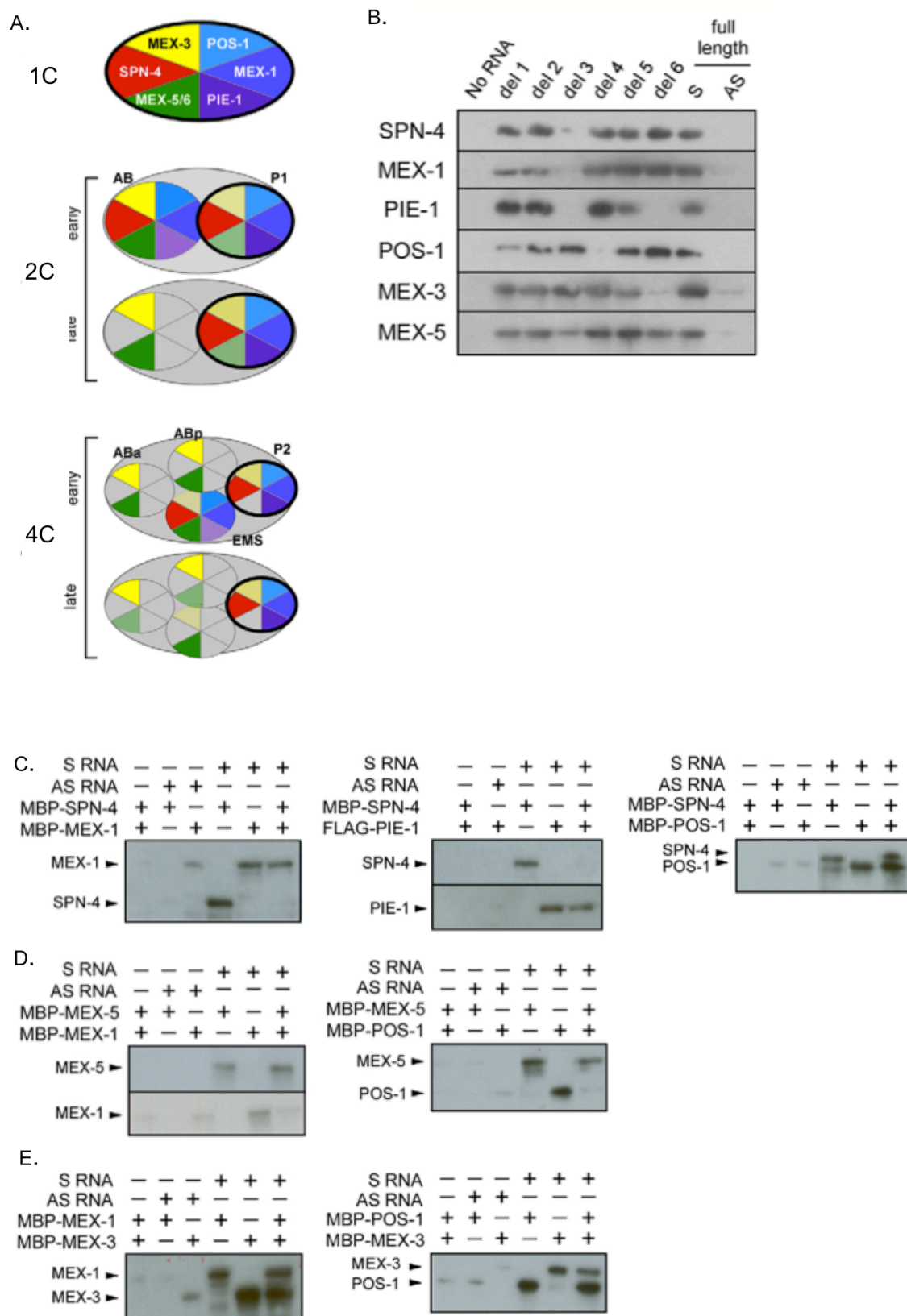
The seven regulators of GFP::H2B<sup>mom-2</sup> expression in embryos are themselves provided as maternal proteins and are all present in the 1-cell embryo. Beginning with the first embryonic division, these seven proteins exhibit dynamic and different spatiotemporal localization patterns (Figure 3.9A) (Draper et al. 1996; Mello et al. 1996; Guedes and Priess 1997; Tabara et al. 1999; Schubert et al. 2000; Ogura et al. 2003). To better understand how translation of *gfp::h2b<sup>mom-2</sup>* transcripts is determined in cells

possessing a combination of both positive and negative regulators, I performed the following two sets of experiments. First, I divided the *mom-2* 3'UTR into six equal regions in order to map binding sites for each protein. I generated *mom-2* 3'UTR RNAs deleted for one of the six regions (del1 - del6 RNAs) and performed binding assays with each of the RBPs (Figure 3.9B). SPN-4, MEX-1, and PIE-1 binding was abolished with del3 RNA, suggesting that Region 3 contains sequence essential for their binding. PIE-1 binding appears to require sequence present in Region 6 as well. The same assay revealed that Region 4 and 6 are essential for POS-1 and MEX-3 binding, respectively. Region 4 and Region 6 contain a predicted POS-1 and MEX-3 binding site, respectively (Farley et al. 2008; Pagano et al. 2009). Consistent with multiple putative MEX-5 binding sites across the entire *mom-2* 3'UTR, no single region was found essential for MEX-5 binding (Figures 7D and S4, Farley et al. 2008; Pagano et al. 2009).

Second, I performed in vitro RNA-binding competition experiments. Both a positive and a negative regulator were mixed with the target biotinylated *mom-2* 3'UTR RNA to determine whether binding to the *mom-2* 3'UTR RNA by one protein is favored over the other. P2 and P3 have a high level of the negative regulator SPN-4, as well as the three positive regulators, PIE-1, MEX-1, and POS-1 (Figure 3.9A). I found that mixing MEX-1 or PIE-1 with SPN-4 immediately prior to the addition of *mom-2* 3'UTR RNA resulted in the binding of only MEX-1 or PIE-1, but not SPN-4, to the RNA. This result and the above 3'UTR domain mapping suggest that PIE-1 and MEX-1 share overlapping binding sites with SPN-4 and that binding of either protein prevents simultaneous binding by SPN-4. In P2 and P3, the high level of MEX-1 and PIE-1 presumably prevents SPN-4 from binding to the *mom-2* 3'UTR, thereby antagonizing the

SPN-4 repressive effect. In a similar competition RNA-binding experiment, I found that binding of POS-1 and SPN-4 to the *mom-2* 3'UTR RNA was not affected by the presence of the other protein, consistent with the domain mapping showing these two proteins bind to distinct regions of the 3'UTR (Figure 3.9B and C).

EMS, the somatic sister of P2, does not express GFP::H2B<sup>*mom-2*</sup>. Levels of SPN-4, MEX-1, POS-1, MEX-5, and MEX-6 are all initially high in EMS, whereas the first three proteins are degraded by the proteasome later in the cell cycle (Figure 6A). I found that when MEX-1 or POS-1 was pre-mixed with MEX-5 in the RNA binding reaction, only MEX-5, but not MEX-1 or POS-1, was pulled down by the *mom-2* 3'UTR RNA (Figure 3.9D). The clear binding preference for MEX-5 over MEX-1 or POS-1, along with the persistence of MEX-5/6 levels in EMS can explain the repression of *gfp::h2b<sup>mom-2</sup>* translation in EMS. Competition binding showed no preference in binding to the *mom-2* 3'UTR RNA for MEX-3 versus MEX-1 or POS-1 (Figure 3.9E), consistent with these three proteins binding preferentially to different regions shown in Figure 3A.



**Figure 3.9: Competitive binding to the *mom-2* 3' UTR**

A) Schematic of MEX-3 (yellow), SPN-4 (red), MEX-5/6 (green), POS-1 (aqua), MEX-1 (blue) and PIE-1 (purple) localization in early embryos at the indicated stages. Each protein is represented in each blastomere by a 1/6 section of that blastomere where shading indicates protein levels. Germline blastomeres are in thick outline. B) *In vitro* RNA pulldowns using RNAs corresponding to either the full-length or deleted versions (del1-del6) of the *mom-2* 3'UTR as illustrated on the right. S and AS denote sense and antisense RNAs, respectively. POS-1, SPN-4, MEX-1, MEX-3 and MEX-5 were assayed using anti-MBP antibody, whereas PIE-1 was assayed using anti-Flag antibody. (C-E) Competition *mom-2* 3'UTR RNA binding assays. S and AS denote sense and antisense RNAs, respectively.

## VII. Discussion

In this chapter I show that a large set of maternally-supplied RBPs is required to set up proper MOM-2/Wnt signaling in the *C. elegans* embryo, thereby specifying normal intestinal development and spindle orientation. The 3'UTR of the *mom-2* transcript is sufficient to confer localization information for both the *gfp::h2b<sup>mom-2</sup>* mRNA and the corresponding GFP::H2B<sup>*mom-2*</sup> protein. The *gfp::h2b<sup>mom-2</sup>* mRNA is translationally repressed properly in oocytes and early embryos, and correctly translated in known Wnt signaling cells. I have identified nine maternally-supplied RBPs which can all bind in vitro to an RNA corresponding to the *mom-2* 3'UTR, and which suffice to explain correct spatiotemporal translation of *gfp::h2b<sup>mom-2</sup>* transcripts in vivo. In vitro experiments reveal overlapping binding sites and a hierarchy of binding preferences to the *mom-2* 3'UTR among these RBPs.

### Common themes governing translational control via the 3'UTR

My studies on the *mom-2* and *zif-1* 3'UTRs reveal two common mechanisms for how positive and negative regulators control translation of these maternal mRNAs via the

3'UTR. First, in the absence of all positive and negative regulators identified to date, the transcripts are translated. Unless additional positive regulator(s) required for translation of *gfp::h2b<sup>mom-2</sup>* are identified, these results suggest that these maternal mRNAs are inherently capable of being translated. Repression of each transcript in oocytes and embryos is maintained by spatially and temporally distinct sets of repressors. Translation occurs when repression is relieved or antagonized by a positive regulator (Figure 3.10A). My results suggest competitive binding of 3'UTR sequences as a likely mechanism for relieving translational repression in vivo. I cannot, however, rule out additional mechanisms by which positive-acting RBPs could interfere with repression, such as by binding and sequestering a negative-acting RBP or by masking binding sites for inhibitory microRNAs. Second, the spatiotemporal translational readout of a reporter appears to be controlled by the combination of competitive binding between positive and negative acting RBPs for specific 3'UTR sequences, and the blastomere-specific localization of particular RBPs.

#### Regulation in oocytes

I show that OMA-1/2 are required in oocytes to repress *gfp::h2b<sup>mom-2</sup>* mRNA translation, and are capable of continued repression when ectopically present in post 1-cell embryos. This can explain the Wnt-defective phenotype associated with *oma-1(zu405d)* embryos (Figure 3.10A). These results also explain the curious phenotypes of *gsk-3(RNAi)* and *gsk-3(nr2047ts)* embryos. In the canonical Wnt pathway, GSK-3 negatively regulates the pathway by phosphorylating b-catenin and promoting its degradation (Wu and Pan 2010). However, *gsk-3(RNAi)* and *gsk-3(nr2047ts)* embryos partially resemble *mom-2* mutant embryos, for both b-catenin-dependent and b-catenin-



independent phenotypes, suggesting a positive role upstream of b-catenin in the *C. elegans* Wnt pathway (Schlesinger et al. 1999; Shirayama et al. 2006). Our lab and others have shown that *gsk-3(RNAi)* and *gsk-3(nr2047ts)* embryos express ectopic OMA-1 protein, which likely underlies their Wnt-defective phenotypes (Nishi and Lin 2005; Shirayama et al. 2006). My demonstration here that OMA proteins repress translation of *gfp::h2b<sup>mom-2</sup>* suggests that previously characterized Wnt-defective phenotypes in *gsk-3(RNAi)* and *gsk-3(nr2047ts)* embryos likely result from compromised expression of the Wnt/MOM-2 ligand (Figure 3.10C).

