UNDERSTANDING THE SEQUENCE-SPECIFICITY AND RNA TARGET RECOGNITION PROPERTIES OF THE OOCYTE MATURATION FACTOR, OMA-1, IN *CAENORHABDITIS ELEGANS*

A Dissertation Presented

By

EBRU KAYMAK

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Biochemistry and Molecular Pharmacology

SIGNATURE PAGE

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DEDICATION

This thesis is dedicated to my family. To my dear mother, Turkan Kaymak, and my father Orhan Kaymak.

Sevgili annecigime ve babacigima..

Sizin emeginiz ve desteginiz olmadan bu basariyi elde edemezdim. Hep yanimda oldugunuz icin ve elinizden gelenin en fazlasini yaptiginiz icin cok tesekkur ederim.

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Thanks to all the labmates in the Ryder lab. John Pagano started me with purifications and in vitro selection experiments through my rotation. His guidance was very appreciated. Brian Farley has been a great mentor throughout the years. He showed me everything I know to do with *C. elegans*. Ruth Zearfoss has been my troubleshooting person. Thanks to her for always being available to help with valuable suggestions.

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ABSTRACT

Maternally supplied mRNAs encode for necessary developmental regulators that pattern early embryos in many species until zygotic transcription is activated. In *Caenorhabditis elegans*, post-transcriptional regulatory mechanisms guide early development during embryogenesis. Maternal transcripts remain in a translationally silenced state until fertilization. A suite of RNA-binding proteins (RBP's) regulate these maternally supplied mRNAs during oogenesis, the oocyte-to-embryo transition, and early embryogenesis. Identifying the target specificity of these RNA-binding proteins will reveal their contribution to patterning of the embryo. We are studying post-transcriptional regulation of maternal mRNAs during oocyte maturation, which is an essential part of meiosis that prepares oocytes for fertilization. Although the physiological events taking place during oocyte maturation have been well studied, the molecular mechanisms that regulate oocyte maturation are not well understood.

OMA-1 and OMA-2 are essential CCCH-type tandem zinc finger (TZF) RBP's that function redundantly during oocyte maturation. This dissertation shows that I defined the RNA-binding specificity of OMA-1, and demonstrated that OMA-1/2 are required to repress the expression of 3′UTR reporters in developing oocytes. The recovered sequences from *in vitro* selection demonstrated that OMA-1 binds UAA and UAU repeats in a cooperative fashion. Interestingly, OMA-1 binds with high affinity to a conserved region of the *glp-1* 3′UTR that is rich in UAA and UAU repeats. Multiple RNA-binding proteins

regulate translation of GLP-1 protein, a homolog of Notch receptor. In addition to previously identified RBP's, we showed that OMA-1 and OMA-2 repress *glp-1* reporter expression in *C. elegans* oocytes.

Mapping the OMA-1 dependent regulatory sites in the *glp-1* mRNA and characterizing the interplay between OMA-1 and other factors will help reveal how multiple regulatory signals coordinate the transition from oocyte to embryo but the abundance of OMA-1 binding motifs within the *glp-1* 3′UTR makes it infeasible to identify sites with a functional consequence. I therefore first developed a strategy that allowed us to generate transgenic strains efficiently using a library adaptation of MosSCI transgenesis in combination with rapid RNAi screening to identify RBP-mRNA interactions with a functional consequence. This allowed me to identify five novel mRNA targets of OMA-1 with an in vivo regulatory connection. In conclusion, the findings in this dissertation provide new insights into OMA-1 mediated mRNA regulation and provide new tools for *C. elegans* transgenesis. Development of library MosSCI will advance functional mapping of OMA-1 dependent regulatory sites in the target mRNAs. Extending this strategy to map functional interactions between mRNA targets and RNA-binding proteins in will help reveal how multiple regulatory binding events coordinate complex cellular events such as oocyte to embryo transition and cell-fate specification.

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  Kaymak, E., Wee, LM and Ryder, SP (2010) Structure and function of nematode RNA-binding proteins

- Most of the text and all figures in Chapter II and portions of the text in Chapter IV, are published as a scientific article in the *Journal of Biological Chemistry*. No permission is needed for the authors to use this material.

  Kaymak, E. and Ryder, S.P (2013) RNA recognition by the *C. elegans* oocyte maturation determinant, OMA-1

- Most of the text and all figures in Chapter III and portions of the text in Chapter IV are submitted as a scientific article in the journal *Developmental Dynamics*

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- Appendix B is adapted from the following publication (permission taken from author if properly cited):

  Elewa, A., Shiriyama, M., **Kaymak, E**, Harrison, P.F., Powell, D.R., Du, Z., Chute, C.D., Woolf, H., Yi, D., Ishidate, T., Srivnivasan, J., Bao, Z. Beilharz, T.H., Ryder, S.P., Mello, C.C. (2015) POS-1 promotes endo-mesoderm development by inhibiting the cytoplasmic deadenylation of *neg-1* mRNA. *Dev. Cell*

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  Tamburino, A.M., **Kaymak, E.**, Shrestha, S., Ryder, S.P. Walhout, A.J.M. (2015) PRIMA: an RNA-centered protein-RNA interaction mapping assay (submitted)

ABBREVIATIONS

BRE	Bruno Response Element
CPE	Cytoplasmic Polyadenylation Element
CPEB	Cytoplasmic Polyadenylation Element Binding Protein
DTC	Distal Tip Cell
DSL	Delta/Serragate/Lag-2
4E-BP	eIF4E Binding Protein
EMSA	Electrophoretic Mobility Shift Assay
FP	Fluorescence Polarization
FBE	FBF Binding Element
GBM	GLD-1 Binding Motif
mRNA	messenger RNA
MZT	maternal-zygotic transition
OBM	OMA-1 binding motif
PRE	POS-1 Recognition Element
RBP	RNA-Binding-Protein
STAR	Signal Transduction and Activation of RNA
SCR	Spatial Control Region
TGF-ß	Transforming Growth Factor Beta
TCE	Translational Control Element
TZF	Tandem Zinc Finger
UTR	Untranslated Region
VgLE	Vg1 Localization Element

PREFACE

Part of the work described in Chapter I was published as a review article in the journal *Current Opinion in Structural Biology* written by Sean Ryder, me and Liang Meng Wee.

All of the work presented in Chapter II was published in a research article in the *Journal of Biochemistry* written by Sean Ryder and me. All of the work was done in the Ryder Lab.

The work presented in Chapter III is submitted as a research article to the journal of *Developmental Dynamics* and is in revision. I am the primary author in this article. All of the work was done in the Ryder Lab.

The work presented in Appendix A is unpublished. Library of reporter constructs used in Appendix A were cloned by Charlene Pizzimente. All the work is done in the Ryder Lab. Figure 5.1 used in this appendix was produced by me.

The work presented in Appendix B is published in the journal of *Developmental Cell* by Ahmed Elewa in the lab of Craig Mello. Figures 5.2, 5.3 and 5.4 used in this appendix were produced by me.

The work presented in Appendix C is submitted by Alex Tamburino in the lab of Marian Walhout. Figure 5.5 used in this appendix was produced by me.

glp-1 3´UTR reporter strain was made by Brian Farley. OBM1 and OBM2 mutations were also introduced by Brian Farley.

nos-2 3´UTR reporter strain was made by John Pagano.

OMA-1::GFP strain used in Figure 1.6 was purchased from the Caenorhabditis Genetics Center (CGC). The strain identifier is TX189.

CHAPTER I: INTRODUCTION

Universal Characteristics of Metazoan Early Development

Sexual reproduction in metazoans requires the formation of haploid gametes: a larger oocyte and a smaller sperm. Fusion of these two haploid gametes is required to generate a diploid zygote and initiate the development of an organism. How a differentiated oocyte transitions into a totipotent embryo has been a crucial developmental question in the field of embryology.

To ensure a successful development, meiotic divisions in the oocytes must be completed before zygote formation. Therefore, precise regulation of meiosis during oocyte development is necessary to couple meiotic events to fertilization. An evolutionarily conserved feature of oocyte development is meiotic arrest, whereby immature oocytes arrest at meiosis I until a signal triggers completion of meiosis. The process of oocyte maturation is the process that releases the meiotic arrest upon extrinsic cues and prepares the oocyte for fertilization (McCarter et al., 1999; Yamamoto et al., 2006). Maturation is an essential step for proper early embryonic development. Physiological changes that occur during oocyte development have been well characterized. Nuclear envelope breakdown, meiotic spindle assembly, and cortical cytoskeleton rearrangement morphologically characterize oocyte maturation (Horner and Wolfner, 2008; McCarter et al., 1999). The duration of this arrested state varies between organisms: human oocytes can arrest for decades, mouse oocytes arrest for months, *Drosophila* oocytes arrest for hours and nematode worm *C.*

elegans oocytes arrest for approximately twenty-three minutes (Kishimoto, 2003; McCarter et al., 1999; Nishiyama and Tachibana, 2010; Sagata, 1996; Stetina and Orr-Weaver, 2011; Whitaker, 1996). As oocytes develop during oogenesis, information required for programming early development is stored in oocytes. This phenomenon is conserved across metazoans and understanding the molecular basis of this established program in oocytes has been one of the key developmental questions studied. Although oocyte maturation, fertilization and early embryogenesis events remain poorly understood in molecular terms, a developmental theme unifies the oocyte-to-embryo transition across all metazoans studied: developmental control of this transition relies on post-transcriptional regulation of maternal messenger RNAs (mRNAs) (Farley and Ryder, 2008; Richter, 1991; Tadros and Lipshitz, 2005). Remarkably, there is little or no transcription during the oocyte-to-embryo transition but after the maternal to zygotic transition (MZT) state in the embryos, transcriptional regulation of the zygote's genome takes over the control of developmental events (Blackwell, 2004; Cao et al., 2006; Schier, 2007; Stitzel and Seydoux, 2007). Therefore, until initiation of zygotic transcription, maternal mRNAs that are transcribed during early embryogenesis are essential for programming of oocyte-to-embryo transition and early development.

Regulation of Oocyte-to-Embryo Transition in Metazoan Model Organisms

Several of the key general principles in the field of post-transcriptional control of maternal mRNAs during oocyte-to embryo transition were addressed in detail using the model organisms *Xenopus* and *Drosophila* (Richter and Lasko, 2011).

Xenopus is a model organism that has been used extensively to study the key concepts of oocyte development and different modes of post-transcriptional control in oocytes (Ferrell, 1999; Tunquist and Maller, 2003). The oocytes of *Xenopus* are large and easy to extract in large quantities, as well as easy to manipulate and to microinject. As seen in other organisms, *Xenopus* oocytes arrest at prophase I of meiosis I. At this stage, the oocytes are transcriptionally active to produce mRNAs that are required for maturation and embryonic cell divisions. As oocytes continue to grow, these mRNAs are kept in a masked state meaning that they are not translationally active (Richter, 1991; Spirin, 1966). Once oocyte maturation is initiated, by the hormone progesterone in this case, synthesis of new proteins is required and this necessitates selective activation of maternal mRNAs (Tunquist and Maller, 2003). A well characterized mechanism of maternal mRNA activation in *Xenopus* is cytoplasmic polyadenylation. mRNAs containing *cis*-regulatory sequences termed cytoplasmic polyadenylation elements, (CPEs) in their 3′ untranslated region (3′UTR) are translationally repressed through binding of a complex of two proteins: CPE-binding protein (CPEB) and Maskin (Lin et al., 2010). Maskin is an eIF4E-binding protein (4E-

BP). Since eIF4-E is a cap-binding protein, binding of 4E-BP competes with eIF4-G binding, thus preventing recruitment of 40S ribosomal subunit and translational initiation (Cao et al., 2006; Stebbins-Boaz et al., 1999). Upon initiation of oocyte maturation, CPEB is phosphorylated (Mendez et al., 2000b). This prevents the interaction of CPEB with poly(A) ribonuclease (PARN) which catalyzes the deadenylation reaction (J. H. Kim and Richter, 2006). This permits cytoplasmic adenylation and activation of transcripts required for maturation events, such as cell-cycle progression. Well studied examples of cytoplasmic polyadenylation and subsequent activation of maternal mRNAs during oocyte maturation are the activation of two cell-cycle regulators *cyclin B* and *mos* that are conserved across vertebrates (Cao, 2002; Mendez et al., 2000a; Sheets et al., 1994; Stebbins-Boaz et al., 1996). In mouse oocytes, *cyclin B1* is activated in a similar pathway to *Xenopus* (Hodgman et al., 2001; Tay et al., 2000; Tay and Richter, 2001). Once maturation initiates, cytoplasmic polyadenylation of *cyclin B* mRNA is observed in *Drosophila* as well. In immature fly oocytes, *cyclin B* is repressed by the RNA-binding protein Pumilio. Polyadenylation during maturation is then achieved by a poly(A) polymerase present in the oocytes, Wispy (Benoit et al., 2008; Juge et al., 2002). These examples highlight how post-transcriptional regulation is coupled to polyadenylation mechanisms and is crucial for progression of oogenesis across model species. Activation of Cyclin B is one of the key events that initiate oocyte maturation in most species, including mammals (Barkoff et al., 2000; Sagata, 1996).

Post-transcriptional regulation of maternal mRNAs can also be coupled to localization mechanisms during oogenesis. For example, in *Drosophila*, *oskar* mRNA is localized to the posterior of the oocyte to establish posterior and germ cell fates (Bergsten and Gavis, 1999; Ephrussi and Lehmann, 1992; Kim-Ha et al., 1995). During its localization, in early oogenesis, *oskar* mRNA is repressed by an RNA-binding protein Bruno through interactions via *cis*-regulatory sequences termed Bruno response elements (BRE) (Kim-Ha et al., 1995; Lie and Macdonald, 1999; Snee et al., 2008; Webster et al., 1997). Bruno interacts with a 4E-BP, Cup. Cup, like Maskin, prevents *oskar's* translation by competing with eIF4E-G (Filardo and Ephrussi, 2003; Nakamura et al., 2004; Nelson et al., 2003; Wilhelm et al., 2003; Zappavigna et al., 2004). Once at the posterior pole, in late oogenesis, *oskar* translation is activated by polyadenylation for proper cell fate specification (Chang et al., 1999). Similarly, *bicoid* and *nanos* mRNA localization to the anterior and posterior poles of the oocyte is crucial for anterior-posterior axis formation (Gavis and Lehmann, 1992; Irion et al., 2006; Lasko, 2012). Importance of maternal mRNA localization patterns has been shown in frog oocytes too. In *Xenopus*, establishment of dorsal-ventral axis relies on correct localization of maternal mRNAs to animal and vegetal poles during late oogenesis (Melton, 1987; Mowry and Cote, 1999; Mowry and Melton, 1992). For example, restricting *Vg1* mRNA, encoding a transforming growth factor beta (TGF-β) molecule, to the vegetal pole is crucial for specification of endoderm and mesoderm cell-fate (Birsoy, 2006; Thomsen and Melton, 1993; Weeks and

Melton, 1987). There is a sequence element in the 3′UTR of this mRNA that is necessary and sufficient for its localization, the Vg1 localization element (VgLE) (Mowry and Melton, 1992). Two different RNA binding proteins (VgRBP60/hnRNPI and Vg1RBP/Vera interact with two distinct sequence elements within VgLE and these interactions are required for the localization of *Vg1* mRNA (Cote et al., 1999; Deshler et al., 1998). *Vg1* is translationally repressed prior to its localization to the vegetal pole. This repression requires a *cis*-regulatory sequence termed the translational control element (TCE) (Otero et al., 2001; Wilhelm et al., 2000) presumably via *trans*-acting partner(s) that mediate this repression (Colegrove-Otero et al., 2005). These examples highlight how post-transcriptional regulation is coupled to mRNA localization mechanisms and is crucial to axis formation and cell-fate specification events across model species.