#### Regulation in one- and two-cell embryos

Repression of *gfp::h2b<sup>mom-2</sup>* translation in 1- and 2-cell embryos requires both SPN-4 and MEX-3 (Figure 3.10A and B). I showed in chapter 2 that repression of the *zif-1* 3'UTR reporter in early germline blastomeres also requires both MEX-3 and SPN-4. In *spn-4(RNAi);mex-3(zu155)* embryos, the *zif-1* reporter is expressed precociously as early as the 2-cell stage. High levels of both MEX-3 and SPN-4 coexist within the same cell only in the 1-cell and (briefly) early 2-cell embryo (Draper et al. 1996; Ogura et al. 2003), thereby limiting translational repression of their joint targets to only these very early stages. Repression would be relieved after the 2-cell stage when MEX-3 and SPN-4 no longer coexist. This distinctive mode of translational repression in the 1-cell embryo highlights the unique molecular nature of the 1-cell embryo, with protein regulators of different developmental pathways coexisting within a common cytoplasm with maternally-supplied mRNAs that need to be translated in a blastomere-restricted manner (Robertson and Lin 2013).

I showed that MEX-3 can bind to IFET-1, suggesting a possible mechanism by which MEX-3 could inhibit *mom-2* translation (Güven-Ozkan et al. 2010). MEX-3, bound to the *mom-2* 3'UTR, and IFET-1, bound to the 5' cap (Li et al. 2009), could form a closed loop that inhibits translation through a IFET-1/MEX-3 interaction. SPN-4 has been shown to physically interact with MEX-3 (Huang et al. 2002; Ogura et al. 2003) and therefore it is possible that SPN-4 also represses translation of MEX-3 target mRNAs by stabilizing an inhibitory loop involving MEX-3 and IFET-1.

#### Regulation after the two-cell stage

In 4-cell embryos, high levels of SPN-4 and MEX-3 are detected in reciprocal patterns, with SPN-4 in both P2 and EMS, and MEX-3 in ABa and ABp (Figure 3.9A) (Draper et al. 1996; Ogura et al. 2003). The P2 blastomere also has high levels of MEX-1, PIE-1, and POS-1. A low level of MEX-3 in conjunction with the competitive binding advantage of PIE-1 and MEX-1 over SPN-4 can explain why *mom-2* is translated in P2. (Figure 3.10A and B). Two observations suggest that POS-1 promotes translation of *gfp::h2b<sup>mom-2</sup>* mRNA through a mechanism different from that by MEX-1 and PIE-1. First, while both PIE-1 and MEX-1 share overlapping binding sites with SPN-4, POS-1 appears to bind to a different region. Second, *pos-1* RNAi in either *pie-1* or *mex-1* mutant embryos enhances the defects in GFP::H2B<sup>*mom-2*</sup> expression. By contrast, little or no enhancement was observed following simultaneous depletion of *mex-1* and *pie-1*. With the caveat that this conclusion is based on experiments using RNAi, it is consistent with POS-1 promoting translation of *gfp::h2b<sup>mom-2</sup>* mRNA through a mechanism different from that of MEX-1 and PIE-1.

My data suggest a model that repressed translation of *mom-2* in EMS results from high levels of MEX-5/6, coupled with their competitive binding advantage over MEX-1 and POS-1 for the *mom-2* 3'UTR. The MEX-5 binding advantage could derive from higher numbers of putative binding sites in the *mom-2* 3'UTR. In addition, MEX-5/6 could contribute to *mom-2* translational repression in somatic blastomeres in at least two other ways. First, MEX-5/6 activity is required to keep the level of *mom-2* mRNA low in somatic blastomeres, likely by promoting its degradation. Second, it has been shown that MEX-5/6 activity is required to restrict PIE-1, MEX-1, and POS-1 to the germline blastomeres (Schubert et al. 2000).

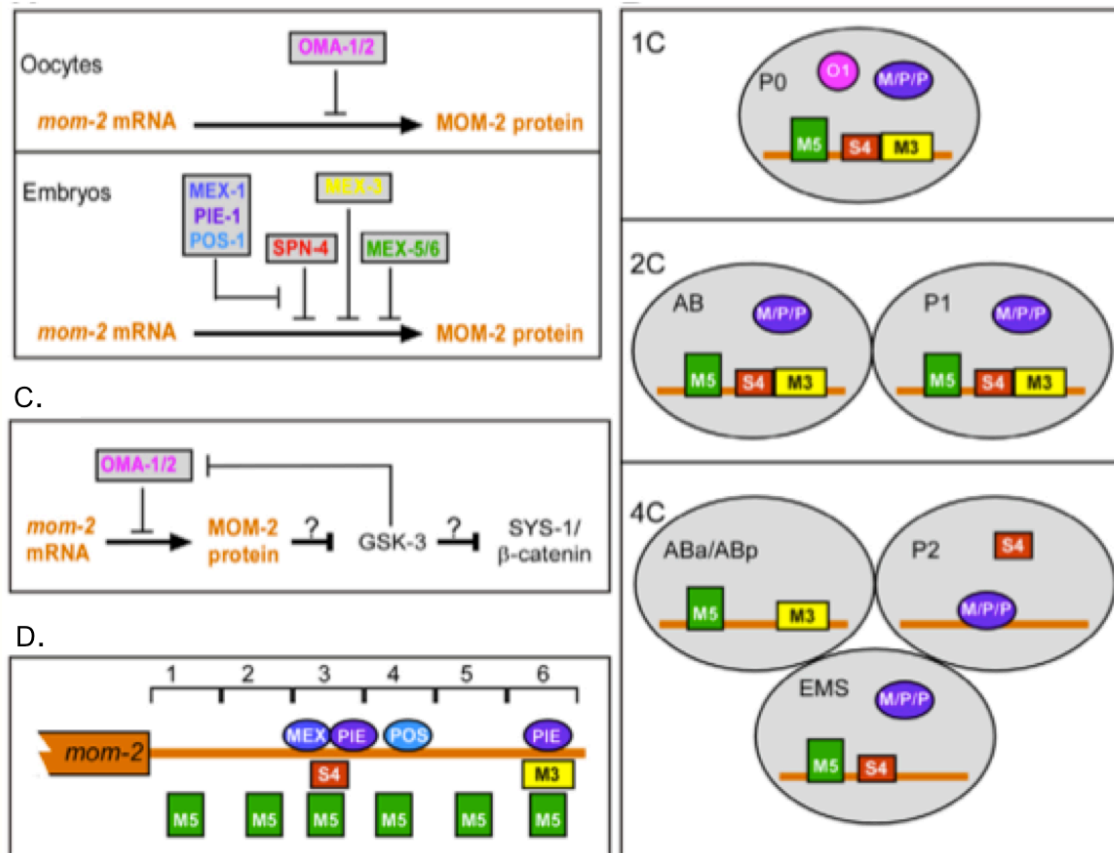
Intriguingly, whereas both POS-1 and MEX-5/6 have essential and antagonistic roles in regulating maternal *zif-1* and *mom-2* translation, they both function in opposite ways on both transcripts. POS-1 is a negative regulator for *zif-1* translation in P2. The preference for MEX-5/6 binding to the 3'UTR over POS-1 appears to drive translation of *zif-1* in EMS. The contrary is true for *mom-2*, where POS-1 promotes translation in P2 and MEX-5/6 represses translation in EMS. It is currently unclear how POS-1 and MEX-5/6 can exhibit opposite functions within the same cell in the translational regulation of different maternal transcripts.

### 3'UTRs as post-transcriptional enhancers

In newly fertilized embryos, regulating gene expression at the level of translation provides certain advantages over transcriptional regulation. First, it uncouples protein expression from transcription, which needs to be repressed for correct specification of germline blastomeres in both flies and worms (Nakamura and Seydoux 2008). Second,

because it does not require transcription, translational control is faster, and therefore much better suited to a developmental program where cell divisions occur every 10-15 minutes.

The importance of 3'UTRs and RBPs bound to them in regulating translation of germline transcripts is well established in *C. elegans* and *Drosophila* (Wilkie et al. 2003; Piccioni et al. 2005; Merritt et al. 2008). However, the mechanism by which the cohort of maternally-supplied RNA-binding proteins specifies cell fate in early *C. elegans* embryos via translational regulation of multiple shared targets remains largely unknown. My results from chapters 2 and 3 leads me to propose that the 3'UTR of maternal mRNAs contains a combinatorial code which determines the precise topography of RBPs, both positive and negative, that are bound to it. Therefore, the spatiotemporal expression pattern of any particular maternal mRNA is determined primarily by the precise combination of RNA-binding regulators present in the cell that are capable of binding to the 3'UTR sequence. By reading the combinatorial code provided by the 3'UTR, this cohort of RNA-binding proteins specify cell fate through translational control. This is reminiscent of transcriptional enhancers, whose binding sites provide a combinatorial code for cell-specific transcription regulators, which specify cell fate through transcriptional control. Future studies will more precisely define the combinatorial code(s) associated with maternal mRNAs, as well as interrogating RBP codes associated with somatic transcripts.



**Figure 3.10: Model of maternal *mom-2* translational control.**

A) Proposed functions of each tested RBP in the translation of *mom-2* mRNA in oocytes and embryos. MEX-1, PIE-1 and POS-1 positively regulate *mom-2* translation via antagonizing repression by the other proteins. (B) Proposed regulation of *mom-2* translation in each early blastomere (gray ovals) as a result of the hierarchical binding of the tested regulators. O1, OMA-1; M/P/P, MEX-1, PIE-1 and POS-1; M5, MEX-5/6; S4, SPN-4; M3, MEX-3. Brown line, *mom-2* 3'UTR. (C) The function of GSK-3 in a canonical Wnt signaling pathway and the proposed negative-feedback loop between GSK-3, OMA-1/2 and *mom-2* translation. Question marks denote regulations that have not been demonstrated in *C. elegans* embryos. (D) Schematic of the *mom-2* 3'UTR and proposed binding regions of each protein based on the *in vitro* mapping data shown in Fig. 3A.

## CHAPTER 4: A NOVEL PATHWAY OF MATERNAL mRNA DEGRADATION

### Summary

Based on RNA localization, *C. elegans* has two different classes of maternal mRNAs, class I and class II. Class I transcript levels remain constant throughout early embryonic development whereas class II transcript levels begin to get degraded by the 4-cell stage embryo, and at that time preferentially in somatic blastomeres. It is currently unclear how class II maternal messages get degraded temporally and spatially.

Utilizing in situ hybridization and qPCR, I have shown that zygotic transcription and components of the endogenous siRNA pathway are required for the timely degradation of at least some class II maternal RNAs. In addition, I and others have identified an endogenous locus containing 2 117mer repeats and approximately 200 25mer repeats (termed *vet-5*). There is evidence that RNA is transcribed from both repeat regions in the *vet-5* locus, preferentially in early and late embryos, and that endogenously generated siRNAs map to this locus. *vet-5* gets zygotically transcribed beginning at the 4-cell stage embryo in somatic blastomeres only and its localization and expression levels are disrupted upon removal of siRNA pathway components. The *vet-5* locus is therefore a prime candidate as a template of siRNA production required for the targeted degradation of class II maternal RNAs.

Deletion of specific endogenous-specific siRNA factors does not perturb the exogenous RNAi pathway. Therefore I introduced *vet-5* exogenously into worms defective for the endogenous siRNA pathway to determine if exogenous *vet-5* is sufficient to target class II maternal mRNAs for degradation. I found that either the

117mer repeat or 25mer repeat regions of *vet-5* is sufficient for degradation of at least some class II maternal mRNAs. I propose that the *vet-5* repetitive locus gets zygotically transcribed, precisely at the time that maternal mRNA is first degraded, thereby providing a pre-amplified pool of siRNAs that can rapidly target endogenous class II maternal RNAs for degradation.

## Introduction

Maternal provided mRNAs are critical for early *C. elegans* embryogenesis. Without zygotic transcription, embryonic development occurs without observable defects until approximately the 28-cell stage, at which time gastrulation occurs, thereby highlighting the importance of maternally deposited RNA for early embryonic development (Powell-Coffmann 1996). The importance of zygotic transcription to initiate gastrulation has also been demonstrated in the amphipod *Parhyale hawaiiensis* and *zebrafish*, both of which can rely fully on maternal mRNA until the onset of gastrulation (Alwes et al. 2011; Wagner et al. 2004). Although maternal mRNA is critical for early embryogenesis, many studies have revealed that degradation of maternal mRNA is equally as critical and that the onset of zygotic transcription marks the initiation of maternal mRNA clearance. The precise reasons for maternal mRNA clearance are not known, however it is postulated that elimination of specific maternal mRNAs could be essential for early development and that degradation is required to prevent abnormal mRNA dosage in the early embryo (Tadros and Lipshitz 2009).