The importance of post-transcriptional regulation of gene expression in controlling spatial and temporal translation of maternal mRNAs to ensure proper development has been studied extensively in the nematode worm *Caenorhabditis elegans* as well. Since Sydney Brenner introduced *C. elegans* as a model system to study developmental biology (Brenner, 1974), there have been many studies focused on post-transcriptional regulation in controlling spatiotemporal translation of maternal mRNAs. Ease of storage, large brood size, short life cycle (3 days at 25°C from egg ,through four larval stages, to an adult), facile genetics and a defined cellular lineage make *C. elegans* an attractive

model system to study fundamentals of maternal mRNA regulation in oocyte-to-embryo transition to complement studies in other metazoan systems (Hubbard and Greenstein, 2000; Sulston and Brenner, 1974; Sulston et al., 1983). In addition to the mentioned strengths of using *C. elegans* as a model organism, nematode worms present some unique features to facilitate studying the oocyte-to-embryo transition. *C. elegans* are predominantly hermaphroditic. Both sperm and oocytes are derived from the germline in the same gonad arm. As the organism develops from the final larval stage to an adult stage, gametogenesis switches from sperm production to oocyte production (Corsi et al., 2015; Riddle et al., 1997). This turns worm oocyte-to-embryo transition into an "assembly-line" where an oocyte matures, gets ovulated and fertilized in about 23 minutes, which is remarkably fast compared to other model organisms studied (Greenstein, 2005; Hubbard and Greenstein, 2000). Moreover, the transparency of the worm and the ability to knockdown genes by RNAi (Fire et al., 1998) provides a unique advantage to visualize all the developmental changes in oogenesis and early embryogenesis in a living organism, in real time, under a microscope. Fluorescent protein reporters have proven to be especially powerful tools for studying gene expression patterns and for following protein localization in *C. elegans* because the transparency of the worm facilitates the use of fluorescent markers as a means of determining gene expression patterns by direct observation under a fluorescent microscope. Upon introduction of GFP and the technology to make transgenic worm strains led to generation of strains

expressing GFP fusions with different promoters and/or different 3′UTRs to study gene regulatory events (Chalfie et al., 1994). This facilitated investigation of gene expression regulation and the role of *cis*-regulatory elements in patterns of expression, protein localization, and cellular developmental events in *C. elegans*.

C. elegans Germline Development, Oocyte Maturation and Early Embryogenesis

C. elegans contain two gonad arms that connect to a common uterus. Each gonad arm contains mitotically dividing primordial germ cells in the distal tip region **(Figure 1.1)**. These proliferating cells then exit the distal region and enter meiosis. During the meiotic transition, the primordial germ cells lose their cell membrane and nuclei migrate to the wall of the gonad arm where they share a cytoplasmic syncytium (Hirsh et al., 1976). In this syncytial region of the gonad, transcription of maternal mRNAs required for oogenesis and early embryogenesis occurs (Gibert et al., 1984). As nuclei transition through meiosis, they enter the prophase stage of meiosis I and arrest. Around the loop region of the gonad, gonad arm makes a turn of about 180 degrees and a small portion of the germ cell nuclei recellularize to form oocytes (Hirsh et al., 1976). The rest of the nuclei are degraded through apoptosis (Gumienny et al., 1999). At this loop region transcription ceases and cytoplasmic streaming deposits maternal mRNAs and proteins into developing oocytes (Nadarajan et al., 2009; Wolke et al., 2007).

Immature oocytes then develop sequentially in the proximal gonad arm. Developing oocytes remain at this stage of meiosis until oocyte maturation occurs **(Figure 1.1)** (Corsi et al., 2015; Farley and Ryder, 2008; Greenstein, 2005; Hubbard and Greenstein, 2005). The oocyte proximal to the spermatheca, which is the organ that stores sperm, is the first oocyte that matures, ovulates and becomes fertilized. This cycle repeats approximately every 23 minutes (Kimble and Crittenden, 2007; McCarter et al., 1999).

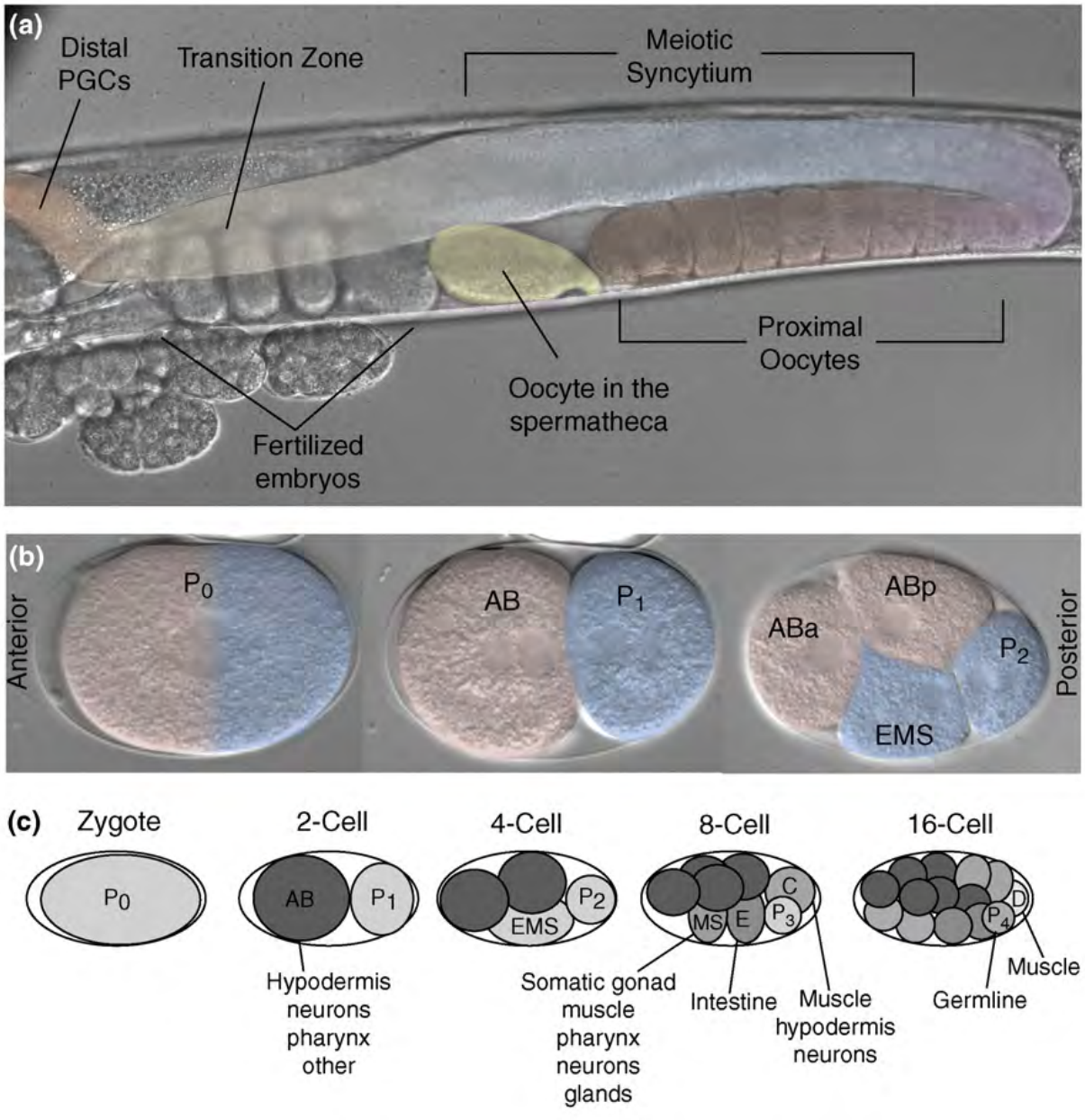

(a)
Distal PGCs
Transition Zone
Meiotic Syncytium
Proximal Oocytes
Oocyte in the spermatheca
Fertilized embryos
(b)
Anterior
Posterior
P0
AB
P1
ABa
ABp
P2
EMS
(c)
Zygote
2-Cell
4-Cell
8-Cell
16-Cell
P0
AB
P1
P2
EMS
MS
E
C
P3
P4
D
Muscle
Hypodermis
neurons
pharynx
other
Somatic gonad
muscle
pharynx
neurons
glands
Intestine
Muscle
hypodermis
neurons
Germline

Figure 1.1 Anatomy of *C. elegans* hermaphrodite reproduction

a) A single gonad arm from a hermaphrodite worm is shown. The gonad is highlighted in false color. The distal arm contains mitotically dividing progenitor cells (red). There is a transition (orange) from mitosis to meiosis concurrent with a transition from a single celled state to a syncytial region (blue). Meiotic nuclei recellularize, first to form spermatocytes in the L4 larval stage that are stored in the spermatheca (yellow) and then switch to form oocytes (purple) at the onset of adulthood. b) Patterns of the first two cellular divisions after fertilization. Anterior and posterior marks are marked. c)Pattern of division and early lineage of embryogenesis. Several founder cells are established early in embryogenesis that go on to form different tissues in the adult.

Maturation of the oocyte proximal to the spermatheca is temporally coupled to its ovulation and fertilization; therefore, it is a key step for coordinated regulation of oogenesis to ensure successful fertilization. How oocyte maturation is triggered is known: the major sperm protein (MSP), a diffusible hormone produced by sperm, initiates *C. elegans* oocyte maturation (Miller et al., 2001). As a response to this trigger the fully-grown oocyte proximal to the spermatheca completes its meiotic divisions, undergoes nuclear envelope breakdown and ovulates into the spermatheca where it is fertilized. The maturing oocyte also undergoes structural changes and becomes ovoid before ovulation. In addition, meiotic spindle assembly begins in the mature oocyte prior to fertilization (McCarter et al., 1999; Ward and Carrel, 1979). Although these hallmark events of oocyte maturation are morphologically well-characterized, molecular mechanisms behind these events that regulate oocyte maturation are poorly understood. Some maternal mRNAs and RNA-binding proteins have been identified as positive effectors of oocyte maturation. These are the maternal mRNAs transcribed by the germ cells at the distal end of the *C. elegans* germline, silenced, and then activated after fertilization. Sperm entry then initiates a cascade of events, like the completion of meiosis, reorganizes the cytoskeleton and establishes the anterior posterior axis (Ward and Carrel, 1979). Point of sperm entry determines the posterior pole of the embryo which undergoes its first mitotic division along this axis (Goldstein and Hird, 1996; Wallenfang and Seydoux, 2000). The first asymmetric cell division gives rise to a

larger and a smaller blastomere. The larger one is the anterior blastomere (AB) and the smaller one is the posterior blastomere (P_1). The anterior blastomere is the first founder cell. The AB founder cell specifies pharynx, neurons and hypodermis. The posterior blastomere is the germline progenitor. In turn, division of P1 gives rise to another founder cell, EMS, and another germline blastomere P_2. The posterior cell divides two more times in this pattern producing a founder cell and a germline blastomere with each division. Each founder cell then differentiates into a specific tissue type before gastrulation. Ultimately, the last P-lineage cell divides symmetrically into two primordial germ cells that establish the entire germline (Sulston et al., 1983).

Transcriptional activity ceases when the oocytes enter the prophase arrest and transcription is not initiated in the somatic blastomeres until the four-cell stage embryo (Blackwell and Walker, 2006; Seydoux, 1996; Seydoux and Fire, 1994; Walker et al., 2007). Asymmetric expression of maternal transcripts drives cellular differentiation before transcription begins in the embryo (Pellettieri and Seydoux, 2002). Therefore, post-transcriptional regulation of maternal mRNAs transcribed in the syncytial region contributes significantly to regulate the complex events of meiosis, oogenesis in the germline and early cell divisions in the embryo (Farley and Ryder, 2008; Stitzel and Seydoux, 2007). RNA-binding proteins play a crucial role in the regulation of maternal transcripts. In *C. elegans*, there are around 500 annotated RNA-binding proteins with known RNA-binding

domains; such as, RRM, KH, CCCH, PUF and Piwi/Argonaute/Zwille (PAZ) (Kimble and Crittenden, 2007; Lee and Schedl, 2006; Tamburino et al., 2013).

Nematode RNA-Binding Proteins

RNA regulation is pervasive and impacts nearly every aspect of gene expression. RNA molecules function both as regulators and targets in diverse pathways to ensure appropriate decoding of the genome. RNA-binding proteins are central to this form of regulation. They act as effectors of RNA stability and translation efficiency, they guide transcripts to defined locations within a cell, they control the fidelity of gene decoding, and they function as cofactors to promote the activity of functional and structural RNA molecules.

Forward and reverse genetic experiments indicate that many play distinct roles in germline development, gametogenesis, and early embryogenesis, where regulation of maternal RNAs plays a primary role (Farley and Ryder, 2008). Below is an outline of representative structures from the PUF, and TZF families, and highlights of data that identifies the basis for specialized function in the expanded set of nematode homologs.

The PUF family: (Kaymak et al., 2010)

PUF proteins in nematode germline development

The *fem-3* binding factor (FBF) was the first Pumilio homolog identified in *C. elegans* (Zhang et al., 1997). Pumilio and FBF together comprise the

founding members of the PUF family of RNA-binding proteins. FBF is encoded by two nearly identical genes, *fbf-1* and *fbf-2*. Together, they act to maintain the population of progenitor cells in the distal region of the germline and promote switch from spermatogenesis to oogenesis at the onset of adulthood (Crittenden et al., 2002; Zhang et al., 1997) **(Figure 1.1)**. FBF binds in a sequence specific fashion to the 3′UTR of several messenger RNAs, including *fem-3* and *gld-1* (Bernstein et al., 2005b). GLD-1 and FEM-3 promote spermatocyte differentiation, and GLD-1 promotes entry into meiosis (Ahringer and Kimble, 1991; Francis et al., 1995b). FBF represses translation of *gld-1* mRNA in the distal end of the germline, and it represses translation of *gld-1* and *fem-3* mRNA in developing oocytes (Crittenden et al., 2002; Zhang et al., 1997).