Not all maternal mRNAs get degraded upon zygotic transcription activation. For instance, it has been shown in *Drosophila* that 20% of maternal transcripts are already

eliminated before the onset of zygotic transcription (Tadros et al. 2007; De Renzis et al. 2007). In *C. elegans*, it is not known what fraction of maternal mRNAs are dependent on zygotic transcription for their degradation, but it appears that the majority of maternal transcripts remain stable until the initiation of zygotic transcription. Onset of zygotic transcription varies greatly from gene to gene; however the earliest zygotic transcription in *C. elegans* is detected at the 4-cell stage embryo in somatic blastomeres specifically (See Figure 1.8) (Schauers and Wood 1990; Seydoux and Fire 1994).

Maternal mRNAs in *C. elegans* can be grouped into two major classes: class I and class II (See Figure 1.13). Class I mRNAs show uniform localization in early embryos and remain relatively stable throughout development. Class II mRNAs, on the other hand, get degraded specifically in somatic blastomeres in the early embryo. The majority of class II maternal RNAs show rapid degradation beginning at the 4-cell stage embryo, thereby coinciding with the onset of zygotic transcription (Seydoux and Fire 1994). Class I mRNAs mainly encode for housekeeping genes that are required for many aspects of development. Even though class I mRNA levels remain constant during the early embryo it cannot be ruled out that this is due to a combination of maternal mRNA degradation and a simultaneous accumulation of zygotically transcribed mRNA. Class II maternal mRNAs encode for a variety of genes each with distinct functions in the early embryo (Seydoux and Fire 1994).

There are several known mRNA degradation pathways some of which have been studied in *C. elegans*; however a complete block of mRNA degradation has not been achieved. RNA degradation often occurs through the activity of endonucleases, 3'-5' exonucleases, and 5'-3' exonucleases. The 3'-5' exonuclease class is best known for



degrading 5' guanosine capped and 3' poly(A) adenylated mRNAs. The *C. elegans* genome encodes for several 3'-5' exonucleases that exhibit a wide variety of functions, including rRNA processing (Gabel et al. 2008), DNA cross-link repair, and telomerase activity (Meier et al. 2009); however it has not been experimentally tested if 3'-5' exonucleases are important for the degradation of mRNA. In addition to endo/exonucleases, the *C.elegans* genome encodes for 9 *smg* genes that have been implicated in the degradation of mRNAs (Mango 2001; Pulak and Anderson 1993). Though SMG proteins are capable of targeting mRNAs for degradation they appear to function specifically in the non-sense mediated decay pathway for the degradation of unstable mRNA species (Mango 2001; Pulak and Anderson 1993). Recently, it was found that mRNA degradation could occur at the site of cytoplasmic foci termed P-bodies (Parker and Sheth 2007; Gallo et al. 2008). P-bodies are conserved from yeast to mammals and are composed of a core set of proteins including de-capping enzymes, activators of decapping enzymes, deadenylases and 5'-3' exonucleases (Parker and Sheth 2007). P-bodies recruit distinct components in somatic blastomeres versus germline blastomeres, with the decapping activating enzymes LSM-1 and LSM-3 being unique to somatic blastomeres. It is believed that the presence of LSM-1/3 marks somatic blastomeres as being degradation competent (Gallo et al. 2008). Removing P-body components, however, does not lead to a complete block of mRNA degradation (Gallo et al. 2008). This can be explained by functional redundancy between P-body components or redundancy with other mRNA degrading pathways. Depletion of *lsm-1* in yeast inhibits, but does not eliminate mRNA degradation thereby suggesting that redundancy may be common in all eukaryotes (Coller et al. 2001; Tharun et al. 2000).

Another common mechanism of RNA degradation is through RNAi mediated pathways, including miRNAs, piRNAs, and siRNAs. Many miRNAs have been characterized in *C. elegans*, however the majority have been shown to not be essential for development or viability (Alvarez-Saavedra and Horvitz 2009). Some classes of miRNAs are expressed in the early embryo and have been suggested to have an important role during embryogenesis. For example the mir-35-mir-42 class, which encompasses 8 genes with functional redundancy, are expressed in the early embryo after the initiation of zygotic transcription. Deleting several mir-35-mir-42 class members simultaneously leads to embryonic lethality. A direct function of the mir-35-mir-42 miRNAs has not been assigned (Alvarez-Saavedra and Horvitz 2010). Embryonic expression and embryonic lethality has also been reported for mir-51, mir-57, mir-59, and mir-228, with their precise function still unknown (Brenner et al. 2010). The *C. elegans* genome also contains a high abundance of piRNAs. Though mRNA silencing has been implicated by piRNA-mediated mechanisms, the majority of piRNAs appear to target transposons. Recently it was discovered that the silencing response of piRNAs can also act through the siRNA pathway. Some piRNAs can trigger activation of specific siRNA pathways thereby leading to widespread silencing of pseudogenes, transposons, and mRNAs (Bagijn et al 2013).

Endogenous siRNAs have long been implicated in mRNA silencing in *C. elegans* (reviewed in Jamalkandi and Masoudi-Nejad 2011). There are two well characterized siRNA pathways: the endogenous pathway and the exogenous pathway. The exogenous pathway is initiated when dsRNA is exogenously introduced thereby triggering a rapid siRNA mediated silencing response. The endogenous pathway is initiated from

endogenously produced dsRNA, usually from hairpin structures, overlapping genes, transposable elements, or RdRP derived dsRNA (Chung et al. 2008; Czech et al. 2008; Okamura et al. 2008). The endogenous and exogenous pathways utilize many of the same proteins for generating mature siRNAs, but several regulators are unique to each pathway. In fact, mutational analysis has shown that deleting many of the endogenous-specific regulatory proteins leads to an enhanced exogenous RNAi phenotype. Endogenous-specific proteins include various argonautes, such as ERGO-1, and 11 different *eri* (enhanced *RNAi*) genes. Deletion of any of these genes leads to an enhancement of exogenous RNAi. The observation of an enhanced RNAi response suggests that 1) these regulatory proteins are unique to the endogenous siRNA pathway and 2) some siRNA regulatory proteins have overlapping functions for both pathways. Removing an endogenous-specific regulator would inhibit the endogenous pathway thereby allowing regulators with overlapping functions to intensify their activity towards the exogenous pathway leading to an enhancement of exogenous RNAi.

In this chapter I have investigated whether the endogenous siRNA pathway plays a role in the degradation of class II maternal RNAs during the onset of zygotic transcription. I mapped a previously identified early transcript (its transcription can be detected as early as the 4-cell embryo), *vet-5*, to a genomic locus containing a series of repetitive elements. I have suggestions that the endogenous siRNA pathway can generate siRNAs from the *vet-5* locus and that these siRNAs can target class II maternal mRNAs for degradation at the onset of zygotic transcription. Removing components of the endogenous siRNA pathway or eliminating zygotic transcription leads to a delay in degradation of some class II maternal RNAs. When *vet-5* is introduced exogenously in

endogenous siRNA mutants, the observed degradation defects can be rescued. Therefore I propose that repetitive genomic sequences, such as the *vet-5* locus, could be a target for the production of endogenous siRNAs and that this process could reveal a novel mRNA degradation mechanism at the maternal-to-zygotic transition.

## Materials and methods

### Strains

N2 was used as the wildtype strain. Genetic markers are: LGI, *eri-6(mg379)*; LGIII, *pie-1(zu154)*, LGIV, *eri-1(mg366)*; LGV, *ergo-1(tm1860)*. Plasmids used, strain names and transgenes are as follows: WM158 [*ergo-1(tm1860)*], GR1373 [*eri-1(mg366)*], *eri-6(mg379)*, JJ532 [*pie-1(zu154)* *unc-25(e156)/qC1* *dpy-19(e1259)* *glp-1(q339)*].

### Plasmid construction

The full length *vet-5* feeding RNAi plasmid was generated by digesting pRL2273 (GW entry clone containing HindIII and EcoRI cut sites) and pC101 with HindIII and EcoRI and ligating the 2310 bp fragment from pRL2273 and the 474 bp fragment from pC101 through T4 ligase. An LR reaction was done with the ligated plasmid and pRL731[feeding RNAi destination (JA/L4440/GW)]. The resulting plasmid (pRL3257) contains 1 117mer repeat and 4 25 mer repeats.

The 25mer and 117mer feeding RNAi clones were generated through ligation of long primers as follows. 25mer:

RLOLG4386(GGGAGATCTAAATTGGCATTACGGCAGATTCTATAAATTGGCAT

TACGGCAGATTCTATAAATTTGCATTACGGCAGATTCTAA) and RLOLG4387 (GGGGGTACCATAGAATCTGCCGTAATGCCAATTTTATAGAATCTGCCGTAATG CAAATTTATAGAATCTGCCGTAATGCCAATTT), 117mer: RLOLG4388 (GGGAGATCTCAATCCGAAAGTTCGGAAAACGATCGCCGACGAGAGCGACGA TGAACGATCGGGATGGATGGATCGGGAGGATCGGAATGTATGAAAGGA) and RLOLG4389 (GGGGGTACCCCCATTCCCATCGTTGTTTCGTCCCGATCCTTTCATACATTCCG ATCCTCCCGATCCATCCATCCCGATCGTTCATCGTCGCTCTCGTCG). Primers were mixed and placed in a 1 liter beaker containing 600 ml boiling water. The water was allowed to cool to room temperature (~3 hours) and dNTP mix and tag polymerase were added followed by incubation at 72 degrees for 10 minutes. The product was purified through a qiagen gel extraction column to eliminate unligated primer and ligated with BglII/KpnI digested pRL731 to generate pRL3297 (25mer) and pRL3298 (117mer).

### RNA interference and imaging

Feeding RNAi was performed by feeding L1 larvae for 2 days at 20°C (Timmons and Fire 1998) except for *ama-1* RNAi which was performed by feeding L3 larvae for 24 hours at 20°C. All images were acquired, processed, and quantified as described (Güven-Ozkan et al. 2008).

### qPCR

For the qPCR assays worms were cut open at 23.6°C and embryos were collected at the 1- and 2-cell stage and 4-cell stage separately. 1- and 2-cell stage embryos were

snapfrozen on liquid nitrogen instantly and 4-cell embryos were snapfrozen after being allowed to develop for 18 minutes to reach 12-cell stage. 10 freeze/thaws on liquid nitrogen were done and embryos were lysed by douncing. RNA was extracted with an oligo(dT) resin following the streamlined protocol in the Illustra QuickPrep mRNA Purification Kit from GE Healthcare. RT-PCR was performed with oligo(dT) primers following the protocol in the Enhanced Avian RT First Strand Synthesis Kit from Sigma. qPCR was done with class II maternal RNA and actin (ACT-1) and tubulin (TBB-2) specific primers using Bullseye Evagreen Master Mix (MidSci) on the StepOncePlus Real Time PCR system from Applied Biosystems. Fold change was calculated by the following formula:  $2^{-[\Delta CT_{\text{sample}} - \Delta CT_{\text{control}}]}$ . The control used for all graphs was tubulin.

$\Delta CT$  values were derived by:  $CT_{12\text{-cell sample}} - CT_{1\text{-cell sample}}$  thereby setting the 1-cell sample relative to 1.

### Single molecule mRNA FISH

All smFISH experiments were done as described in materials and methods in chapter 2.

The only modification is that the 25mer and 117mer probes were used undiluted.

## **Results**

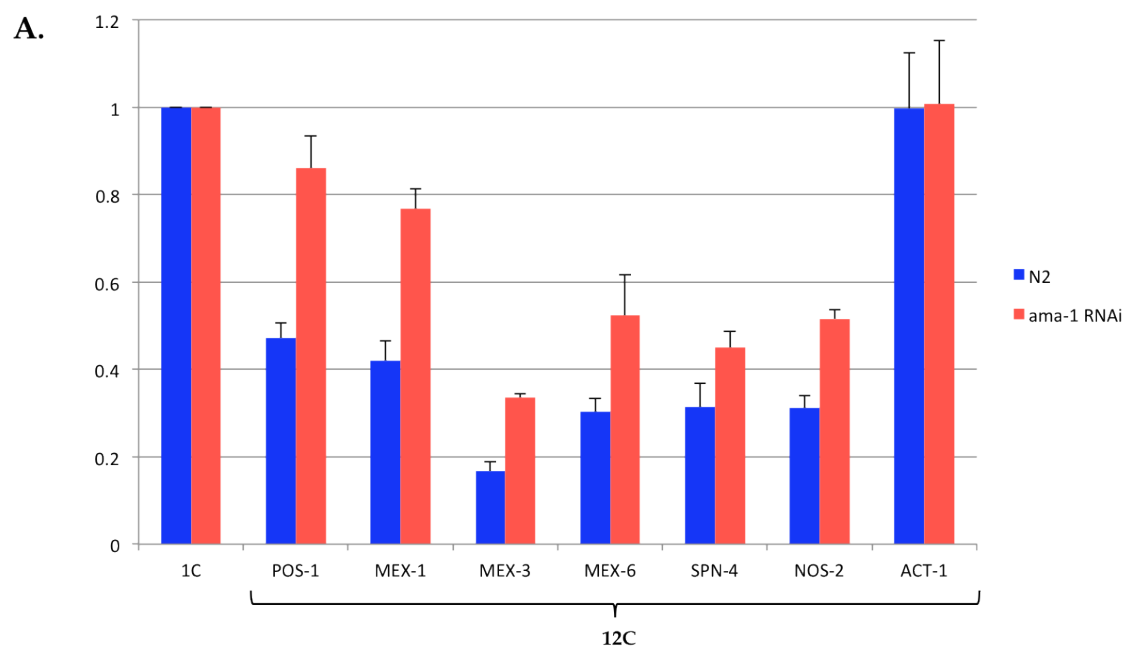
### **I. Blocking zygotic transcription leads to a delay in degradation of some class II maternal mRNAs**

Degradation of maternal messages is partly dependent upon zygotic transcriptional activity in *zebrafish* and *Drosophila* (Giraldez et al. 2006; Ferg et al. 2007; Benoit et al. 2009; Bushati et al. 2008). It is currently unclear if zygotic

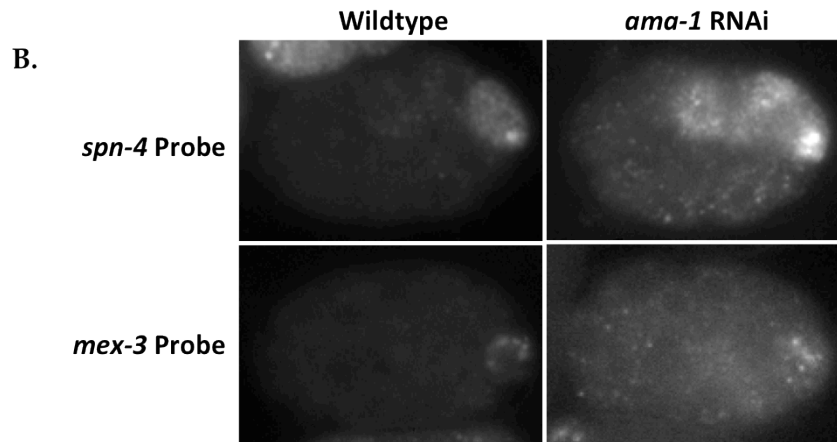
transcription plays a role in the clearance of maternal mRNA in *C. elegans*. To investigate this, I removed the Polymerase II component *ama-1* through feeding RNAi. *ama-1* encodes the large subunit of RNA polymerase II and has been mutationally identified and cloned many years ago (Rogalski and Riddle 1988; Rogalski et al. 1988; Bird and Riddle 1989). Mutating *ama-1* leads to a complete block of maternal and zygotic transcription and therefore impairs oogenesis and subsequently fertility. To circumvent this problem I fed worms with anti-sense *ama-1* RNA at the L3 larvae stage, at which time the gonad, containing high levels of maternal RNA, has already been formed. This leads to a failure to initiate gastrulation at the ~28 cell stage and complete cell cycle arrest by the ~100 cell stage indicating oogenesis occurred normally, but zygotic transcription was blocked (Powell-Coffman et al. 1996).

*ama-1* RNAi worms were cut open and embryos collected at the 1-cell and 12-cell stages, thereby yielding samples containing only 1-cell or only 12-cell embryos (approximately 500 embryos/sample). mRNA from these embryo samples was isolated by binding dounced samples to an oligo(dT) resin. cDNA was subsequently prepared with oligo(dT) specific primers. qPCR was performed with primers corresponding to six class II maternal mRNAs, *pos-1*, *mex-1*, *mex-3*, *mex-6*, *spn-4*, and *nos-2*, and two class I maternal mRNAs, *act-1* and *tbb-2*. Transcript levels were compared between 1-cell and 12-cell samples in wildtype and *ama-1* backgrounds. I found that there is a significant decrease in transcript levels from 1-cell to 12-cell for class II maternal mRNAs (but not class I) in wildtype worms and that this decrease was attenuated in *ama-1* RNAi worms (Figure 4.1A). This result suggests that zygotic transcription is, at least in part, required for the timely degradation of class II maternal mRNAs.

Two single molecule RNA fluorescent in situ hybridization (smFISH) probes were generated against the class II maternal mRNAs *mex-3* and *spn-4*. In situ hybridizations were done on wildtype and *ama-1* embryos with the *mex-3* and *spn-4* smFISH probes. smFISH signal for *mex-3* and *spn-4* were nearly identical. In wildtype embryos high and uniform smFISH signal was detected in oocytes and embryos up to the early 4-cell stage. By late 4-cell signal began to decrease in somatic blastomeres and was very low to undetectable in somatic blastomeres by the 14-cell stage. Since somatic blastomere signal was still detected by the 12-cell stage in wildtype embryos, I only scored embryos for persistence of mRNA at the 14 to 20-cell stage. I found that both *mex-3* and *spn-4* mRNA persisted in *ama-1* RNAi mutant embryos (60.0% and 57.1% of embryos displayed a persistence of *mex-3* or *spn-4* mRNA respectively) (Figure 4.1 B and C).







C. Percentage of embryos that show delay in degradation

	<i>spn-4</i> probe	N	<i>mex-3</i> probe	N
N2	0%	21	0%	26
<i>ama-1</i> RNAi	57.1%	22	60.0%	14

**Figure 4.1: Zygotic transcription is required for the timely degradation of some class II mRNAs** A) qPCR of 1-cell embryos vs. 12-cell embryos from wildtype (N2) and *ama-1* RNAi samples. 1C indicates the 1-cell samples and 12C indicates the 12-cell samples. The 1-cell sample has been set to 1. X-axis labels represent the primers used and the Y-axis indicates fold change. Each bar labeled with a specific primer represents the 12-cell sample (12C). Results are normalized against tubulin. B) Fluorescent in situ hybridization micrographs of 16-cell stage embryos with indicated probes in wildtype and the *ama-1* RNAi background. C) Percentage of embryos that showed a delay in degradation based on the in situ hybridization experiments.

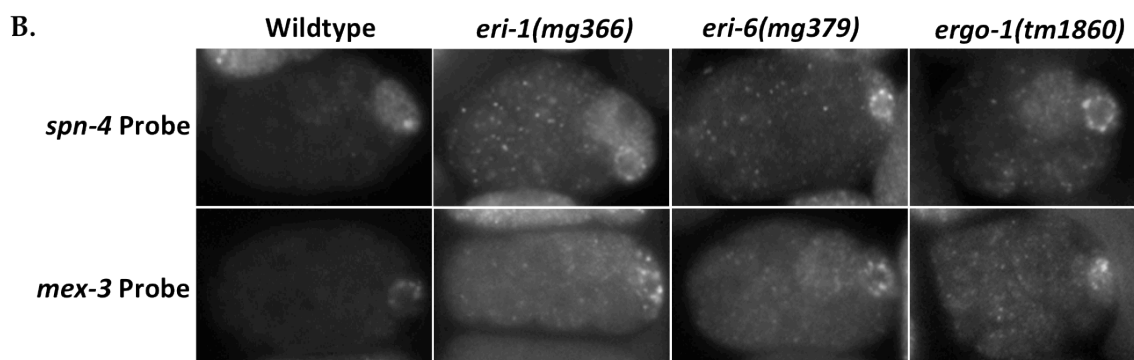
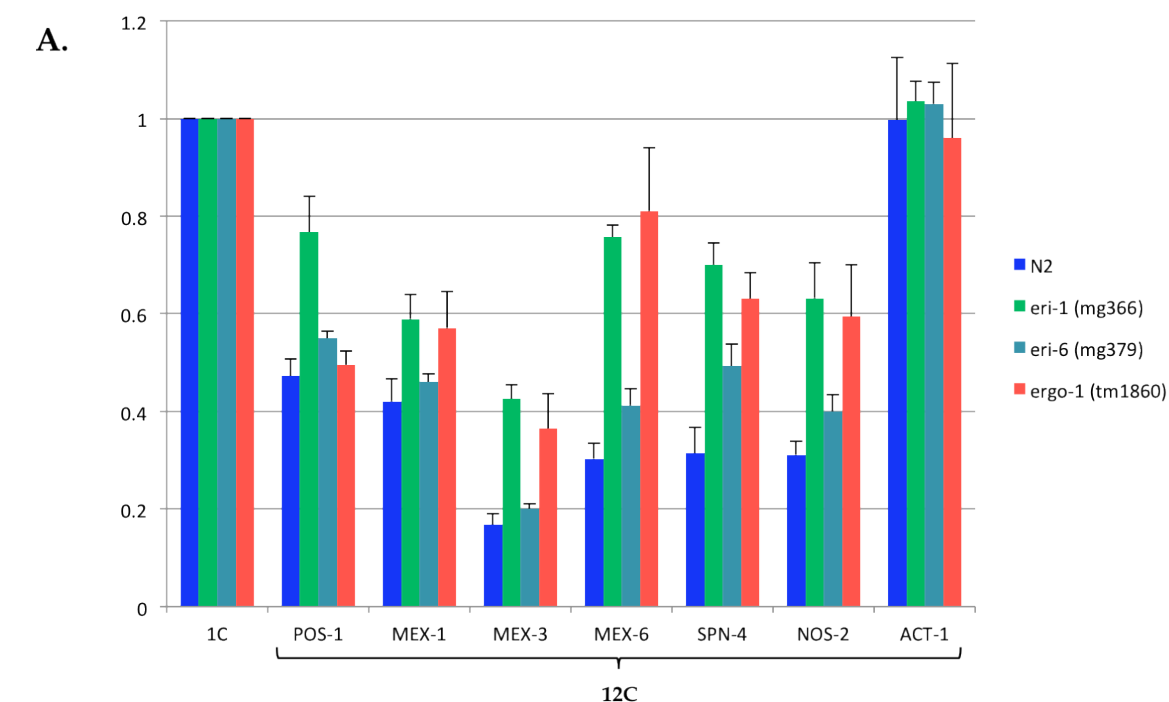
## II. Deleting factors of the siRNA pathway leads to a delay in degradation of some class II maternal RNAs

It has previously been shown that endogenous siRNAs can target mRNAs for post-transcriptional gene silencing in *C. elegans* (reviewed in Jamalkandi and Masoudi-Nejad 2011); however it is currently unclear if siRNAs target maternal mRNAs

specifically during the maternal to zygotic transition. I found that individually deleting the endogenous-specific siRNA factors, *eri-1*, *eri-6*, and *ergo-1*, leads to a persistence of some class II maternal mRNAs. qPCR was carried out as previously described and higher mRNA levels were detected at the 12-cell stage, as compared to wildtype, in each of these mutants for at least some class II maternal RNAs (Figure 4.2A). ERI-6 encodes for a helicase protein that was previously shown to play an important role in passenger strand removal during the production of primary siRNAs (Fischer et al. 2008; Fischer et al. 2011). *eri-6(mg379)* contains a splice mutation and is presumably null. (Fischer et al. 2008). I detected a marginally higher level of mRNA in *eri-6(mg379)* embryos, as compared to wildtype, in 4 out of 6 class II maternal mRNAs tested, *pos-1*, *mex-6*, *spn-4*, and *nos-2* (Figure 4.2A). ERGO-1 is an argonaute protein that was characterized to be important for the production of 26G primary siRNAs specifically in the early embryo (Grishok et al. 2001; Yigit et al. 2006). *ergo-1(tm1860)* contains a large deletion. I detected a clear higher level of mRNA in *ergo-1(tm1860)* embryos, as compared to wildtype, in 5 out of 6 class II maternal mRNAs tested, *mex-1*, *mex-3*, *mex-6*, *spn-4*, and *nos-2* (Figure 4.2A). ERI-1 encodes for a highly conserved Dicer interacting RNase protein and is critical for the activity of Dicer to generate primary siRNAs (Kennedy et al. 2004) *eri-1(mg366)* contains a small insertion and has been shown to be null. I detected a clear higher level of mRNA in *eri-1(mg366)* embryos, as compared to wildtype, in 6 out of 6 class II maternal tested, *pos-1*, *mex-1*, *mex-3*, *mex-6*, *spn-4*, and *nos-2* (Figure 4.2A).