Ten additional *puf* genes, termed *puf-3* to *puf-12*, are present in the *C. elegans* genome. Most have distinct biological functions defined by phenotypic differences, mRNA target specificity, or expression pattern. Three of these genes—*puf-5*, *puf-6*, and *puf-7*—are redundantly required for embryonic viability and oocyte maturation (Lublin and Evans, 2007). They prevent premature translation of *glp-1* mRNA in oocytes. PUF-8 promotes mitosis in germline progenitor cells, similar to FBF, but binds to RNA with different sequence specificity and as such likely regulates a distinct set of target mRNAs. PUF-9 regulates hunchback-like (*hbl-1*) mRNA in the hypodermis and ventral nerve cord(Nolde et al., 2007). RNAi screens reveal important roles for PUF-3, PUF-4, PUF-10, PUF-11, and PUF-12 in oogenesis and early embryonic development,

but their critical mRNA targets have not been identified. (Fraser et al., 2000; Sönnichsen et al., 2005). In the following sections, we review a recently published crystal structure of FBF and highlight biochemical experiments that define differences in RNA recognition in this family (Wang et al., 2009).

Biochemical insights into PUF binding specificity

Wickens and co-workers have dissected the RNA binding properties of several PUF proteins (Bernstein et al., 2005a; Koh et al., 2009; Opperman et al., 2005; Stumpf et al., 2008). The consensus sequence recognized by FBF, termed the FBF binding element (FBE), is 5′-UGURNNAUA-3′ (Bernstein et al., 2005b). The FBE is nine nucleotides in length and is partially degenerate at three positions. FBEs are present in the 3′UTR of *fem-3*, *gld-1*, and several other mRNAs regulated by FBF in the germline. Mutation of the FBE in the 3′UTR of *fem-3* leads to de-repression of FEM-3 and failure to switch from spermatogenesis to oogenesis (Ahringer and Kimble, 1991).

PUF-8 and PUF-9, on the other hand, recognize an eight nucleotide consensus identical to that bound by human Pum1 (5'-UGUANAUA-3') termed the Nanos Response Element (NRE) (Opperman et al., 2005; Wang et al., 2002). The NRE is similar to the FBE but is a single nucleotide shorter. This difference is critical, as FBF discriminates between these two elements by more than 30-fold. Intriguingly, the specificity of PUF-8 can be converted to that of FBF by

swapping a 64-amino acid fragment in the middle of the PUF domain, demonstrating that this region is critical for specificity.

PUF-5 and PUF-6/7 recognize a longer, partially degenerate consensus motif termed the PUF-5 Binding Element (5BE: 5′-CyCUGUAyyyUGU-3′, where y is a pyrimidine) (Stumpf et al., 2008). PUF-11 binds three sets of RNA targets, 5′-CUGUGAAUA-3′, 5′-CUGUANAAUA-3′ and 5′-NUGUNAAAUA-3′, suggesting multiple modes of RNA recognition through a mechanism that is not immediately apparent (Koh et al., 2009). Clearly, these experiments show that the nematode PUF family has diverged to expand the repertoire of sequences recognized by the PUF domain. Recent crystal structures begin to address the molecular basis for this variance.

Crystal structures of PUF proteins

The first structures of a PUF domain, including *Drosophila* Pumilio and human Pum1, were determined independently in 2001 (Edwards et al., 2001; Wang et al., 2001). The structures revealed an architecture of eight repeat motifs comprised of three alpha helices. The repeats pack against each other to form an extended curved structure that vaguely resembles a banana. A subsequent structure of human Pum1 bound to RNA demonstrates that the concave surface comprises the RNA binding interface, where each repeat recognizes a single nucleotide **(Figure 1.2A)** (X. Wang et al., 2002). The amino acids that face the concave surface define the nucleotide specificity at each repeat, which has been

reviewed previously (Lu et al., 2009). This architecture immediately suggests a model where PUF proteins bind to RNA with modular specificity, such that changing the order of the repeats could modify RNA-binding specificity. Several experiments with chimeric PUF proteins support this model and suggest this domain is particularly amenable to protein engineering (Koh et al., 2009; Opperman et al., 2005; Stumpf et al., 2008).

All of the nematode PUF proteins are comprised of eight repeats, but many bind to a consensus element that contains more than eight nucleotides. To gain insight into the structural basis for recognition of longer elements by this domain, Hall and coworkers crystallized FBF-2 in complex with six different RNA sequences, including four naturally occurring sites (Wang et al., 2009). This study reveals that FBF has an elongated structure with less curvature relative to other PUF domain proteins **(Figure 1.2B)**. This elongated structure enables a single base to flip out and point away from the protein without affecting interactions with the other eight nucleotides. Thus, a slight variance of the curvature of the overall structure, governed by repeats 4-6, has a profound impact on the RNA-binding specificity.

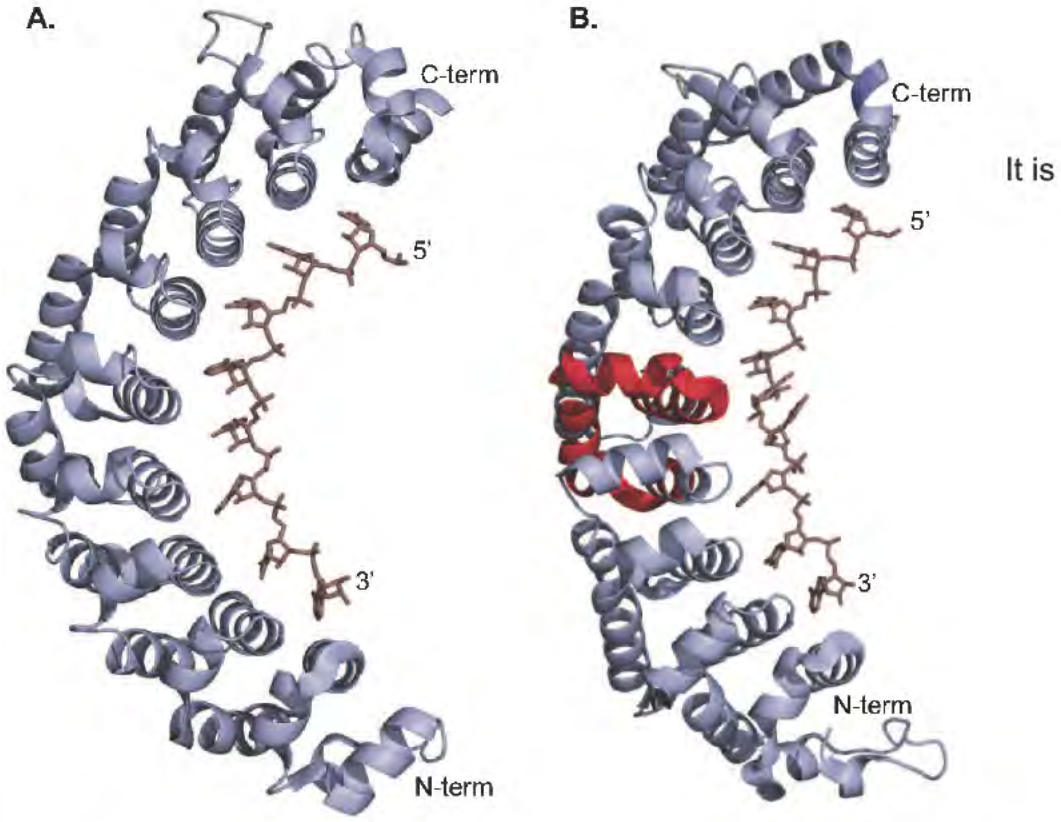


Figure 1. 2 Crystal structure of Puf domain proteins bound to RNA

a) The structure of human Pum1 bound to NRE. Each PUF repeat specifies a single nucleotide (Wang et al. 2002). b) Structure of *C. elegans* FBF bound to the FBE, similar to the NRE but containing an additional nucleotide. Here, reduced curvature of the protein enables recognition of a nine nucleotide consensus with an eight repeat PUF domain (Wang et al, 2009). One nucleotide flips away from the binding surface of the protein. The region of the protein that confers FBF like specificity when swapped into PUF 8 is shown in red.

It is possible that curvature driven base flipping accounts for the multiple modes of RNA recognition by PUF-11 (Koh et al., 2009). Each mode contains two conserved regions, including a UGU trinucleotide and an element comprised of AAAUA. These may represent eight "core" interactions with this protein. Each mode contains an insertion of one or more nucleotides into a distinct position

within this core. If the inserted nucleotides flip out, a model similar to FBF would resolve the multiple modes of binding. The incomplete degeneracy of the inserted nucleotides may be partially explained by differential stacking free energy with neighboring nucleotides. A similar model could be proposed for PUF-5/6/7, where eight nucleotides are specified unambiguously, and five more nucleotides are partially degenerate (Stumpf et al., 2008). More structural work is needed to assess this hypothesis and define the basis for the variance in PUF specificity. It is also important to assess whether conformational flexibility contributes to binding specificity.

TTP-like CCCH tandem zinc finger proteins: (Kaymak et al., 2010)

TZF proteins in C. elegans early embryogenesis

TTP is a mammalian RNA-binding protein that regulates the immune response by promoting the turnover of the mRNA encoding the pro-inflammatory cytokine TNF-alpha (Farley et al., 2008; Pagano et al., 2009). TTP is an AU-rich element (ARE) binding protein, which coordinate the stability of mRNAs containing extended repeats of UAUU in their 3′UTRs. TTP has two $CX_8CX_5CX_3H$ zinc finger motifs. Each motif binds to a single UAUU repeat (Hudson et al., 2004).

There are several TTP homologs in the *C. elegans* genome, many of which are required for worm fertility. A cascade of TZF proteins, including OMA-

1/2, MOE-3, MEX-5/6, MEX-1, POS-1, and PIE-1, guide the progression from the oocyte to embryo. OMA-1/2 and MOE-3 are partially redundant factors that promote oocyte maturation, and inhibit embryonic gene expression prior to fertilization (Detwiler et al., 2001; Mello et al., 1992; Tabara et al., 1999). MEX-5/6 are required for anterior patterning in the early embryo (Schubert et al., 2000). They are translated from maternally supplied mRNA shortly after fertilization, and migrate to the anterior of the embryo prior to the first cellular division. POS-1, PIE-1, and MEX-1 are also translated after fertilization, but accumulate in the posterior of the embryo in a pathway that depends upon MEX-5/6 anterior localization (Cuenca et al., 2003; Reese et al., 2000). All three proteins are required for posterior patterning and segregation of germline and somatic lineages, but have non-redundant functions (Mello et al., 1992; Tabara et al., 1999).In addition to these well studied examples, there are eight additional TZF genes in the *C. elegans* genome. DCT-13 and possibly Y116A8C.20 promote germline tumor formation in a sensitized genetic background, while CCCH-1, CCCH-2, CCCH-5, F38C2.7, Y116A8C.19, and C35D6.4 have no known function (Pinkston-Gosse and Kenyon, 2007).

NMR structure of a TZF family protein

Wright and coworkers determined the solution structure of the Tis11D bound to the RNA sequence 5´-UUAUUUAUU-3´ **(Figure 1.3A)** (Hudson et al., 2004). Tis11D is a mammalian paralog of TTP that regulates mRNA stability in

response to growth factors (Varnum et al., 1991). It binds to RNA with identical specificity to TTP. The structure reveals that each $CX_8CX_5CX_3H$ finger motif independently recognizes the four nucleotide sequence UAUU. A conserved motif with the sequence (R/K)YKTEL lies upstream of the first cysteine of each finger. This region makes numerous contacts with the RNA. These are primarily comprised of hydrogen bonds between the protein backbone and the Watson-Crick edges of the bases, and van der Waals interactions that specify the shape of the base at each position. In addition, the side chains of two conserved aromatic amino acids form stacking interactions between adjacent RNA bases at two positions within each finger. These amino acids are essential for high affinity binding, and may contribute to specificity through differential stacking propensity. This structure has thus far provided our only glimpse into RNA recognition by this class of RNA-binding proteins, and as such serves as the primary frame of reference for the interpretation of experiments for related factors.

Biochemical insights into nematode TZF binding specificity

In most cases, the RNA-binding activity of nematode TZF proteins has not been investigated in detail. The two exceptions are MEX-5 and POS-1, which bind to RNA but with different specificity compared to TTP, Tis11D, and each other (Farley et al., 2008; Pagano et al., 2007). MEX-5 binds with high affinity but relaxed specificity to any uridine rich sequence, including polyuridine. This contrasts with TTP which binds >80-fold more tightly to AREs than polyuridine.

POS-1 binds with high affinity to a consensus termed the POS-1 recognition element (PRE: 5'-UA($U_{2\ 3}$)RD($N_{1\ 3}$)G-3', where R is any purine, D is A, G, or U, and N is any base). Compared to TTP binding sequence, the PRE is more degenerate and specifies three purines instead of two.

In Tis11D, three contiguous amino acids in each finger form an adenosine recognition pocket: glutamate, leucine, and the first cysteine of the CCCH motif (**Figure 1.3B**) (Hudson et al., 2004). The glutamate side chain accepts a hydrogen bond from the exocyclic amine of the adenosine. The leucine and the cysteine are conserved in both MEX-5 and POS-1, but the glutamate is not. In MEX-5, the analogous amino acids are arginine in the first finger and a lysine in the second. Mutating both to glutamate confers TTP-like specificity to MEX-5, suggesting they are critical specificity determinants (Pagano et al., 2007). In POS-1, an alanine and a valine occupy the analogous amino acids. It is not clear how these amino acids contribute to the differences in POS-1 specificity, or how this protein specifies three purines compared to two. Structural data are needed to resolve this problem. One nematode TZF protein, CCCH-1, has two glutamate residues in the analogous position similar to TTP. The rest have basic residues, small hydrophobic residues, or some combination thereof. It is expected that CCCH-1 will bind to RNA with TTP-like specificity, and that the others will bind to RNA with hybrid specificity, but this has not been experimentally demonstrated.