I performed in situ hybridizations with *mex-3* and *spn-4* probes on *eri-1*, *eri-6*, and *ergo-1* mutants worms. 9.5% and 12.7% of embryos showed persistence of *spn-4* and

*mex-3* mRNA respectively in *eri-6* mutants, 26.7% and 28.6% of embryos showed persistence of *spn-4* and *mex-3* mRNA respectively in *ergo-1* mutants, and 25.8% and 30.8% of embryos showed persistence of *spn-4* and *mex-3* mRNA respectively in *eri-1* mutants (Figure 4.2 B and C). These results indicate that the siRNA pathway is, at least partly, required for the degradation of class II maternal mRNAs.



### C. Percentage of embryos that show delay in degradation

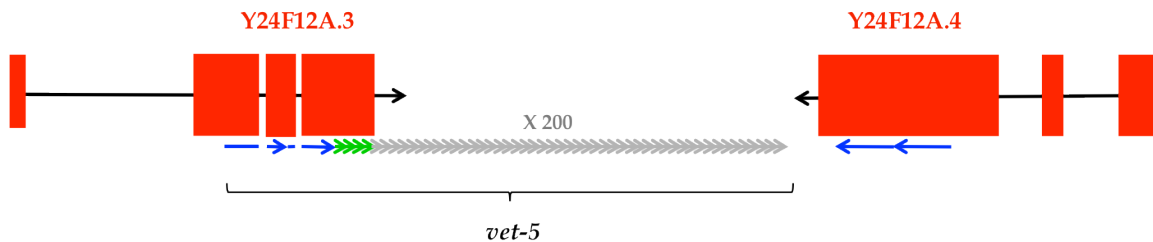
	<i>spn-4</i> probe	N	<i>mex-3</i> probe	N
<i>eri-1</i> ( <i>mg366</i> )	25.8%	31	30.8%	26
<i>eri-6</i> ( <i>mg379</i> )	9.5%	21	12.9%	31
<i>ergo-1</i> ( <i>tm1860</i> )	26.7%	15	28.6%	14

**Figure 4.2: The siRNA pathway is required for the timely degradation of some class II mRNAs.** A) qPCR of 1-cell embryos vs. 12-cell embryos in indicated genetic backgrounds. 1C indicates the 1-cell samples and 12C indicates the 12-cell samples. The 1-cell sample has been set to 1. X-axis labels represent the primers used and the Y-axis indicates fold change. Each bar labeled with a specific primer represents the 12-cell sample (12C). Results are normalized against tubulin. B) Fluorescent in situ hybridization micrographs of 16-cell stage embryos with indicated probes in indicated backgrounds. C) Percentage of embryos that showed a delay in degradation based on the in situ hybridization experiments.

### III. Very Early Transcript *vet-5* maps to a highly repetitive locus

Zygotic transcription in *C. elegans* is first detected in the 4-cell stage embryo and at that time only in somatic blastomeres (Schauer and wood 1990; Seydoux et al. 1996). Sixteen total very early transcripts, referred to as *vet* genes, were originally identified from which only 6 have been cloned as cDNAs due to the existence of a putative open reading frame (Schauer and Wood 1990; Pettitt and Wood 1993). Based on in situ hybridization data 5 out of the 6 cDNAs show RNA localization mainly in the cytoplasm, whereas one, termed *vet-5*, has strong nuclear localization (See Figure 1.8)(Seydoux and Fire 1994). The *vet-5* cDNA was originally cloned by the Bill Wood lab and contains 2 117mer repeats followed by 4 25mer repeats. For the remainder of this dissertation I will refer to this piece of cDNA as *vet-5c*. I mapped *vet-5c* to the genomic locus Y24F12A.3, and found that the 4 25mer repeats contained within the cDNA are followed by another ~200 25mer repeats, with little variation in sequence. The full genomic locus (2 117mer

repeats and ~200 25 mer repeats) will be referred to as *vet-5*. Another gene, presumably a pseudogene, Y24F12A.4 is transcribed from the anti-sense strand downstream of *vet-5*. The Y24F12A.4 locus contains 2 117mer repeats that are similar in sequence to the 117mer repeats in *vet-5* (Figure 4.3).



**Figure 4.3: The endogenous Y24F12A.3 and Y24F12A.4 loci**

Cartoon showing the structure of the Y24F12A.3 and Y24F12A.4 loci. Blue arrows indicate the 117mer repeats, green and grey arrowheads indicate the 25mer repeats. The two 117mer repeats and four green 25mer repeats represent the *vet-5* cDNA originally cloned by the Bill Wood lab. Grey arrowheads represent the additional ~200 25mer repeats in the genomic locus. Red boxes are exons.

### 1. Transcription is detected from the *vet-5* locus

Consistent with the existence of cloned cDNA from the *vet-5* locus, transcription is detected from the genomic *vet-5* locus based on RNA-seq data produced by the Waterston lab. High transcript reads are detected in the 117mer region with lower reads being detected in the 25mer region. In addition, consistent with *vet-5* presumably playing a role in the early embryo, transcription reads are significantly higher in embryos as compared to adult worms. Transcript reads are also detected from the Y24F12A.4 locus in the 117mer region, although it cannot be determined whether these reads map to the

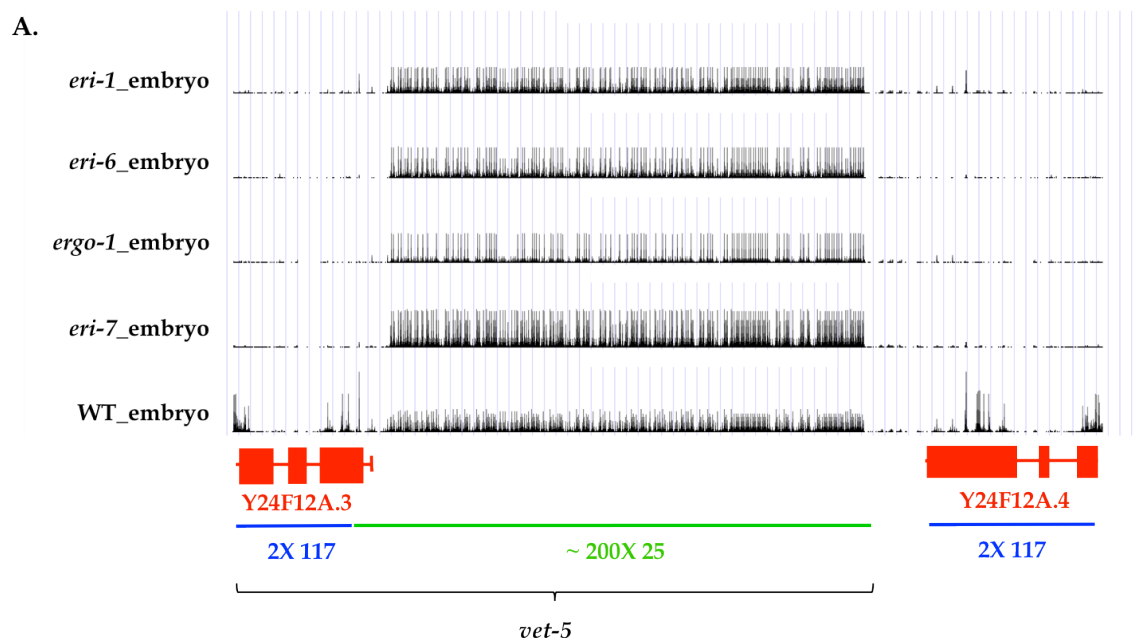
*vet-5* locus or the Y24F12A.4 locus since the 117mer region in both loci are nearly identical in sequence.

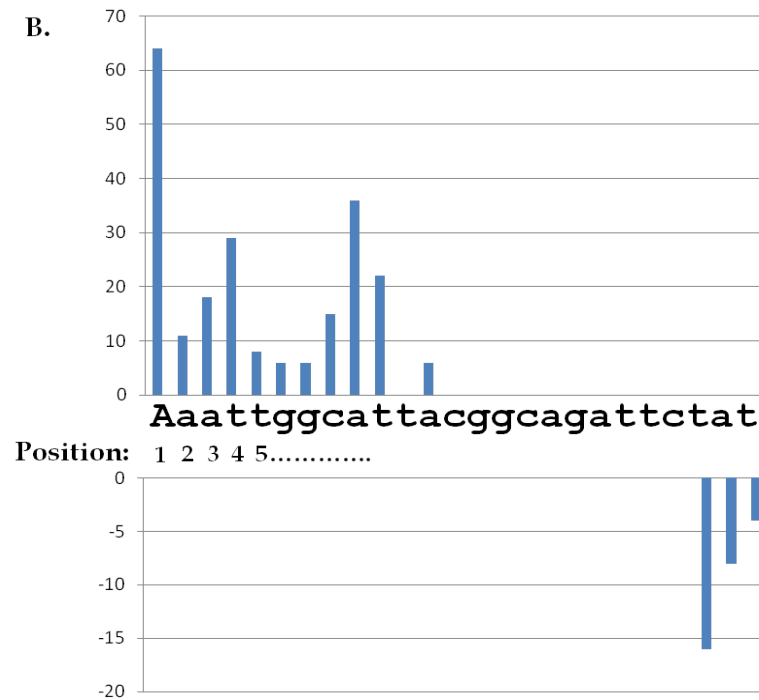
## **2. siRNAs are derived from the Y24F12A.3 locus**

The *C. elegans* genome produces siRNAs through multiple different pathways from over half of its total genes. The Ruvkun lab investigated which loci produce ERI dependent siRNAs. Small RNA reads from all coding genes, pseudogenes and transposons were plotted as normalized reads in wildtype versus *eri-7*, *eri-6*, *eri-1* and *ergo-1* mutant embryos (Fischer et al. 2011). *eri-6* and *eri-7* are two adjacent, divergently transcribed genes that produce separate pre-messenger RNAs. The pre-messenger RNAs from *eri-6* and *eri-7* are both required to generate a functional mRNA molecule; therefore removing one out of the two simulates a double mutant (Fischer et al. 2008). siRNAs derived from 80 loci, which encode primarily coding genes and pseudogenes, were depleted in *eri-7*, *eri-1*, and *ergo-1* mutant embryos as compared to wildtype by >67%. Interestingly, one of the target loci depleted of siRNA reads is Y24F12A.3 (*vet-5*) (Fischer et al. 2011). In collaboration with Zhenyu Xuan from the University of Texas at Dallas, I further analyzed the small RNA data set and found that siRNAs were specifically depleted from the 117mer region, and not the 25mer region, in *eri-1*, *eri-6*, *eri-7*, and *ergo-1* mutant embryos (Figure 4.4A). It should be noted, however, that the repeats within 25mer region of *vet-5* are present in the *C. elegans* genome in at least 3 other loci. Therefore it cannot be determined if the persistence of small RNA reads in the mutants accurately map to the *vet-5* locus or to one of the other loci.

### 3. Small RNAs from the 25mer region show distinct phasing

Next I determined if siRNAs from the 25mer region of *vet-5* show distinctive phasing. I investigated this by utilizing the small RNA data set from the Fischer et al. 2011 paper and, in collaboration with Zhenyu Xuan, mapped the RNA reads to individual start sites within the repeat region. We found that the majority of siRNAs map to position 1 (25.7%) on the sense strand as annotated by the first nucleotide of the 25mer repeat region. A smaller percentage mapped to positions 2, 3, 4, 5, 6, 7, 8, 9, 10, and 12 (4.4%, 7.2%, 11.6%, 2.8%, 2.0%, 2.0%, 6.0%, 14.1%, 8.4%, and 2.0% respectively). A small number of reads also map to the anti-sense strand at positions 1, 2, and 3. (Figure 4.4B).