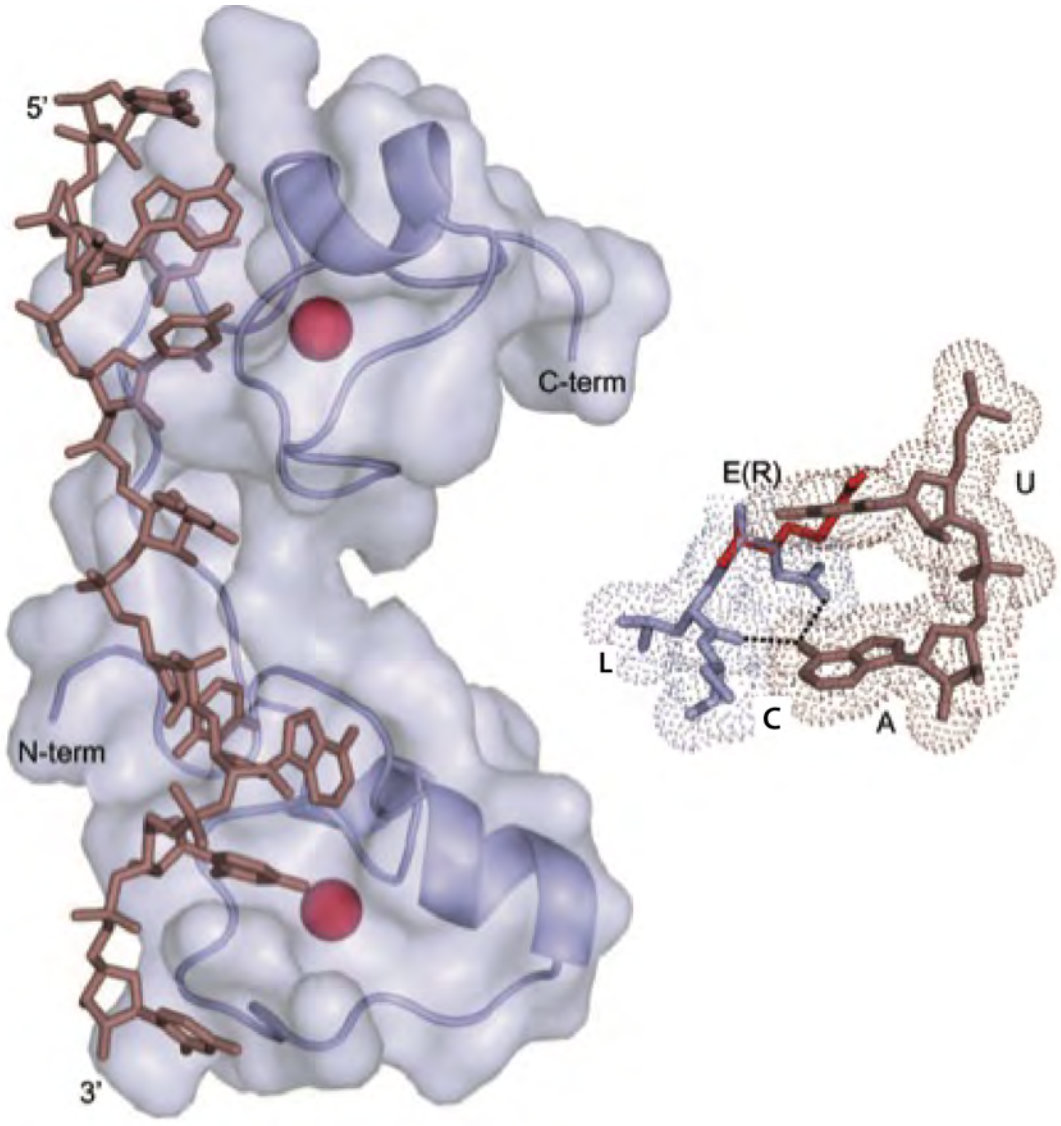


Figure 1. 3 NMR Structure of human Tis11D bound to RNA

Each zinc finger domain independently recognizes a UAUU through a combination of base specific hydrogen bonding interactions and stacking interactions driven by aromatic side chains. The inset shows recognition of adenosine in the N terminal finger. Three amino acids, glutamate, leucine and cysteine, come together to form an adenosine recognition pocket. In MEX 5, the glutamate is replaced with an arginine (red), which is proposed to flip away from the adenosine and form non specific interactions with the backbone of adjacent nucleotides (Hudson et al., 2004).

K homology (KH) family of RNA-binding proteins:

KH domain proteins in C. elegans germline development and embryogenesis

KH domain is an evolutionarily conserved domain first identified in heterogeneous nuclear ribonucleoprotein particle (Valverde et al., 2008). There are several homologs of this family of proteins in *C. elegans* and most are involved in oogenesis or cell-fate specification in early embryogenesis. One such protein is MEX-3. MEX-3 is a dual KH-domain RNA-binding protein that is required for anterior cell-fate specification (Draper et al., 1996). *mex-3 (muscle excess)* mutant embryos show abnormal development at the anterior blastomere of the two-cell embryo where excess muscle tissue is produced instead of hypodermal and pharyngeal tissues (Draper et al., 1996). This is due to spatial and temporal regulation of a homeodomain transcription factor PAL-1 that functions in specification of muscle cell fates in the embryo (Draper et al., 1996). MEX-3 represses *pal-1* mRNA in the anterior of the embryo and the 3′UTR of *pal-1* was shown to be sufficient for this repression (Hunter and Kenyon, 1996). *nos-2* mRNA is another target of MEX-3. *nos-2* encodes a Nanos homolog which is required for primordial germ cell development (Jadhav et al., 2008). In the germline, the absence of both MEX-3 and another protein GLD-1, also a KH domain protein, leads to transdifferentiation of germ cells into somatic cells (Ciosk et al., 2006). Hence, both of these KH domain proteins are required to maintain germ cell identity in the germline.

GLD-1 is another RNA-binding protein with a KH domain, but it also has a STAR (signal transduction and activation of RNA) domain (Jones et al., 1996; Vernet and Artzt, 1997). GLD-1 is essential for progression of meiosis of germ cell nuclei in the distal end of the germline to subsequently ensure proper oogenesis (Francis et al., 1995b; Jones et al., 1996). Several mutations that alter or inhibit GLD-1 function are within the STAR domain (Francis et al., 1995b; Jones and Schedl, 1995). *gld*-1 null mutants show ectopic proliferation of germ cells leading to a germline tumor and failed oogenesis (Francis et al., 1995b; 1995a). Consistent with its role in promoting meiotic progression, GLD-1 is expressed at its most abundant level in the transition zone of the germline where it acts to repress *glp-1* mRNA, a *C. elegans* encoded homolog of Notch receptor (Austin and Kimble, 1987; Farley and Ryder, 2012; Francis et al., 1995b; Marin and Evans, 2003). Repression of *glp-1* by GLD-1 is required for proper timing of the mitosis-meiosis switch (Kadyk and Kimble, 1998; Marin and Evans, 2003). Other targets of GLD-1 are also identified (Lee and Schedl, 2001; Min-Ho Lee and Schedl, 2004; Ryder et al., 2004). For example, GLD-1 also represses *pal-1* in regions of the germline where there is no MEX-3 expression (Mootz et al., 2004). Repression of *rme-2*, which regulates yolk updake by oocytes, by GLD-1 is required for successful oogenesis (Grant and Hirsh, 1999; Lee and Schedl, 2001). GLD-1 also functions in male sex determination, as GLD-1 mediated repression of *tra-2*, which is required for the switch to oogenesis, contributes to male sex determination (Doniach, 1986; Jan et al., 1999; Kuwabara et al., 1992).

Crystal structure of GLD-1 dimerization domain and STAR domain

Conventional KH domain bears a $\beta_1\alpha_1\alpha_2\beta_2\beta_3\alpha_3$ fold with a GxxG loop connecting the first two alpha helices (Valverde et al., 2008). RNA recognition is achieved via van der Waals forces, electrostatic and hydrophobic interactions in a binding cleft that accommodates four nucleotides (Valverde et al., 2008). GLD-1 has a STAR domain which is a maxi-KH domain flanked by two conserved regions (Ryder et al., 2004; Vernet and Artzt, 1997). KH domain of GLD-1 is flanked by a dimerization domain, QUA1, at the N-terminus and by another domain QUA2 at the C-terminus (Chen et al., 1997; Ryder et al., 2004; Vernet and Artzt, 1997). Crystal structure of the QUA1 dimerization domain was solved first. The structure showed that the domain possesses a helix-turn-helix motif where each protomer is perpendicular to the other (Beuck et al., 2012; 2010). Crystal structure of the whole STAR domain was then solved with the RNA sequence: 5′-CUAACAA-3′ (Teplova et al., 2013). This structure showed that the KH and the QUA2 domains interact to form a hydrophobic surface to accommodate the RNA-bases whereas the positively charged regions of the domains surround the sugar-phosphate backbone of the RNA molecule. QUA2

domain interacts with the two residues at the 5′ end of the RNA whereas the KH domain interacts with the remaining residues (Teplova et al., 2013) **(Figure 1.4)**.

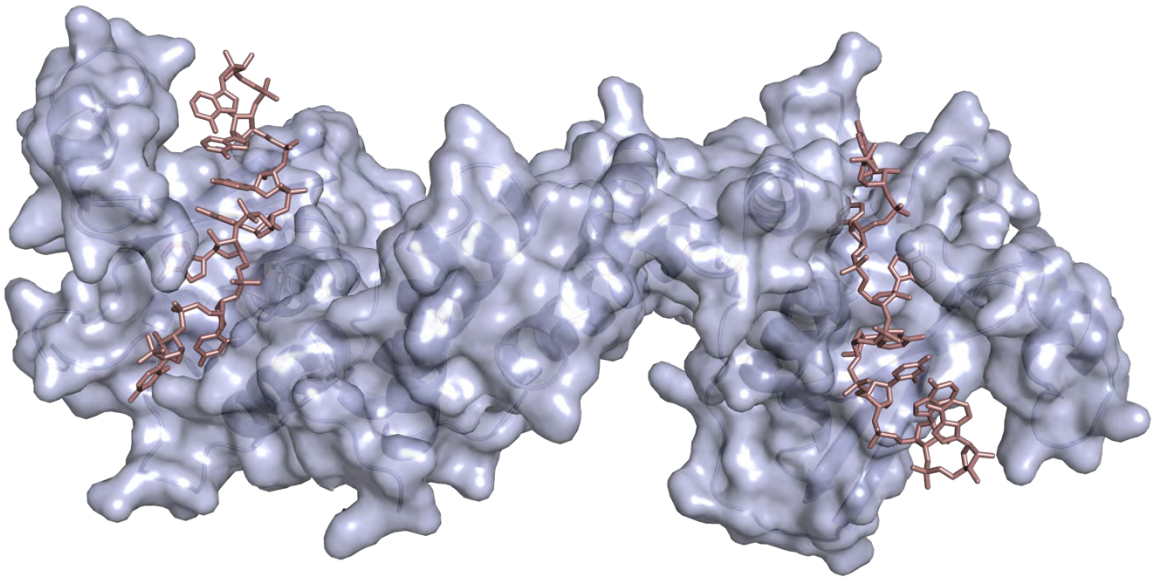


Figure 1. 4 GLD-1 Crystal Structure bound to RNA

Each protomer of the dimer is shown bound to CUAACAA RNA sequence. The RNA sits in a hydrophobic surface produced by the KH and QUA2 domains. The backbone of the RNA molecules interacts with the positively charged residues of the domains (Teplova et al., 2013).

Biochemical insights into MEX-3 and GLD-1 binding specificity

The sequence specificities of the KH domain proteins, MEX-3 and GLD-1 have been studied in detail (Pagano et al., 2009; Ryder et al., 2004). The RNA-binding sequence specificity of MEX-3 was biochemically identified. MEX-3 consensus sequence is DKAG$N_{0\ 8}$UHUA, where D is A, G or U; K is G or U, N is any base, and H is A, C or U. In fact, this sequence is present in the 3′UTR of

pal-1 and *nos-2*. MEX-3 consensus sequences in the *nos-2* 3´UTR was shown to be required for the repression of this target. *nos-2* 3´UTR has two MEX-3 binding sites. Mutating each half-site AUAG to CCCC led to disruption of the spatial and temporal regulation of the UTR reporter expression (Pagano et al., 2009). MEX-3 consensus sequence is also present in 30% of the annotated *C. elegans* 3′UTR's suggesting an interplay with other factors to specify functional targets (Pagano et al., 2009).

GLD-1 recognizes a hexameric sequence UACU(A/C)A (Ryder et al., 2004). STAR family members have an extended KH domain, which might permit a tighter consensus than MEX-3. One of the conserved domains, QUA2, flanking the KH domain adds an alpha-helix extension through which two to three additional nucleotides can be recognized. Recently it was shown that the GLD-1-binding motifs (GBMs) are responsible for association of GLD-1 with hundreds of maternal transcripts (Wright et al., 2010). GBMs were also shown to be functional for some targets of GLD-1. Mutating the GBMs present in the *rme-2* and *glp-1* 3´ UTRs led to de-repression of reporter expressions in the syncytial regions of the germline (Farley and Ryder, 2012; Wright et al., 2010).

To conclude, the function of RNA-binding proteins is dictated by their structure. It is important to understand how structural changes in the RNA-binding domains define the basis for novel function. While genetics and biochemical experiments can identify the critical sequence elements, they cannot in most cases address how these elements contribute to novel function in a

mechanistic sense. Structural studies can provide key insights needed to understand biological function.

C. elegans germline has been shown to have four times the number of RNA-binding proteins as compared to the soma (Wang et al., 2009). In line with this finding, there are a suite of RNA-binding proteins that have essential functions in regulation of complex events of germline development, oogenesis and early embryonic cell divisions, as discussed above. To summarize, these proteins include, OMA-1/2, MOE-3, MEX-5/6, PUF-5/6/7, GLD-1, POS-1, MEX-1 and PIE-1, which regulate oogenesis (OMA-1, OMA-2, MOE-3, PUF-5/6/7) (Bernstein et al., 2005a; Detwiler et al., 2001; Lublin and Evans, 2007; Shimada et al., 2002) and embryogenesis (GLD-1, MEX-5/6, POS-1, MEX-1 and PIE-1) (Mello et al., 1992; Schubert et al., 2000; Tabara et al., 1999). As alluded to above, *C. elegans* germline development and early embryogenesis relies extensively on post-transcriptional regulation via specific RNA-binding proteins that regulate when and where maternal mRNAs are translated. Different modes of post-transcriptional regulation involve RNA-binding proteins. In some instances, RBPs bind small RNAs, such as microRNAs, to target the RBP to its cognate RNA for regulation (Ghildiyal and Zamore, 2009; Pasquinelli, 2012). In other instances, RBPs directly interact with specific sequence elements in 5′ untranslated regions (5′UTR) or 3′ untranslated regions (3′UTR) of the mRNA (de Moor et al., 2005; Evans and Hunter, 2005; Glisovic et al., 2008; Lunde et al., 2007). Since the 5′UTRs are trans-spliced to a common leader sequence for the

majority of transcripts in *C. elegans*, 3′UTR mediated regulation of gene expression is prevalent (Blumenthal, 2012; 1995). The theme of 3′UTR mediated regulation of maternal transcripts is commonly seen in the *C. elegans* germline (Merritt et al., 2008).

3′UTR Governed Expression Pattern of Transcripts in *C. elegans* Germline

It is possible to integrate fluorescent reporters into worms to study post-transcriptional regulatory events (Praitis et al., 2001; Frøkjaer-Jensen et al., 2012; 2008). The Seydoux lab used this technology and showed that maternally encoded transcripts expressed in the germline show specific patterns of reporter expression. They also showed that the 3′UTRs of germline expressed transcripts are sufficient to govern their expression patterns in the germline, oocytes and early embryos (Merritt et al., 2008). In this study, expression patterns of fluorescent transgenic reporter strains were analyzed under two different conditions and compared for a group of germline expressed genes. One set of reporters contained gene specific promoters and an unregulated 3′UTR. These reporter strains showed ubiquitous GFP expression in the germline and oocytes for all the genes tested. In contrast, when the reporters bearing a pan-germline promoter and gene specific 3′UTR were analyzed, a patterned reporter expression was observed. Moreover, for all the germline specific genes studied, the pattern of reporter expression mostly matched the pattern of expression of

the endogenous protein. By contrast, sperm-expressed genes showed promoter governed expression pattern in the sperm.