**Figure 4.4: small RNA reads map to the *vet-5* locus**

A) Mapping of siRNAs derived from the RNA-seq dataset in Fisher et al 2011. The position of the Y24F12A.3 and Y24F12A.4 loci are indicated in red, the 117mers in blue and the 25mers in green. Peaks indicate siRNAs that map to the region. Peaks in wildtype vs. mutant are not to scale. B) Phasing of small RNAs derived from the 25mer region based on Fisher et al 2011 RNA-seq data of wildtype embryos. Y-axis represents the number of small RNAs, 249 total smRNAs were detected. Positive and negative Y-axis indicates reads mapping to the sense and anti-sense strand respectively.

#### IV. *vet-5* RNA can be detected by smFISH

Previous in situ hybridization experiments revealed that *vet-5c* is primarily localized to the nucleus (Seydoux and Fire 1995). I generated two smFISH probes, one against the 117mer region of *vet-5c* and one against the 25mer region of *vet-5c*. Both probes showed similar patterns in wildtype embryos. Signal was not detected until the 4-cell stage embryo and at that time only in somatic blastomeres. Strong nuclear foci were

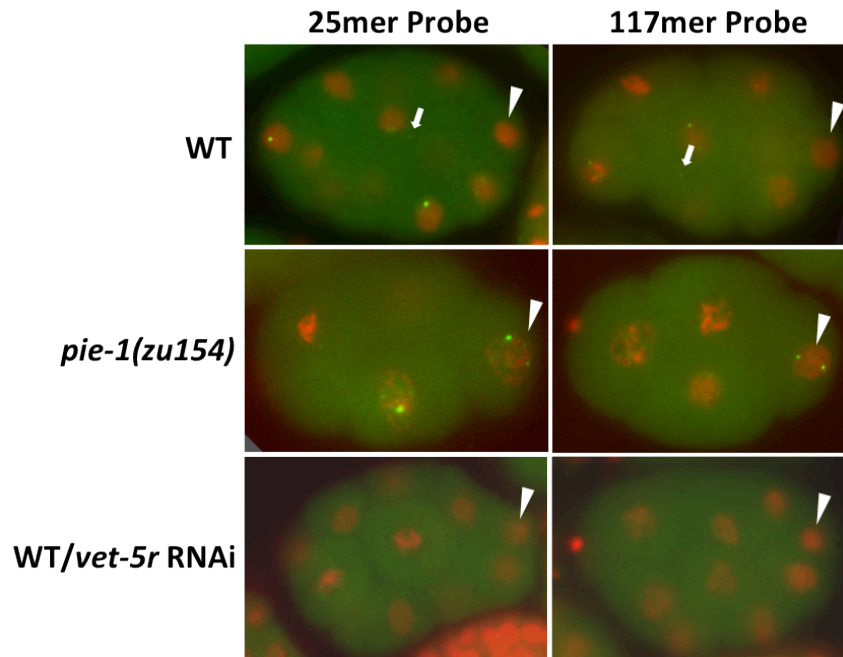


detected in addition to several small cytoplasmic foci (Figure 4.5). Signal remained detectable in all somatic blastomeres throughout embryonic development.

### **1. smFISH probes specifically detect RNA**

I am confident that the signal I detected with the 25mer and 117mer smFISH probes represent RNA and not genomic DNA. First of all, signal is only detected in somatic blastomeres in wildtype embryos, but in all blastomeres in *pie-1(zu154)* embryos (Figure 4.5). This is an expected result as the function of PIE-1 is to keep germline blastomeres transcriptionally repressed. Second, the signal is abolished or greatly reduced in embryos from worms treated with *vet-5* RNAi (Figure 4.5). The *vet-5* RNAi clone generated (will be referred to as *vet-5r*) contains one 117mer repeat and four 25mer repeats and is therefore likely to knock down endogenous *vet-5*.

The abolishment of smFISH signal in *vet-5r* RNAi embryos suggest that the smFISH probes are only able to detect full-length *vet-5* RNA, rather than siRNAs derived from the *vet-5* locus. This observation is further supported by the fact that smFISH probes have much higher specificity for larger RNA fragments. One smFISH probe consists of ~48 20 nucleotide fragments, each containing a specific fluorophore, that stagger along the length of its target. Shorter RNA species, such as a 25 nucleotide fragment, are therefore highly limited in the specificity and strength of signal that can be generated (Harterink et al. 2011).



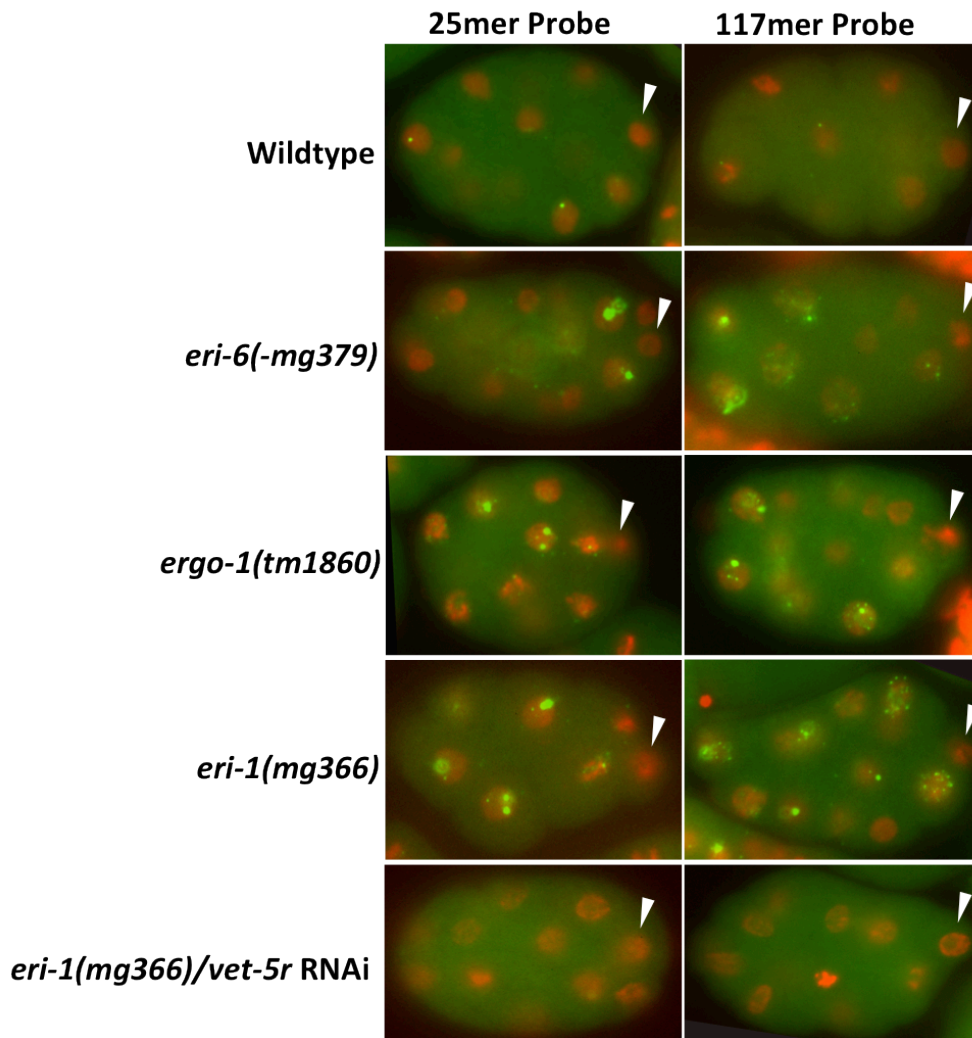
**Figure 4.5: Endogenous *vet-5* can be detected by smFISH**

Fluorescent in situ hybridization micrographs of embryos in indicated backgrounds with smFISH probes specific to the 25mer region and 117mer region. smFISH signal is green and nuclei, as marked by DAPI staining, are red. Arrows point to cytoplasmic foci detected and arrowheads point to the germline blastomere.

## 2. Increased *vet-5* RNA is detected in siRNA mutants

I performed in situ hybridizations with the 25mer and 117mer smFISH probes on the siRNA mutants *eri-1*, *eri-6*, and *ergo-1*. smFISH signal was again similar for the 25mer probe and the 117mer probe in every mutant tested and each mutant showed a similar localization pattern. Detected signal was significantly higher, as compared to wildtype, with very strong nuclear foci and many smaller cytoplasmic foci. In addition when I introduced *vet-5r* RNAi in any of the mutants, signal was low to non-detectable (Figure 4.6). This result indicates that *vet-5* RNA is affected when the endogenous siRNA pathway is disrupted. Consistent with the previous result that the smFISH probes

only detect full-length *vet-5* RNA, I propose that disrupting the siRNA pathway prevents the generation of siRNAs from the *vet-5* locus leading to an increase in full-length *vet-5*.



**Figure 4.6: *vet-5* expression is affected in siRNA mutants**

Fluorescent in situ hybridization micrographs of embryos in indicated backgrounds with smFISH probes specific to the 25mer region and 117mer region. smFISH signal is green and nuclei, as marked by DAPI staining, are red. Arrowheads point to the germline blastomere.

## V. Exogenous *vet-5* can rescue degradation defects in some class II maternal RNAs

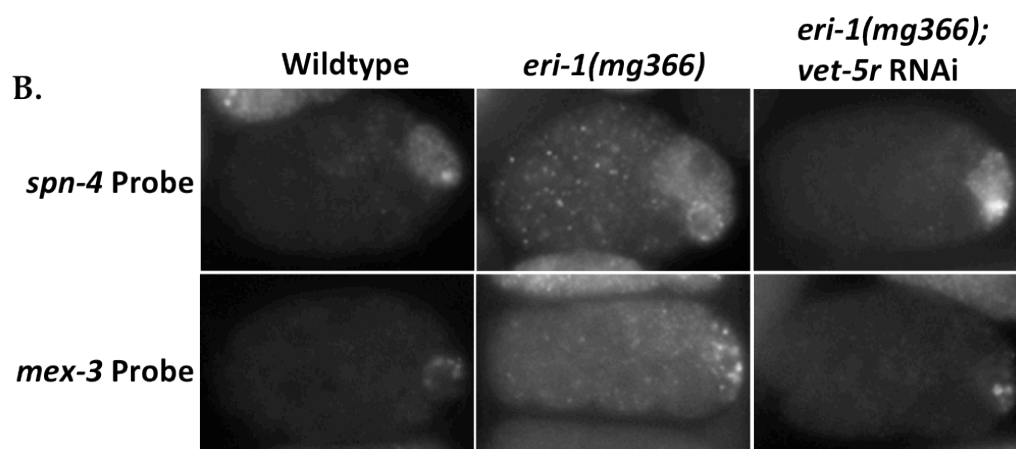
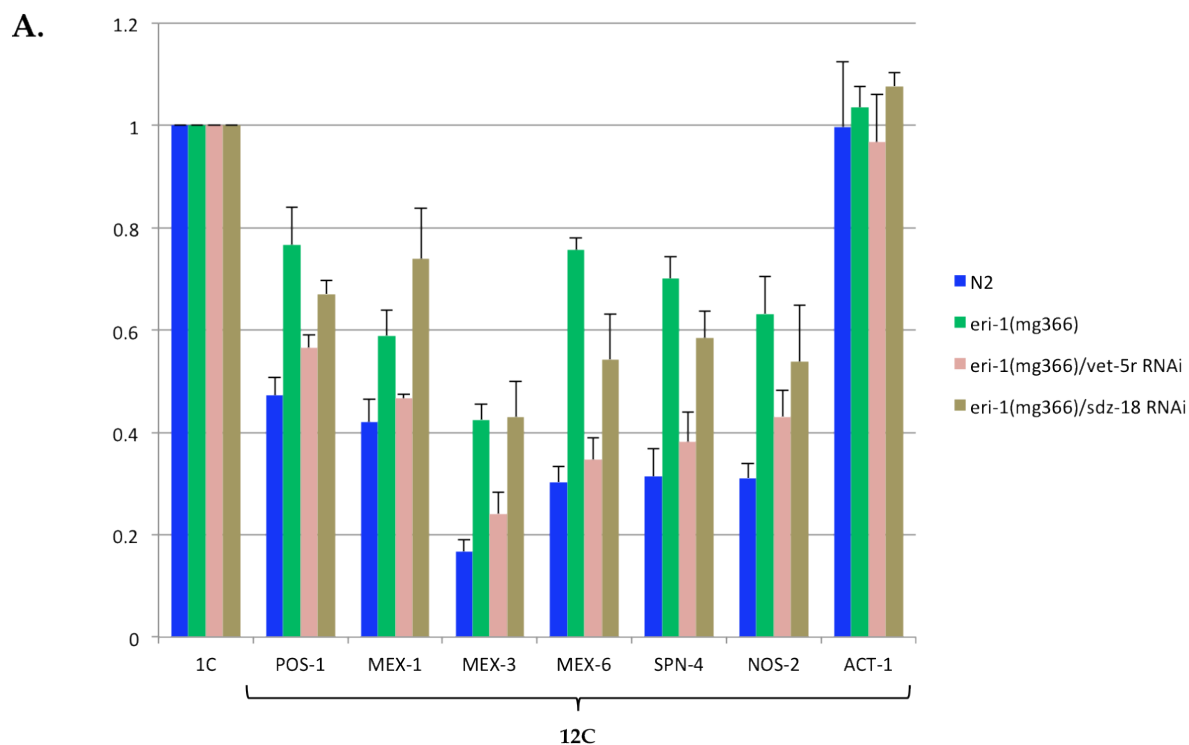
The endogenous and exogenous siRNA pathways in *C. elegans* are regulated by a distinct set of regulatory proteins (see Figure 1.15). Specifically, 11 enhanced RNAi (ERI) proteins have been discovered to act specifically in the endogenous siRNA pathway. To investigate if *vet-5* plays a role in the endogenous siRNA pathway I exogenously introduced *vet-5* in the endogenous-specific siRNA mutant *eri-1*. Though *eri-1* is required to generate endogenous siRNAs, the exogenous pathway remains unaffected in an *eri-1* mutant; therefore siRNAs can presumably still be generated from exogenously introduced *vet-5* in *eri-1* mutants.