Patterned germline reporter expressions showed that many germline expressed genes are post-transcriptionally regulated through their 3′UTRs. This regulation is conferred through RNA-binding proteins. Consistently, there are a number of RNA-binding proteins that contribute significantly to oogenesis and early embryogenesis by regulating maternal mRNAs in the germline and early embryos. A number of the RNA-binding proteins regulating maternal transcripts and their cognate RNA targets are known, such as GLD-1, FBF-1/2, PUF-5/6/7, POS-1, MEX-3 (Bernstein et al., 2005b; Crittenden et al., 2002; Farley and Ryder, 2012; Lublin and Evans, 2007; Pagano et al., 2009; Ryder et al., 2004; Wright et al., 2010). These proteins have been shown to repress their regulatory targets spatially and temporally in germline development and early embryos, as discussed above. Studies have identified regulatory targets these proteins. However, for most of these and other RNA-binding proteins, direct regulatory targets are not known. It is likely that there are additional regulatory factors, especially in the oocytes since oocytes are rich in maternal mRNAs and proteins that are precisely regulated during oocyte-to-embryo transition to ensure proper embryonic development as well as oocyte development. Very little is known about the network of RNA-binding proteins and their direct targets required for *C. elegans* development.

To begin understanding which RNA-binding proteins regulate which mRNAs during oocyte-to-embryo transition to contribute to the pattern of gene expression, it is necessary to dissect the direct interaction sites between RNA-binding proteins and their cognate targets that are functionally relevant to a pattern of expression observed at the oocyte-to-embryo transition. One such example of a maternal transcript that has a patterned expression at this transition is *glp-1*. *glp-1* mRNA is translationally repressed during throughout oogenesis but it becomes translated in early embryos.

glp-1 Signaling

glp-1 was first identified in a genetic screen for sterility. Loss of function mutants of *glp*-1 have only approximately 10 germ cells in their gonads, as compared to an average of around 230 germ cells in the distal mitotic zone of wild-type adults (Austin and Kimble, 1987; Kimble and Crittenden, 2007). This results in failure of formation of oocytes and sterility in hermaphrodites. Another screen for maternal-effect embryonic lethal genes also identified *glp*-1 (Priess et al., 1987). Embryos produced in the absence of maternal *glp-1* died during embryogenesis due to a failure in producing pharyngeal tissue. A gain-of-function mutation in *glp-1* was subsequently isolated. This dominant mutation resulted in continuous proliferation of the mitotic germ cells leading to a tumorous germline (Berry et al., 1997).

GLP-1 belongs to the Notch family of transmembrane receptors. Notch signaling pathway is highly conserved in a variety of model organisms and was shown to contribute to stem cell maintenance (Andersson et al., 2011; Bray, 2006). This pathway regulates germline stem cell fate typically by a ligand expressing cell communicating with a receptor expressing cell. In *C. elegans*, the ligand that activates *glp-1* signaling is present at the distal tip cell (DTC) **(Figure 1.5)**, which is a somatic gonad cell at the distal end of the germline, and is responsible for maintaining about 60-80 cells as stem cells. This ligand belongs to the Delta/Serragate/LAG-2 family and it is called LAG-2 in *C. elegans* (Byrd and Kimble, 2009; Fox and Schedl, 2015; Henderson et al., 1994). Once LAG-2 is present, it interacts with the extracellular portion of the type I transmembrane domain and leads to the cleavage of the intracellular domain of GLP-1. This domain then translocates to the nucleus where it activates a transcription factor LAG-1, which then activates downstream genes such as *fbf-2* and *lip-1* (Lamont and Kimble, 2007; Lee et al., 2006; Neves et al., 2007; Yochem and Greenwald, 1989). GLP-1 begins to be expressed at the two-cell stage and is localized to the anterior blastomere(s) (AB) of the two- and four-cell embryos. Its ligand in the embryos is APX-1 (Evans et al., 1994; Mango et al., 1994; Mello et al., 1994). This ligand is present in the single posterior blastomere, P2, at the two- and four-cell stages. Therefore, only the anterior blastomere touching the P2 blastomere shows an activation of transcription factors that specify hypodermal cell fates (Neves et al., 2007). This communication between the posterior and anterior

controls specification of the anterior blastomeres. Through these inductive signaling events, GLP-1 participates in correct cell fate specification patterns in the embryos (Priess, 2005).

As described, GLP-1 protein has a spatially restricted expression pattern. It is present at the distal end of the germline and the anterior cells of the four-cell embryo where it regulates the switch from mitosis to meiosis at the distal end and specifies endodermal cell fates in the anterior cells of embryos (Austin and Kimble, 1987; Crittenden et al., 1994; Evans et al., 1994). However, the mRNA encoding GLP-1 is present throughout the germline and in all stages of the embryos and, thus, post-transcriptionally regulated. In 1994, Evans and colleagues showed that the 3′UTR of the *glp-1* mRNA is sufficient to drive its restricted pattern of expression (Evans et al., 1994). Distinct regions of the UTR that are sufficient for translational repression across different regions of the germline, oocytes and embryos have also been mapped. Initial studies identified a 61-nucleotide region of the UTR termed the spatial control region (SCR) as necessary to confer the endogenous GLP-1 pattern of expression to a reporter (Evans et al., 1994). Follow up experiments identified a 34-nucleotide sub-region containing both repression and de-repression elements is sufficient to generate the *glp-1* translation pattern (Marin and Evans, 2003). One of these regions, called *Glp-1* Repression Element (GRE), contributes to repression in the distal end of the germline but not in oocytes (Evans et al., 1994; Marin and Evans, 2003). On the other hand, another distinct 129-nucleotide element at the 3´- end

of the UTR is required for repression in oocytes (Evans et al., 1994). The GRE element is also required for repression of *glp-1* in the posterior blastomeres of embryos.

Subsequent studies identified RBP binding sites within this region that are sufficient for repression. For example, the RNA-binding proteins, POS-1 and GLD-1 directly repress *glp-1* 3′UTR through *cis*–acting sequence elements containing overlapping binding sites across a conserved region of the UTR (Farley and Ryder, 2012). Other RBP's discussed earlier contribute to repression of *glp-1* at distinct developmental locations of the germline and embryos. GLD-1 represses *glp-1* in the syncytial region of the germline as well (Marin and Evans, 2003). PUF-5/6/7 are required to repress *glp-1* around the re-cellularization region of the oocytes (Lublin and Evans, 2007). MEX-3, GLD-1 and POS-1 are involved in repressing *glp-1* in the embryos (Farley and Ryder, 2012; Marin and Evans, 2003; Ogura et al., 2003; Pagano et al., 2009) **(Figure 1.5).** Since *glp-1* 3′UTR has a sequence element that confers translational repression in developing oocytes and one-cell embryos, it is likely that there are a different group of proteins participating in the GLP-1 expression pattern during oocyte-to-embryo transition.

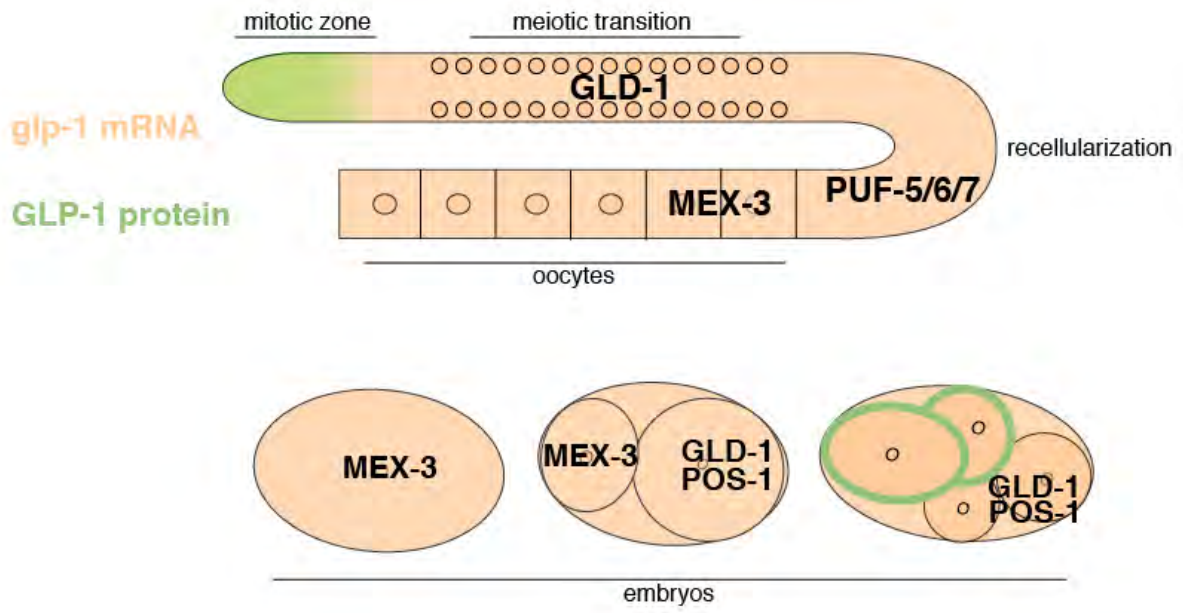


Figure 1. 5 Repression of glp-1 in the germline and embryos

Schematic representation of the *C. elegans* germline and early embryos is shown with different regions annotated.
glp-1 mRNA is present throughout the germline and in all cells of the embryos (shown in tan). The protein has a restricted pattern of expression (shown in green). GLP-1 is present at the distal end of the germline and in the anterior cells of the four-cell embryo. At other regions, RNA-binding proteins participate in the repression of *glp-1*. These RNA-binding proteins are labelled in the schematic at their corresponding regions of activity.

Two redundant proteins that are abundantly present in developing oocytes, OMA-1 and OMA-2, are candidate regulatory proteins to ensure successful oocyte maturation by regulating specific maternal transcripts, such as *glp-1* mRNA, present in the oocytes (Detwiler et al., 2001).

OMA-1 and OMA-2 as Post-Transcriptional Regulators in Oocyte-to-Embryo Transition

oma-1 was identified in a genetic screen for embryonic lethal phenotypes. A gain of function mutation, *zu405*, was identified and mapped to a region that did not show any obvious phenotype by RNAi (Lin, 2003). However, when this region was knockeddown simultaneously with a highly related homologue of this sequence, sterility was observed in the worms treated with double knockdown. The sterility phenotype was shown to be due to defects in oocyte maturation. Accordingly, these genes resulting in the observed phenotype were named *oma-1* and *oma-2* (standing for oocyte maturation defective). Subsequently, null alleles of *oma*-1 and *oma*-2 were found in a two-step dominant suppressor screen for the *zu405* embryonic lethality (Detwiler et al., 2001; Lin, 2003).

oma-1 and *oma-2* are redundantly required for oocyte maturation. Worms homozygous for *oma-1* and *oma-2* null alleles are sterile (Detwiler et al., 2001). They produce both sperm and oocytes but no embryos; they have an empty uterus. The gonad arm fills with a higher number of oocytes as compared to wild-type worms. In addition, the oocytes of these worms are larger than wild-type oocytes (Detwiler et al., 2001). However, worms homozygous for either *oma-1* null or *oma-2* null show no phenotypic effect. Worms homozygous for reduction of function alleles of *oma-1* and *oma-2* also do not show a fully penetrant oocyte

maturation defect and do produce embryos. However, these embryos have cleavage defects and fail to hatch (Detwiler et al., 2001).

OMA-1 and OMA-2 are closely related to each other. They share 64% amino acid sequence identity and they are both translated in the oocytes. OMA-1 and OMA-2 are expressed in proximal oocytes and their abundance increases in growing oocytes reaching a maximum level in maturing oocytes and rapidly decreasing following the first mitotic division of the one-cell embryo (Detwiler et al., 2001) **(Figure 1.6)**.

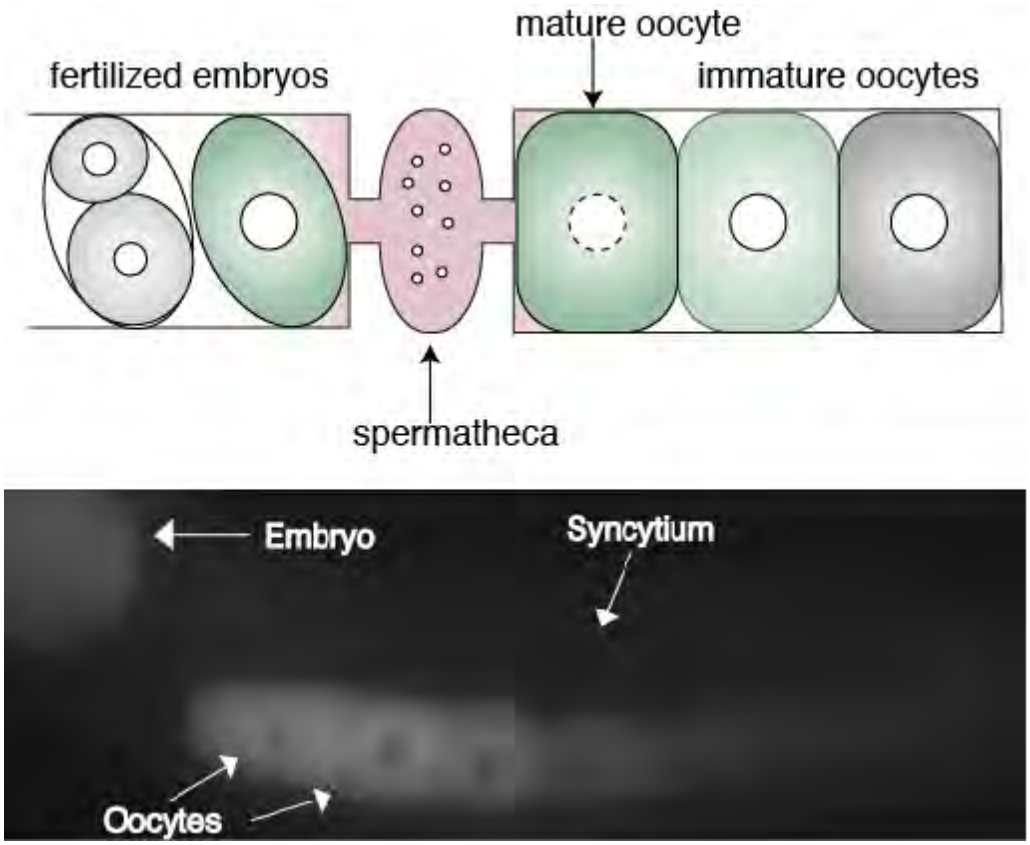


Figure 1. 6 Expression pattern of OMA-1

The top panel shows a schematic of *C. elegans* oocyte maturation where [OMA-1] is denoted in green. OMA-1 is present in developing oocytes. Its concentration is highest in the mature oocyte and in one-cell embryo.
Bottom panel is an image of the gonad of a transgenic strain expressing OMA-1::GFP. The described pattern of OMA-1 expression is observed in this strain too.