### 1. Exogenous full-length *vet-5* can rescue degradation defects

Transcript levels of 6 out of 6 class II maternal mRNAs tested persisted in *eri-1* mutants as shown by qPCR. I repeated qPCR with *eri-1* mutant worms that were fed with *vet-5r* RNAi. All class II mRNAs tested, *pos-1*, *mex-1*, *mex-3*, *mex-6*, *spn-4*, and *nos-2* displayed degradation kinetics similar to wildtype (Figure 4.7A). To rule out a *vet-5*-independent effect due to activation of exogenous RNAi, qPCR was repeated with an unrelated RNAi feeding clone, *sdz-18*. All class II maternal mRNAs tested displayed degradation kinetics similar to *eri-1* mutants in *eri-1/sdz-18* RNAi embryos (Figure 4.7A).

In situ hybridizations were performed with *mex-3* and *spn-4* smFISH probes on *eri-1/vet-5r* RNAi embryos. The results were consistent with the qPCR data. *mex-3* mRNA persisted in only 13.6% (n=22) of *eri-1/vet-5* RNAi embryos, as compared to 30.8% (n=26) in *eri-1* alone. *spn-4* mRNA persisted in only 5.6% (n=18) of *eri-1/vet-5*

RNAi embryos, as compared to 25.8% (n=31) in *eri-1* alone (Figure 4.7B and C). The qPCR and in situ hybridization results indicate that exogenously introduced *vet-5r* is sufficient to target at least some class II maternal mRNAs for degradation.



### C. Percentage of embryos that show delay in degradation

	<i>spn-4</i> probe	N	<i>mex-3</i> probe	N
<i>eri-1</i> ( <i>mg366</i> )	25.8%	31	30.8%	26
<i>eri-1</i> ( <i>mg366</i> ); <i>vet-5r</i> RNAi	5.6%	18	13.6%	22

#### Figure 4.7: Exogenous *vet-5* can rescue the degradation defect

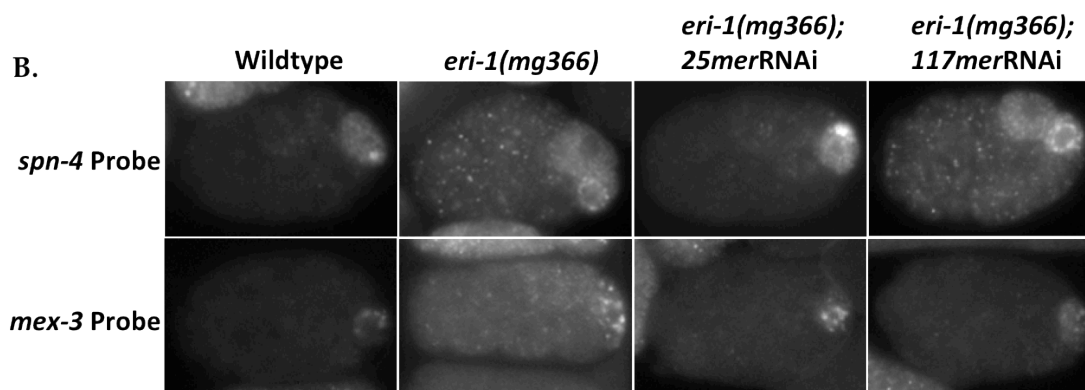
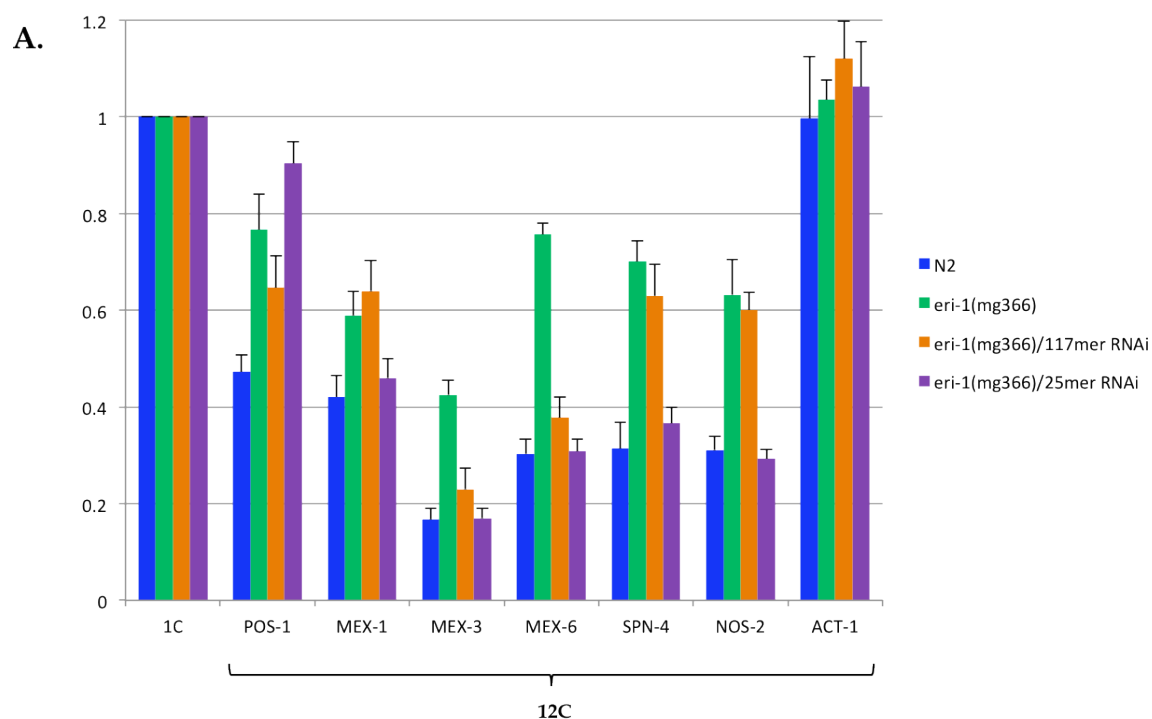
A) qPCR of 1-cell embryos vs. 12-cell embryos in indicated genetic backgrounds. 1C indicates the 1-cell samples and 12C indicates the 12-cell samples. The 1-cell sample has been set to 1. X-axis labels represent the primers used and the Y-axis indicates fold change. Each bar labeled with a specific primer represents the 12-cell sample (12C). Results are normalized against tubulin. B) Fluorescent in situ hybridization micrographs of 16-cell stage embryos with indicated probes in indicated backgrounds. C) Percentage of embryos that showed a delay in degradation based on the in situ hybridization experiments.

## 2. The 117mer and 25mer repeats show selection for target mRNAs

To investigate if the 117mer region or the 25mer region of *vet-5* is responsible for the degradation of mRNAs I generated feeding RNAi clones specific to each separate repeat (will be referred to as *117mer* RNAi and *25mer* RNAi). qPCR experiments were repeated on *eri-1* worms fed with either *117mer* RNAi or *25mer* RNAi. Transcript levels persisted in *eri-1/117mer* RNAi 12-cell embryos for 3 out of the 6 class II maternal RNAs tested, *mex-3*, *mex-6*, and weakly *pos-1*. Whereas transcript levels persisted in *eri-1/25mer* RNAi 12-cell embryos for 5 out of the 6 class II maternal RNAs tested, *mex-1*, *mex-3*, *mex-6*, *spn-4*, and *nos-2* (Figure 4.8A).

In situ hybridizations were performed with the *mex-3* and *spn-4* smFISH probes to confirm the qPCR results. Consistent with qPCR, *mex-3* mRNA persisted in only 14.3% (n=14) of *eri-1/25mer* RNAi embryos and 16% (n=25) of *eri-1/117mer* RNAi embryos as compared to 30.8% (n=26) in *eri-1* alone (Fig 4.7B and C). Also consistent with qPCR,

*spn-4* mRNA persisted in only 13.6% (n=22) of *eri-1/25mer* RNAi, but 28.6% (n=21) of *eri-1/117mer* RNAi embryos as compared to 25.8% (n=31) in *eri-1* alone (Figure 4.8B and C). The qPCR and in situ hybridization results suggest that the 117mer region and 25mer region target a specific subset of maternal mRNAs for degradation.



**C. Percentage of embryos that show delay in degradation**

	<i>spn-4</i> probe	N	<i>mex-3</i> probe	N
<i>eri-1</i> ( <i>mg366</i> )	25.8%	31	30.8%	26
<i>eri-1</i> ( <i>mg366</i> ); 25mer RNAi	13.6%	22	14.3%	14
<i>eri-1</i> ( <i>mg366</i> ); 117mer RNAi	28.6%	21	16.0%	25

**Figure 4.8: The 117mer and 25mer regions degrade a subset mRNAs**

A) qPCR of 1-cell embryos vs. 12-cell embryos in indicated genetic backgrounds. 1C indicates the 1-cell embryo samples that have been set to 1 and 12C indicates the 12-cell samples. X-axis labels represent the primers used and the Y-axis indicates fold change. Each bar labeled with a specific primer represents the 12-cell sample (12C). Results are normalized against tubulin. B) Fluorescent in situ hybridization micrographs of 16-cell stage embryos with indicated probes in indicated backgrounds. C) Percentage of embryos that showed a delay in degradation based on the in situ hybridization experiments.

## VI. Discussion

Early embryonic development is mainly dependent on maternally provided mRNA. Regulation of maternal mRNA has been studied extensively, as discussed in chapters 2 and 3 and by others, to ensure precise spatio and temporal expression of their protein counterparts. However, maternal mRNA does not persist in the embryo and tightly regulated degradation mechanisms must be in place to allow for the timely degradation of maternal mRNA. The degradation of maternal mRNA has been linked with the onset of zygotic transcription and is believed to be important to control mRNA dosage in the early embryo (Baugh et al. 2003; Tadros et al. 2007; De Renzis et al. 2007; Piko and Clegg 1982; Alizadeh et al. 2005; Tadros and Lipshitz 2009).

In *C. elegans* two classes of mRNAs exist, class I mRNAs that remain relatively stable and persist throughout early development in all blastomeres, and class II mRNAs that are degraded in somatic blastomeres, mostly beginning at the 4-cell stage embryo



(Seydoux and Fire 1994). Zygotic transcription is first detected at the 4-cell stage in somatic blastomeres specifically, thereby coinciding with the onset of maternal mRNA degradation and suggesting a possible link between zygotic transcription and maternal mRNA clearance (Schauers and Wood 1990; Seydoux and Fire 1994). Although several mechanisms have been implicated in maternal mRNA degradation in *C. elegans*, including endo/exonucleases and P-body components, a complete block of degradation has been unsuccessful. This can be explained by functional redundancy between pathway components, which is commonly observed in degradation pathways in other species such as yeast (Coller et al. 2001; Tharun et al. 2000) or it can be explained by the existence of a yet undiscovered degradation pathway.

Small regulatory RNAs are continuously being discovered and it is becoming evidently clear that they play an important role in many aspects of RNA surveillance. Gene silencing through the action of regulatory RNAs was first discovered in *C. elegans* by Andrew Fire and Craig Mello in 1998 when they discovered that the introduction of exogenous double stranded RNA can silence endogenous genes (Fire et al. 1998). Shortly thereafter silencing in plants was shown to be accompanied by the appearance of short endogenous RNAs that match the sequence of their target (Tomari and Zamore 2005). It is now clear that three main categories comprise these short RNA species: piRNAs, siRNAs, and miRNAs. miRNAs appear to be the main regulators of endogenous genes and siRNAs appear to be, for the majority, defenders of the genome in response to invasive nucleic acids. However, in recent years it has been discovered that siRNAs also play an important role in the regulation of endogenous genes (Simmer et al. 2002) and

removing parts of the endogenous siRNA pathway leads to selective inhibition of mRNA degradation (Fischer et al. 2011).

Whether the siRNA pathway plays a role in degradation of maternal RNAs at the maternal-to-zygotic transition was not known. In this chapter, I have investigated whether zygotic transcription, in connection with the siRNA pathway, is responsible for the clearance of class II maternal RNAs. When I knocked down either zygotic transcription or several components of the siRNA pathway (*eri-1*, *eri-6*, or *ergo-1*) I observed a persistence of some class II maternal RNAs. The question then became if a specific zygotic transcript is responsible for the degradation of class II maternal mRNAs.

Several early zygotic transcripts, all beginning in the 4-cell embryo, have been detected. One of these transcripts, *vet-5*, displays strong nuclear localization as shown by in situ hybridization (Schauer and Wood 1990; Seydoux and Fire 1994). This is unlike the localization of most mRNAs, which usually reside in the cytoplasm. The cloned cDNA of this transcript contains 2 117mer repeats and 4 25mer repeats. I mapped the *vet-5* cDNA to a genomic locus and discovered that the 4 25mer repeats are followed by another ~200 25mer repeats with little sequence variation and that, based on RNA-seq data, transcription is detected from this region in early and late embryos. Small RNA sequencing experiments have revealed that siRNAs map to this locus (Fischer et al. 2011) suggesting that the repeat region could serve as a pre-amplified pool of endogenous siRNAs. Using smFISH probes, I was able to reproduce the known nuclear localization pattern of *vet-5* and in addition I was able to detect low cytoplasmic signal beginning at the 4-cell stage embryo. Interestingly, *vet-5* smFISH signal increased dramatically in

siRNA mutant embryos. All these results suggest the siRNA pathway could generate siRNAs from the *vet-5* locus to target class II maternal RNAs for degradation.

All the siRNA mutants used only disrupt the endogenous siRNA pathway; therefore siRNAs can presumably still be generated from exogenously introduced *vet-5* in these mutants to target mRNAs for degradation. Consistent with our hypothesis, introducing exogenous *vet-5* RNA led to a rescue of the mRNA persistence observed in siRNA mutants indicating that *vet-5* is sufficient to target mRNAs for degradation. I also exogenously introduced the 117mer and 25mer repeat regions of *vet-5* separately and found that each repeat only targets a subset of mRNAs.

To further characterize if siRNAs generated from the repeats in the *vet-5* locus can target class II maternal mRNAs for degradation, bio-informatics will need to be carried out. It is of great interest to determine if the repeat sequence is complementary to known class II maternal mRNA sequences. In addition, if no sequence complementarity can be found to maternal mRNAs, I cannot rule out the possibility that *vet-5* has an indirect effect on maternal mRNA degradation. For instance, processed siRNAs from the *vet-5* locus may be targeting protective enzymes, such as poly(A) polymerases or 5' cap activators thereby indirectly leading to mRNA degradation. In addition, piRNAs have been shown to indirectly degrade mRNAs by activating the siRNA pathway (Bagijn et al. 2013). *vet-5* repeats could therefore also act as piRNAs to activate the endogenous siRNA pathway.

Though my results show a strong indication that *vet-5* can target class II maternal mRNAs for degradation through the siRNA pathway, a complete block in degradation is not achieved. This can be explained by functional redundancy with other degradation

pathways since the exosome and p-body pathways have also been implicated in mRNA degradation (Parker and Sheth 2007; Gallo et al. 2008). Further double knockdown experiments will need to be carried out to explore this possibility. Another possibility that can explain the lack of a complete block of degradation is the lack of a 100% penetrant phenotype. My smFISH results indicate that only a fraction of embryos (no more 30.8%) show a degradation defect in siRNA mutants. My qPCR data is therefore an underestimation of the degradation defect as it was carried out from a pool of ~500 embryos. A less than a 100% penetrant phenotype could be due to functional redundancy within the siRNA pathway itself, but will need to be further explored. Overall my results provide strong underlying insights into an additional degradation mechanism utilized to degrade class II maternal RNAs at the maternal-to-zygotic transition.

## CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

### Post-transcriptional regulation of maternal mRNA

Maternally provided mRNAs are critical for embryogenesis and guide much of the early developmental events including, but not limited to, cell fate specification, timing of cell divisions, cell-cell interactions and cell signaling. Maternal mRNA is transcribed in high abundance and localizes ubiquitously throughout the adult germline. When sperm fertilizes an oocyte the maternal mRNA packaged within the oocyte is passed on to the zygote and is present ubiquitously throughout the early embryo. The proteins that these mRNAs encode for have unique and critical roles during embryogenesis and their function is only required in a subset of blastomeres. Therefore mechanisms must be in place to allow for precise spatio-temporal expression of proteins derived from maternal mRNA.

Previous studies have shown that the main mechanism of maternal mRNA regulation occurs post-transcriptionally through their 3' UTR (Merritt et al. 2008). I have carried out comprehensive studies to determine how two maternally provided mRNAs, the CUL-2 containing E3 ligase subunit *zif-1* and the Wnt ligand *mom-2*, are regulated through their 3' UTR to ensure precise spatio-temporal protein expression. These studies have revealed two main common themes in post-transcriptional regulation of maternal mRNAs. 1) In the context of RBPs when both positive and negative regulators are removed, target mRNA is translated. 2) Binding to the 3' UTR by negative and positive regulators is often not compatible. These common themes indicate that translation is actively being repressed by RBPs and that translation of target mRNA is enabled when an

activating RBP prevents the binding of a repressive RBP. Translation in a particular blastomere of a specific mRNA is then determined by the combinatorial expression of activating and repressive RNA-binding regulators. The 3' UTR therefore acts much like an enhancer element required for transcriptional regulation where the transcriptional output is dependent on the precise combination of transcription factors present in a cell at a particular time that are capable of binding to the enhancer element.

ZIF-1 and MOM-2 protein are first detected, in a reciprocal pattern, at the 4-cell stage embryo. ZIF-1 is expressed in the three somatic blastomeres whereas MOM-2 is expressed in the germline blastomere P2. Interestingly, *zif-1* and *mom-2* are regulated by a similar set of RBPs despite their reciprocal expression pattern. Some RBPs display a similar role in the regulation of both targets. For instance OMA-1/2 are required for post-transcriptional repression in the oocytes and MEX-3 and SPN-4 together function as repressors in the 1- and 2- cell embryo for both targets. Interestingly, however, POS-1 and MEX-5/6 have opposing roles in regulating *zif-1* and *mom-2* expression. POS-1 acts as a repressor of *pos-1* expression and an activator of *mom-2* expression. MEX-5/6, on the other hand, acts as an activator of *zif-1* expression and a repressor of *mom-2* expression. Consistent with the observation that the 3' UTR contains an embedded “code” to direct post-transcriptional regulation, it can be postulated that the code within a particular 3' UTR determines if a regulator will have an activating or repressing role.

Cofactor binding to known regulators can also account for target-specific activation/repression. Our lab has shown that OMA-1 functions in the oocytes to negatively regulate at least three target mRNAs; in addition to *zif-1* and *mom-2* our lab has identified one additional target that will be published elsewhere. However, OMA-1 is

also known to bind to the *mei-1* 3' UTR. This is an interesting observation since *mei-1* is actively translated in oocytes, but repressed in the early embryo, an expression profile reciprocal to the other three OMA-1 targets. Our lab, through a suppressor screen, has identified several putative OMA-1 cofactors that may function to switch OMA-1 from a repressor on one target to an activator on another target. I have preliminary data indicating that some of these cofactors bind to OMA-1 as well as to specific target mRNAs. These results suggest that the regulatory function of RBPs could be determined by the target-specific recruitment of cofactors. The OMA-1 suppressor screen provides an excellent starting point to dissect this hypothesis further.

Deciphering 3' UTR codes would allow one to determine translational output simply based on the 3' UTR sequence. Recognizing when and where mRNA is translated based on direct sequence can be a powerful tool to understand aberrant protein expression in disease. In addition to deciphering 3' UTR codes, it is also of interest to determine mechanisms of repression. I have shown that at least two repressors from my studies, OMA-1 and MEX-3, appear to act through IFET-1 thereby generating a repressive loop. Currently it is unclear how other repressors function to negatively regulate protein expression.

### **Degradation of maternal mRNA**

In addition to spatio-temporal regulation of protein expression, the degradation of maternal mRNAs is also critical during embryogenesis. Depending on the species, at some point during early embryonic development, a maternal mRNA clearance event occurs, which is often dependent on zygotic transcription. It has been postulated that the

clearance of maternal mRNA is important for regulating mRNA dosage in the early embryo. I have studied how *C. elegans* maternal mRNAs get degraded in somatic blastomeres at the maternal-to-zygotic transition. I have shown that degradation of maternal mRNA is dependent on zygotic transcription and the endogenous siRNA pathway, although I was unable to get a full block in degradation. An endogenous locus containing 2 117mer and ~200 25 repeat elements, termed *vet-5*, to which endogenous siRNAs have been mapped (Fisher et al. 2011) appears to be sufficient to degrade at least some maternal mRNAs. In addition, the 117mer and 25mer repeats appear to act on a unique subset of target mRNAs.

Further studies will need to be carried out to determine the mechanism of *vet-5* dependent degradation. If siRNAs are generated from the *vet-5* locus then one would presume that maternal mRNAs contain a sequence complementary to the siRNA sequence. Bio-informatics will need to be performed to explore this possibility. It cannot be ruled out at this point however that *vet-5* can also have an indirect effect on mRNA degradation. siRNAs produced by the *vet-5* locus could instead target protective mRNA enzymes or production of *vet-5* associated small RNAs can activate additional maternal mRNA-specific degradation pathways. Overall, however, my results have a strong indication that a siRNA mediated pathway may act on repetitive endogenous loci and that the subsequent generation of siRNAs is important for the degradation of maternally provided mRNAs.

Since a full block of mRNA degradation has never been achieved, it would be of interest to block the endogenous siRNA pathway in conjunction with knockdowns of known mRNA degradation machineries. These experiments could reveal functional



redundancy between different degradation machineries. In addition, knockdown of several miRNAs has been shown to cause embryonic lethality, albeit no function has been assigned to these miRNAs. It would therefore be interesting to determine if the miRNAs pathway plays a role in mRNA degradation at the maternal-to-zygotic transition. Overall, the existence of multiple mRNA degradation machineries highlight the complexity of mRNA degradation and additional studies will need to be carried out to dissect these pathways further.

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