This degradation occurs in a timely manner to prevent embryonic lethality (Lin, 2003; Nishi and Lin, 2005). MBK-2, which is a serine/threonine kinase that marks maternal proteins for degradation during embryogenesis, primes degradation of OMA-1 in one-cell embryos. MBK-2 phosphorylates a threonine residue at amino acid position 239 (T239) but this phosphorylation does not directly initiate degradation of OMA-1. Another serine/threonine kinase, GSK-3 phosphorylates OMA-1 at T339 before degradation begins (Nishi and Lin, 2005; Shirayama et al., 2006). It is thought that MBK-2 serves as a priming phosphorylation for GSK-3.

Gain-of-function mutant of *oma*-1 prevents its timely degradation by interfering with phosphorylation of OMA-1 by a kinase MBK-2. This mutation has a leucine instead of a proline at amino acid 240, which is one amino acid downstream of the MBK-2 phosphorylation site (Lin, 2003). It was shown that this mutation impairs MBK-2 phosphorylation. Consequently, OMA-1 is not degraded through a proteasome pathway leading to its persistence in embryos (Nishi and Lin, 2005).

Phosphorylation of OMA-1 at T239 not only primes OMA-1 for degradation, but also is suggested to give OMA-1 a different role in one-cell embryos. Phosphorylated OMA-1 can interact with a TATA-binding protein associated factor, TAF-4, which is an essential component of RNA polymerase II transcription machinery. Therefore, OMA-1 can act as a transcriptional repressor

in one-cell embryos by sequestering this factor in the cytoplasm and changing its cellular localization (Guven-Ozkan et al., 2008).

OMA-1 and OMA-2 have two $CX_8CX_5CX_3H$ type tandem zinc finger (TZF) domains found in the mammalian homolog tristetraprolin (TTP) (Blackshear, 2002; Lai et al., 1999). *C. elegans* has a number of TZF proteins, as described earlier, including MEX-5 and POS-1 that have been shown to bind mRNAs in a sequence-specific manner (Farley et al., 2008; Pagano et al., 2007). By analogy, OMA-1 and OMA-2 may function during oocyte maturation by regulating specific target maternal mRNAs at the oocyte-to-embryo transition since a common theme observed in regulation of the transition in different species is post-transcriptional regulation of maternal mRNAs. Consistent with this hypothesis, a number of mRNA's that are repressed through OMA-1/2 mediated mechanisms have been identified. These include *nos-2*, a *C. elegans* Nanos homolog, and *mei-1*, a katanin subunit, and *zif-1*, a subunit of E3 ubiquitin ligase (Guven-Ozkan et al., 2010; Li et al., 2009; Subramaniam and Seydoux, 1999). Whether the identified targets of OMA-1/2 are directly or indirectly regulated by these factors is not known yet. The pleiotropic phenotype of OMA-1/2 knockdown suggests that these proteins have multiple targets in the oocytes. Additionally, the absence of any delay between oocyte maturation and fertilization in *C. elegans* makes the oocyte-to-embryo transition very fast (~20 minutes). This suggests that tight regulation of multiple maternal mRNAs governs this dramatic transition in early development.

Scope of This Thesis

The overall goal of the research presented in this thesis was to understand how OMA-1/2 contributes to regulation of oocyte-to-embryo transition through post-transcriptional regulation of maternal mRNAs. The first step I took to characterize OMA-1/2 mediated regulation of various mRNA targets was to determine the targets that are directly bound and functionally regulated by OMA-1/2. In Chapter II, I present my work to define the RNA-binding sequence specificity of OMA-1/2 and my identification of a novel regulatory target of OMA-1 activity: *glp*-1. In Chapter III, I present our approach to improve the rate of making transgenic worm strains. I used this approach to generate and characterize several new 3´UTR reporter lines, revealing specific patterns of reporter expressions in the germline. I used the new strains to perform a targeted RNAi screen that allowed me to identify additional mRNA targets of OMA-1/2 which will ultimately help us gain insight into regulation of a complex developmental event, oogenesis.

CHAPTER II: RNA RECOGNITION BY THE *C. ELEGANS* OOCYTE MATURATION DETERMINANT OMA-1

Summary

Background: OMA-1 is required for oocyte maturation in *C. elegans* and may function by regulating maternal mRNAs.

Results: SELEX and biochemical studies reveal that OMA-1 binds with high affinity UA(A/U) motifs. Reporter experiments demonstrate that OMA-1 regulates *glp-1* via its 3′ UTR in live worms. The *glp-1* mRNA encodes the *C. elegans* homolog of Notch, required for early cell fate specification events.

Conclusion: OMA-1 is a sequence specific RNA-binding protein that represses important maternally supplied mRNAs during oocyte maturation via direct binding to the 3′ UTR.

Significance: Identification of key OMA-1 regulatory targets will help reveal its important contribution to establishment of the maternal load of mRNAs during oogenesis.

Abstract

Maternally supplied mRNAs encode proteins that pattern early embryos in many species. In the nematode *Caenorhabditis elegans*, a suite of RNA-binding proteins regulates expression of maternal mRNAs during oogenesis, the oocyte-to-embryo transition, and early embryogenesis. To understand how these RNA-binding proteins contribute to development, it is necessary to determine how they select specific mRNA targets for regulation. OMA-1 and OMA-2 are redundant proteins required for oocyte maturation—an essential part of meiosis that prepares oocytes for fertilization. Both proteins have CCCH-type tandem zinc finger (TZF) RNA-binding domains. Here, we define the RNA-binding specificity of OMA-1, and demonstrate that OMA-1/2 are required to repress the expression of a *glp-1* 3′UTR reporter in developing oocytes. OMA-1 binds with high affinity to a conserved region of the *glp-1* 3′UTR previously shown to interact with POS-1 and GLD-1, RNA-binding proteins required for *glp-1* reporter repression in the posterior of fertilized embryos. Our results reveal that OMA-1 is a sequence specific RNA-binding protein required to repress expression of maternal transcripts during oogenesis, and suggest that interplay between OMA-1 and other factors for overlapping binding sites helps to coordinate the transition from oocyte to embryo.

Background and Significance

Post-transcriptional regulation of maternal mRNAs governs gene regulation during oogenesis and early embryogenesis in metazoans (Farley and Ryder, 2008; Moore, 2005; Schier, 2007). Genetic studies have identified several RNA-binding proteins required for regulation of maternally supplied mRNAs during oogenesis, the oocyte to embryo transition, and early embryogenesis (Colegrove-Otero et al., 2005; Lee and Schedl, 2006). RNA-binding proteins are important during oocyte development as oocytes of metazoans are loaded with translationally repressed maternal RNAs (de Moor et al., 2005; Spirin, 1966; Standart, 1992). During the oocyte to embryo transition, RNA-binding proteins regulate their cognate RNA targets to coordinate events such as axis formation and cell fate specification (de Moor et al., 2005).

Oocyte maturation is the complex process that prepares oocytes for fertilization (Masui, 2001; Masui and Clarke, 1979; McCarter et al., 1999). Metazoan sexual reproduction requires meiosis to produce fertile oocytes. Meiotic divisions in the oocytes must be completed before zygote formation. Therefore, precise regulation of meiosis during oocyte development is necessary to couple meiotic events to fertilization events. An evolutionarily conserved feature of oocyte development is meiotic arrest, which prepares the oocyte for fertilization (Greenstein, 2005). During oocyte maturation, meiotic arrest is released, (McCarter et al., 1999; Yamamoto et al., 2006) the nuclear envelope

breaks down (McCarter et al., 1999), and the cortical cytoskeleton rearranges morphologically (McCarter et al., 1999). *Caenorhabditis elegans* provides a powerful system to study oocyte maturation due to its transparent body, established cellular lineage and easy genetic manipulation (Brenner, 1974). The oocyte proximal to the spermatheca receives a maturation signal from the sperm prior to ovulation and subsequent fertilization (Miller et al., 2001). This cycle is repeated approximately every 23 minutes (Kimble and Crittenden, 2007; McCarter et al., 1999). Although the hallmark events of oocyte maturation are well understood morphologically, the molecular mechanisms governing these events are poorly understood.

During oogenesis, oocytes are loaded with translationally repressed RNAs. Expression of these RNAs must be coordinated in time and space to ensure correct patterning of the embryo. Genetic studies have identified several RNA-binding proteins required for regulation of maternally supplied mRNAs during oogenesis, the oocyte to embryo transition, and early embryogenesis (Colegrove-Otero et al., 2005; Lee and Schedl, 2006). In order to regulate expression of their cognate mRNA targets, these RBPs must be capable of selecting their targets from a complex pool of mRNA sequences.

The putative RNA binding proteins OMA-1 and OMA-2 are redundantly required for oocyte maturation (Detwiler et al., 2001; Shimada et al., 2002). They are expressed in maturing oocytes with the highest level present in the oocyte

most proximal to the spermatheca. Their expression decreases rapidly following the first mitotic division of the one-cell embryo (Detwiler et al., 2001) **(Figure 2.1)**.

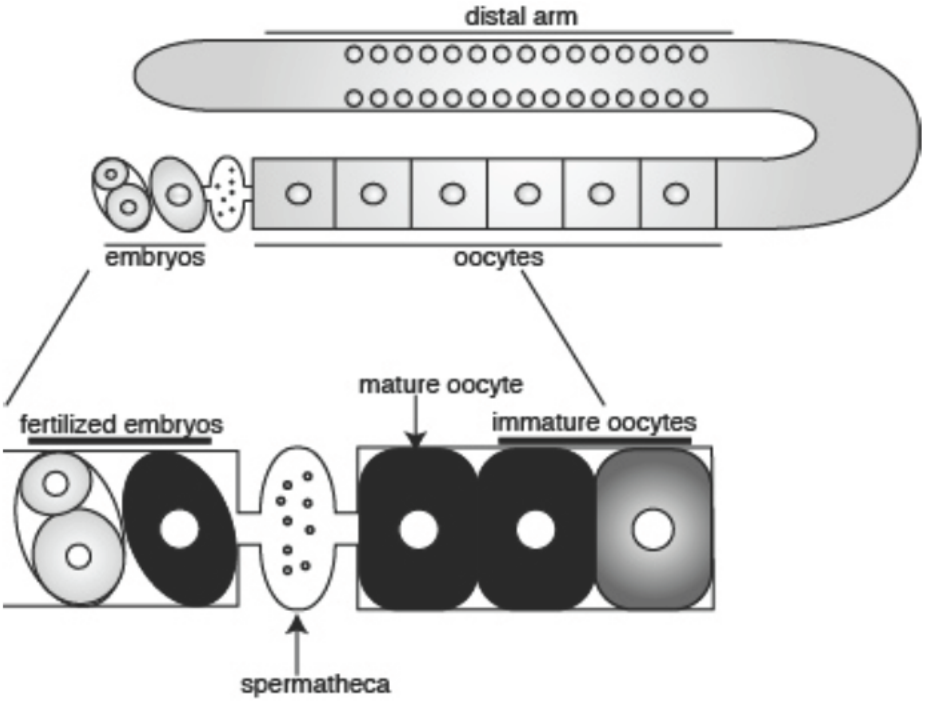


Figure 2.1 Schematic of the *C. elegans* germline

Top: Germline of *C. elegans*.
The syncytial region of nuclei in the distal arm of the gonad, the oocytes, the embryos are shown.
Bottom: Oocyte maturation. The oocyte most proximal to the spermatheca matures first. Nuclear envelope breakdown is a hallmark event. The oocyte completes maturation, is ovulated and fertilized by sperm in the spermatheca. Embryos are then deposited in the uterus. Dark gray color in the oocytes denotes the abundance of OMA 1 and OMA 2.

Rapid turnover of OMA-1 and OMA-2 is required to prevent embryonic lethality (R. Lin, 2003; Nishi and R. Lin, 2005). Worms homozygous for *oma-1* and *oma-2* null alleles are sterile. They produce both sperm and oocytes but no

embryos. The gonad arm fills with a higher number of oocytes as compared to wild-type worms. In addition, the oocytes of these worms are larger than wild-type oocytes (Detwiler et al., 2001).

OMA-1 and OMA-2 have two CCCH type tandem zinc finger (TZF) domains typified by the mammalian homolog tristetraprolin (TTP). TTP has two $CX_8CX_5CX_3H$ motifs that bind to AU-rich elements (AREs) of the mRNA encoding the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) (Blackshear, 2002). Each finger binds one UAUU motif, and the binding event promotes the turnover of the mRNA and leads to regulation of the immune response (Lai et al., 1999). *C. elegans* expresses a number of TZF proteins that regulate oogenesis (OMA-1, OMA-2, MOE-3) (Detwiler et al., 2001; Shimada et al., 2002) or embryogenesis (MEX-5/6, POS-1, MEX-1 and PIE-1) (Farley and Ryder, 2008; Mello et al., 1992; Moore, 2005; Schier, 2007; Schubert et al., 2000; Tabara et al., 1999). Of these, MEX-5 and POS-1 have been shown to bind to RNA with high affinity (Colegrove-Otero et al., 2005; Farley et al., 2008; Lee and Schedl, 2006; Pagano et al., 2007). In contrast, MEX-1 and PIE-1 are proposed to function as transcription factors that bind to DNA (de Moor et al., 2005; Guedes and Priess, 1997; Seydoux et al., 1996; Spirin, 1966; Standart, 1992; Tenenhaus et al., 2001).

OMA-1 and OMA-2 are proposed to function during oocyte maturation by regulating specific target maternal mRNAs at the oocyte to embryo transition. Consistent with this hypothesis, OMA-1 and OMA-2 are required to repress *mei-*

1, *zif-1* and *nos-2* translation. The *mei-1* gene encodes a katanin (a heterodimeric microtubule severing protein) subunit. Genetic studies showed *mei-1* is necessary for meiotic spindle formation; in the absence of *mei-1* function meiosis fails (Clark-Maguire and Mains, 1994a; 1994b; de Moor et al., 2005). The *zif-1* gene, on the other hand, encodes a subunit of the E3 ubiquitin ligase complex. ZIF-1 is required in embryos for proper asymmetric segregation of cell fate regulators through *zif-1* dependent proteolysis (DeRenzo et al., 2003; Guven-Ozkan et al., 2010; Masui, 2001; Masui and Clarke, 1979; McCarter et al., 1999). *nos-2* is a Nanos homolog and is required for primordial germ cell development (Subramaniam and Seydoux, 1999). OMA-2 was shown to repress *nos-2* translation by interacting with its 3′UTR through a UGCUAAUAAU sequence element (Jadhav et al., 2008). How OMA-1/2 represses *mei-1*, *zif-1* and *nos-2* mRNA translation, or whether OMA-1 regulates additional maternal transcripts, is not known. We set out to define the RNA-recognition properties of OMA-1/2 in order to gain insight as to how mRNA targets are selected for regulation.

Experimental Procedures

OMA-1 expression and purification

The sequence encoding amino acids 1-182 of OMA-1 was cloned into pMal-ac (New England Biolabs). This construct was transformed into BL21(DE3) cells. The protein was then expressed after inducing the cells with 1 mM

isopropyl 1-thio-β-D-galactopyranoside (IPTG) and 100 µM zinc acetate ($Zn(OAc)_2$) for 3 hours, at 37°C. The protein was expressed with an N-terminal maltose-binding protein (MBP) tag. The cells were then lysed in 200 mM NaCl, 50 mM Tris pH 8.8, 2 mM dithiothreitol (DTT), 100 µM $Zn(OAc)_2$ and EDTA-free protease inhibitor tablet. OMA-1 was then purified using an amylose (New England Biolabs) affinity column. Protein fractions were eluted in lysis buffer supplemented with 10 mM maltose, Fractions containing OMA-1 fusion were dialyzed into Q-column buffer (20 mM NaCl, 50 mM Tris pH 8.8, 2 mM (DTT), 100 µM $Zn(OAc)_2$). After dialysis, purification was followed by HiTrap Q at 4°C. Elution of the protein fractions was achieved by a salt gradient ranging from a low salt buffer (20 mM NaCl, 50 mM Tris pH 8.8, 2 mM dithiothreitol (DTT), 100 µM $Zn(OAc)_2$) to a high salt buffer (1 M NaCl, 50 mM Tris pH 8.8, 2 mM dithiothreitol (DTT), 100 µM $Zn(OAc)_2$). Final purification was done using a source 15Q (GE Healthcare) ion exchange column at 4°C. Elution was achieved through the same salt gradient as in the HiTrap Q column purification. Pure fractions were determined by Coomassie-stained SDS-PAGE and purified OMA-1 was dialyzed into storage buffer (25 mM Tris, pH 8.0, 25 mM NaCl, 2 mM DTT, 100µM $Zn(OAc)_2$) and stored at 4°C.

In Vitro RNA Selection

RNA library design and *in vitro* selection protocols were adapted from a protocol described previously (Pagano et al., 2009). The initial double stranded DNA library was amplified from the template 5'- GGGAAGATCTCGACCAGAAG-

(N30)-TATGTGCGTCTACATGGATCCTCA with a forward (5' – CGGAATTCTAATACGACTCACTATAGGGAAGATCTCGACCAGAAG - 3') and reverse (5' – TGAGGATCCATGTAGACGCACATA - 3') primer pair using three cycles of PCR. Binding reactions of the RNA pools to OMA-1 were performed in 200 µL of selection buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 0.01 mg/ml tRNA, 0.01% Igepal CA-630, 2mM DTT, 100 uM $Zn(OAc)_2$. Between 5-800 nM of purified MBP-OMA-1(1-182) was equilibrated with the pool of RNA sequences in selection buffer for 1 hour. Then OMA-1 was immobilized on amylose resin (New England Biolabs). At each round of selection, lowering the protein concentration from 800nM, to 200 nM, 20 nM, and 5 nM successively increased the stringency. OMA-1 bound to RNA was eluted from amylose resin with 10 mM maltose in selection buffer, at room temperature. Selected RNA was phenol/chloroform extracted, ethanol precipitated and resuspended in 10 µL of TE buffer. RNA was then reverse transcribed and amplified with 15 rounds of PCR using the SuperScript III One-Step RT-PCR kit with Platinum Taq (Invitrogen). The new DNA pool was then *in vitro* transcribed to generate the next RNA pool that will enter the following round of selections. We performed 4 rounds of selection. The DNA was cloned using StrataClone PCR cloning kit (Stratagene).

Preparation of Fluorescently Labeled RNA

Synthesized oligonucleotides (Integrated DNA Technologies) were 3'-end labeled with fluorescein 5-thiosemicarbazide (Invitrogen) as previously described (Pagano et al., 2011).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift experiments and data analysis were carried out as previously described with a few modifications (Farley et al., 2008; Pagano et al., 2011; 2007). Varying concentrations of purified OMA-1 were equilibrated with 3 nM of labeled RNA in equilibration buffer (0.01% IGEPAL, 0.01 mg/ml tRNA, 50 mM Tris, pH 8.0, 100 mM NaCl, 2mM DTT, 100uM $Zn(OAc)_2$) for 3 hours. Samples were loaded on a 5% native, slab polyacrylamide gel in 1X TB buffer (89 mM Tris and 89 mM Boric acid, pH 8.3). The gels were run in 1 X TB buffer for 120 minutes at 120 volts and at 4°C. The gels were then scanned using a fluor-imager (Fujifilm FLA-5000) with a blue laser at 473 nm.

oma-1;oma-2 RNAi knockdown

We knockdown *oma-1* and *oma-2* using the RNAi feeding method (Kamath et al., 2003). We cloned the oma-1 and oma-2 open reading frames (ORFs) into the RNAi feeding vector construct L4440 by TA cloning, as previously described (Kamath et al., 2003). These clones were then transformed into HT115(DE3) cells. Once these cells were grown to OD_{600} = 0.4, the cells were induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) at a final concentration of 0.4 mM for 4 hours. The cultures with the *oma-1* RNAi feeding construct and *oma-2* RNAi feeding construct were concentrated 10- fold and mixed at equal proportions. The mixed culture was the seeded onto NGM plates that contain 1mM IPTG and 100 µg/ml Ampicillin. Worms were then bleached onto these plates and maintained at 25°C for 2 days before imaging. As a control

food, we used HT115 strain bacteria transformed with the empty RNAi feeding construct vector, L4440.

Imaging of worm strains

Worms at the appropriate age were picked on to 2% agarose pads in 0.4 mM levamisole. DIC and GFP fluorescence images were taken using an oil-immersion 40X objective on Zeiss Axioscope 2 plus microscope (Carl Zeiss, Jena, Germany). Confocal images were also taken using an oil-immersion 40X objective on Leica DMIRE2 microscope (Leica, Wetzlar, Germany).

Quantifications of the GFP pixel intensities were performed as described previously (Farley and Ryder, 2012; Wright et al., 2010) with minor changes. We used a 20 pixel-wide segmented line that passes through the nuclei of the oocytes to determine the average pixel intensity across this line.

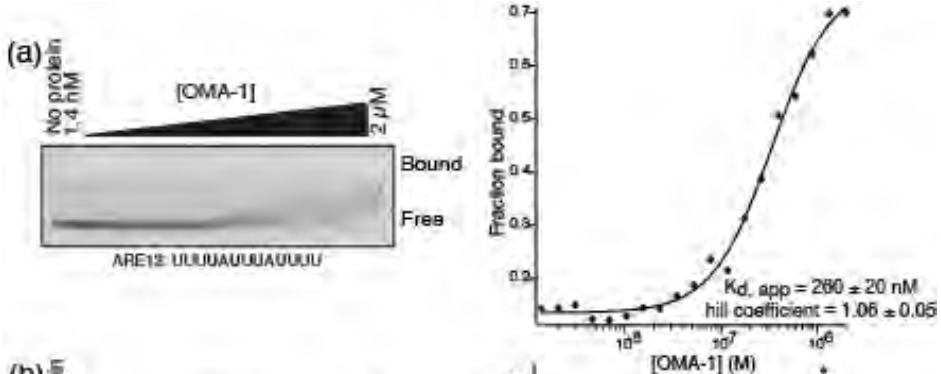
Results

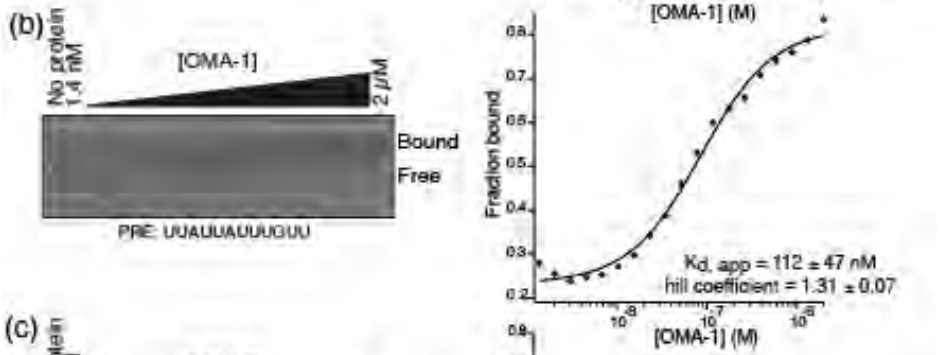
OMA-1 binds with limited affinity to RNA sequences recognized by POS-1, MEX-5, and TTP

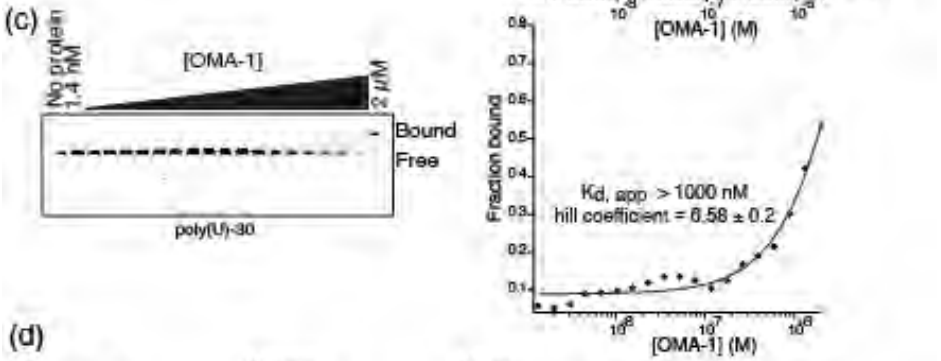
To assess OMA-1 RNA-binding specificity, we first determined the ability of purified recombinant OMA-1 to bind sequences recognized by its mammalian homolog TTP, and by two nematode family members MEX-5 and POS-1. We performed quantitative fluorescent electrophoretic mobility shift assays (F-EMSA) to measure the apparent binding affinity of purified recombinant OMA-1 to the fragment of an AU rich element (ARE13) from the 3′UTR of TNF-α mRNA (recognized by TTP) (Blackshear et al., 2005; Lai et al., 1999); the POS-1 recognition element (PRE) from the 3′UTR of *mex-3* mRNA (recognized by POS-1) (Farley et al., 2008), and polyuridine-30 RNA (recognized by MEX-5) (Pagano et al., 2007). OMA-1 binds with moderate affinity to the TNF-α ARE13 **(Figure 2.2a)** and the PRE **(Figure 2.2b)**, and it binds weakly to polyuridine-30 RNA **(Figure 2.2c)**. However, the affinity of OMA-1 for all three sequences is weaker than the affinity of each sequence for its cognate RNA-binding protein **(Figure 2.2d)**. OMA-1 binds 90-fold more weakly to TNF-α ARE13 RNA compared to TTP, about 3-fold weaker to PRE compared to POS-1, and more than 30-fold weaker to polyuridine-30 compared to MEX-5 **(Figure 2.2d)**. We conclude that while OMA-1 is capable of binding to RNA sequences with variable affinity, its

specificity is not likely to be the same as previously investigated members of the TZF RNA-binding protein family.

To determine whether OMA-1 binds RNA with identical specificity as POS-1, but with lower affinity, we measured OMA-1 binding to three PRE mutants (A3C, U4C, and G10C) that reduce POS-1 binding by >1 kcal/mol (Farley et al., 2008). OMA-1 binding is not affected by the A3C and G10C mutations. By contrast, the U4C mutant binds OMA-1 with reduced affinity ($\Delta\Delta G° = 1.2$ kcal/mol) **(Figure 2.2e).** We conclude that while OMA-1 is capable of binding to RNA sequences with variable affinity, its specificity is not the same as previously investigated members of the TZF RNA-binding protein family.







(d)

Sequence	Kd,app OMA-1 (nM)	Kd,app cognate TZF (nM)	Krel	Reference
ARE	260 ± 20	2.8 ± 0.5	93	Brewer et al., 2004
PRE	112 ± 47	39 ± 6	2.8	Farley et al., 2007
poly(U)-30	>1000	29 ± 6	>33	Pagano et al., 2007

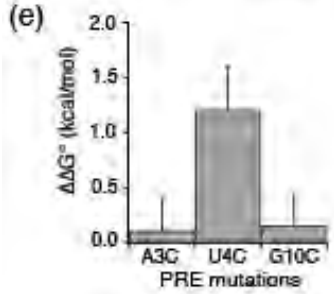


Figure 2.2 OMA-1 is a sequence-specific RNA-binding protein

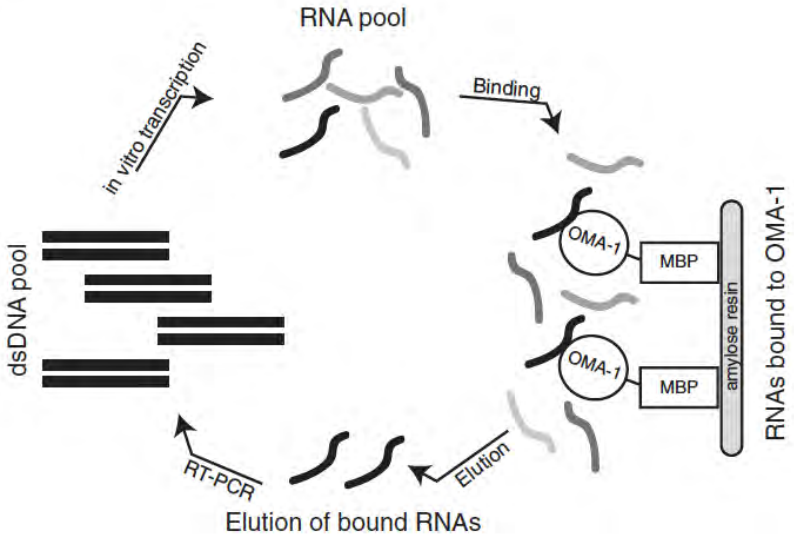
(a) Fluorescent electrophoretic mobility gel shift assay (F EMSA) with the AU rich element of TNF a mRNA (ARE13) and OMA 1. The gel is shown with the bound and free RNA species labeled. Data is fit to the Hill equation. Values reported are the average and standard deviation of three independent experiments. (b) Fluorescent electrophoretic mobility gel shift assay with the POS 1 binding sequence (PRE) and OMA 1. OMA 1 shows weak binding to this sequence. (c) Fluorescent electrophoretic mobility gel shift assay is done with the poly(U) 30, which binds MEX 5, and OMA 1, as described in (a). (d) Table comparing the relative binding affinities of OMA 1 to the RNA sequences recognized by TTP, MEX 5 and POS 1 with respect to their cognate proteins. (e) OMA 1 binds to variants of POS 1 Recognition Element (PRE) differently than POS 1. Each bar shows the change in standard free energy change ($\Delta\Delta G°$) caused by the mutation shown on the *x* axis. The binding affinity of OMA 1 to these variants was measured by fluorescent electrophoretic mobility gel shift assay (F EMSA). This binding affinity is then compared to the binding affinity of OMA 1 to the PRE to calculate the $\Delta\Delta G°$.

OMA-1 SELEX

We hypothesized that OMA-1 binds to RNA with specificity that is different from MEX-5, POS-1, and TTP. To identify sequences that bind OMA-1 with high affinity, we performed an in vitro selection (systematic evolution of ligands by exponential enrichment: SELEX) (Tuerk and Gold, 1990) using synthesized RNA sequences that contain 30 randomized bases, as described previously (Pagano et al., 2009). In the first round of selection, we equilibrated the starting pool with a fragment of OMA-1 that includes the RNA-binding domain (amino acids 1-182) fused to an N-terminal maltose binding protein (MBP) tag. This fusion protein was immobilized on an amylose resin and unbound RNA sequences were washed away. The bound RNA sequences were eluted and amplified to generate a new library of RNA sequences for the next round of selection **(Figure 2.3a)**. F-EMSA was used to monitor the progress of selection. Our results reveal that RNA produced after the fourth round of selection is enriched for sequences that bind OMA-1 compared to the starting pool (data not shown). To identify the sequences within pool 4, we cloned cDNA generated from RNA sequences enriched in this pool and sequenced 69 clones. Of these, 48 contain extended repeats of motif UAA. Two additional sequences were recovered with seven and five copies, respectively. These sequences also contain UAA elements. All but one of the remaining individual sequences also contained UAA elements. Eight of these sequences contain UAU motifs as well, which comprise a portion of the

TTP and POS-1 recognition motifs **(Figure 2.3b)** (Brewer et al., 2004; Farley et al., 2008).

(a)



(b)

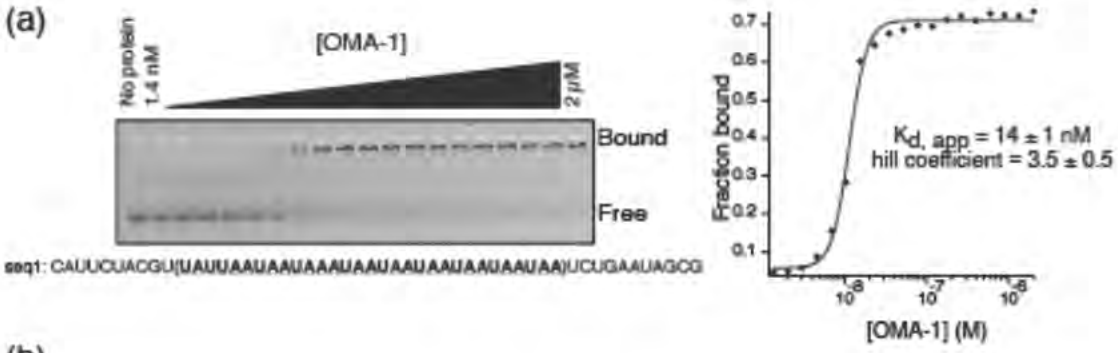
Sequence ID	Sequence	Copy number
seq1	CAUUCUACGU(**UAUUAAUAAUAAAUAAUAAUAAUAAUAAUAA**)UCUGAAUAGCG	48
seq2	GCGCAGAGGGUAGGGGAGU**UAA**GGGAGAAU	7
seq3	CGCGUGACGCUCGCUGAAA**UAAUAA**UGGCC	5
seq4	**UAA**UGA**UAA**GCGGCCAUGGCCAAGAUGCAU	1
seq5	UGUUGGUGCACAUACCAGUGCAGCGUGCAC	1
seq6	UAGU**UAAUAA**GCGUGCCGCAUGAUGUUACC	1
seq7	UAGGGUGUCAAG**UAU**GUAGGA**UAAUAA**UGA	1
seq8	UUUGU**UAAUAAUAA**GAGGCGCCACCACGUAAGG	1
seq9	UCU**UAU**AGAA**UAU**UCGGGGCUCCAGUGGUU	1
seq10	UCGGUCG**UAU**GUUGAA**UAAUAA**UUGCCCAC	1
seq11	UAUGUUGAA**UAAUAA**ACGAUGAGUUACGAC	1
seq12	AUGGUAGGAUCAGCUGUGUUCG**UAU**CCAUC	1
seq13	**UAUAUUAA**UAGAGGCC**UAUAAUAA**GUGACC	1
seq14	AAAUG**UAU**GUAGGU**UAAUAA**UAGAAGCCCAG	1
seq15	G**UAUAU**GUAGAA**UAAUAA**GACUCACUUCGC	1
seq16	AG**UAU**GUUGAA**UAAUAA**GGAUCUGACGACUC	1
seq17	CAAUU**UAU**GUAGAA**UAAUAA**GGCCUUGGCG	1
seq18	U**UAU**GUAGGU**UAAUAA**GAUGUUGGAACACG	1
seq19	UGACCUAGCUUG**UAA**AC**UAU**UGGCGCGCUA	1
seq20	U**UAAUAA**GCGAGCCGCAGUCUGUGGCGUGC	1
seq21	AUCUCGACCAGAAGGUAGUUAGUUAGU	1

Figure 2.3 In vitro selection to identify high affinity binding sequences of OMA-1

(a) Schematic of SELEX (b) List of 69 DNA sequences that were recovered from the selection. The sequences and their respective copy number are also shown. These sequences are enriched in UAA and UAU repeats which are shown in bold and underlined.

To determine whether OMA-1 binds to the recovered aptamer sequences, we performed quantitative F-EMSA binding assays with RNA sequences that were recovered most frequently **(Figure 2.4a)**. Our results showed that OMA-1 binds with highest affinity to the aptamer sequence with the highest number of UAA elements **(Figure 2.4b)**. Binding of OMA-1 to the RNA sequence with repeated motifs results in a Hill coefficient (nH) of 3.5 ± 0.5. This suggests that this interaction between OMA-1 and UAA rich RNA sequences is cooperative. To test whether the UAA elements in these sequences are responsible for OMA-1 binding, we mutated the UAA motifs to CCC and tested the effect of this mutation on binding affinity. We chose seq10 as a representative sequence. Replacing the tandem UAA sequences to CCC in aptamer mseq10 lead to a significant decrease in the binding affinity of OMA-1 (8-fold decrease). We also tested binding of OMA-1 to another variant of seq10 where a UAA element is retained in the center **(Figure 2.4b)**. Together, the data show that OMA-1 binds with high affinity to UAA-rich RNA.

(a)



(b)

Sequence Id	Sequence	$K_{d,app}$ (nM)	Hill coefficient	Number of UAA	UAU
seq1	CAUUCUACGU(UAUUAAUAAUAAAUAAUAAUAAUAAUAAUAA)UCUGAAUAGCG	14 ± 1	3.5 ± 0.5	9	1
seq2	GCGCAGAGGGUAGGGGAGUUAAGGGAGAAU	800 ± 40	1.0 ± 0.2	1	0
seq10	UCGGUCGUAUGUUGAAUAAUAAUUGCCCAC	77 ± 4	1.3 ± 0.3	2	1
mseq10	UCGGUCGUAUGUUGAACCCCCCUUGCCCAC	597 ± 13	1.0 ± 0.1	0	1
m'seq10	UCGGUCGUAUGUUGAAUAACCCUUGCCCAC	486 ± 43	1.5 ± 0.2	1	1

Figure 2.4 UA(A/U) motifs are responsible for the binding of OMA-1 to the aptamer sequences.

Electrophoretic mobility gel shift assay (EMSA) of OMA 1 with the highest ranking RNA sequence with respect to the copy number. This assay is analyzed as described in Figure 1. The assay showed that seq1 binds with a high affinity to OMA 1, as shown by the plot on the right. (b) A table of sequences that includes additional binding assay results with two more sequences recovered from the selection. seq2 is a sequence that has one UAA element. seq10 is a sequence that has 3 UA(A/U) elements. mseq10 is the sequence seq10 in which the UAA elements are replaced by CCC. m'seq10 binds with a similar affinity to mseq10.

OMA-1 binds to multiple fragments of the *glp-1* 3′ UTR

GLP-1 is the *C. elegans* homolog of Notch. It is required for anterior cell fate specification in the early embryo and mitotic proliferation of progenitor cells in the germline (Crittenden et al., 1994; Evans et al., 1994). The mRNA that encodes *glp-1* is found throughout the germline, including oocytes, and in all cells of the early embryo (Evans et al., 1994). Several RNA-binding proteins have been shown to contribute to the asymmetric pattern of GLP-1 expression (Farley and Ryder, 2012; Lublin and Evans, 2007; Wright et al., 2010), but the identity of the factor that represses GLP-1 protein production in maturing oocytes is not known.

The *glp-1* 3′ UTR is densely packed with UA(A/U) motifs, suggesting that OMA-1 may bind to this transcript and repress its translation in oocytes. To determine whether OMA-1 binds to the *glp-1* 3′ UTR directly, we constructed non-overlapping RNA fragments that span the 3′ UTR. Each RNA is approximately 30 nucleotides in length. OMA-1 binds to multiple fragments of the *glp-1* 3′ UTR. OMA-1 binds to fragments 1, 6, and 7 with highest affinity, comparable to the affinity of OMA-1 for the selected aptamer sequences. Fragments 1 and 6 have four UA(A/U) motifs, while fragment 7 has three. OMA-1 binds with moderate affinity to fragments 3, 8, 9, 10, and 11, which contain two or three UA(A/U) motifs. Very weak binding is observed to fragments 2, 4, and 5,

which have one or no motifs present. As such, the affinity of each fragment correlates with the number of UA(A/U) motifs present, with the highest affinity fragments containing four motifs **(Figure 2.5a)**. As with the selected aptamers, binding to the *glp-1* 3′ UTR fragments exhibits positive cooperativity when multiple UA(A/U) motifs are present (for example, fragment 6: nH = 2.2). The results are consistent with the SELEX results that suggest UA(A/U) motifs, which we now term OMA-1 binding motifs (OBMs), are required for high affinity OMA-1 binding.

Fragment 6 corresponds to a sequence that is evolutionarily conserved across nematode species and contains overlapping functional binding sites for POS-1 and GLD-1, RBPs required for *glp-1* silencing in embryos (Farley and Ryder, 2012; Marin and Evans, 2003; Ryder et al., 2004). As such, we decided to investigate the contribution of UA(A/U) motifs to OMA-1 binding to this fragment in more detail **(Figure 2.5b)**.

(a)

glp-1 3'UTR fragments	Sequence	$K_{d,app}$ (nM)	Hill coefficient	Number of UAA	Number of UAU
Frag1	UC**UAU**U**UAA**UUCAU**UAA**UUUUUCAUU**UAU**UG	23 ± 3	1.6 ± 0.1	2	2
Frag2	ACUG**UAU**CCCGGAUGUUUCUUGUCCUCCCAAC	1144 ± 400	1.9 ± 0.5	0	1
Frag3	A**UAU**CUCC**UAA**CUGCUCGGUUCAUUU**UAA**AUA	184 ± 53	1.7 ± 0.1	2	1
Frag4	UGCUCAUCUCACUACAUCACCCAGACACUGGUC	1200 ± 100	1.1 ± 0.5	0	0
Frag5	CCCACAGAGUUUUUUG**UAU**ACUAUUUCGGGUCA	629 ± 76	1.3 ± 0.1	0	1
Frag6	UUUUUCU**UAU**UCUAGAC**UAAUAU**UG**UAA**GCU	32 ± 2	2.2 ± 0.1	2	2
Frag7	A**UAA**GUUGUAGAA**UAA**U**UAU**UGAUCCAAAUCA	62 ± 12	1.5 ± 0.4	2	1
Frag8	GAU**UAA**GAG**UAUAA**GCUUUGUUUUUUCUCCUUU	246 ± 5	2.4 ± 0.5	2	1
Frag9	UCUU**UAUAA**CUUGUUACAAUUUUUGAAAUUCC	152 ± 108	1.6 ± 0.4	1	1
Frag10	CUUUUUUGACAGGCUUU**UAU**UACACUG**UAA**C	161 ± 10	1.8 ± 0.2	1	1
Frag11	UGUGUUUCU**UAU**CUUGCAAACAUU**UAA**UGAA	351 ± 155	1.2 ± 0.3	1	1

(b)

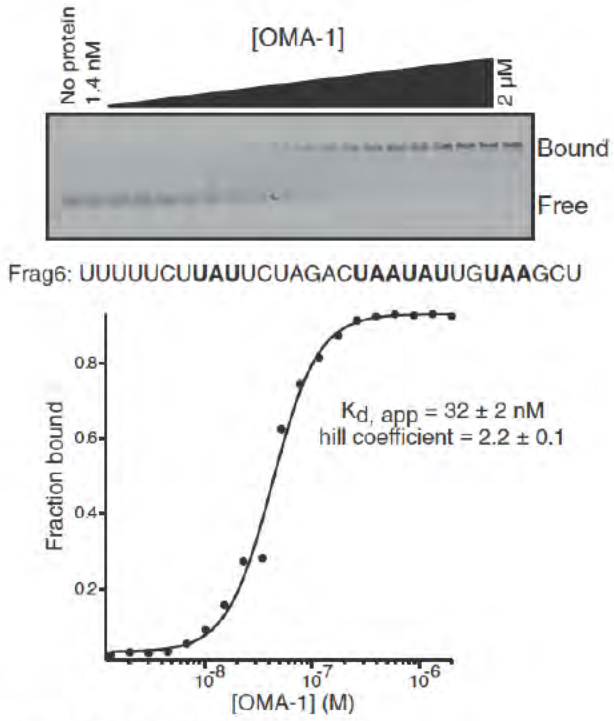


Figure 2.5 OMA-1 binds to the *glp-1* 3′UTR.

(a) EMSA results for OMA 1 binding to fragments of RNA that span the *glp 1* 3′ UTR. Frag1, Frag6 and Frag7 (underlined) are the fragments of the *glp 1* 3′ UTR that bind OMA 1 with the highest affinities. (b) Gel image showing the binding of OMA 1 to frag6, which is a conserved sequence of the *glp 1* 3′ UTR. Below the gel image is the plot of fraction bound of OMA 1 against the concentration of OMA 1. This plot is fit to the Hill equation.

We performed quantitative EMSA to determine the effect of mutating each UA(A/U) motif singly and in combinations to the OMA-1 binding affinity. Mutating each motif in isolation reduces the affinity by 3- to 5-fold **(Table 2.1)**. Mutating three motifs causes a 15-fold decrease in the binding affinity. The two variants of the triple mutation (Triple1 and Triple2) show the same decrease in the binding affinity. Mutating all four of the motifs leads to a 25-fold decrease in OMA-1 binding affinity **(Table 2.1)**.

Table 2. 1 Binding affinities of OMA-1 to variants of the glp-1 3´UTR where the OMA-1 binding motifs are mutated

Sequence ID	Sequence	$K_{d,app}$ (nM)	Hill coefficient	Number of UAA	Number of UAU
WT	UUUUUCUUAUUCUAGACUAAUAUUGUAAGCU	32 ± 4	2.2 ± 0.1	2	2
OBM1	UUUUUCU**CCC**UCUAGACUAAUAUUGUAAGCU	165 ± 14	1.9 ± 0.1	2	1
OBM2	UUUUUCUUAUUCUAGAC**CCC**UAUUGUAAGCU	118 ± 22	2.2 ± 0.5	1	2
OBM3	UUUUUCUUAUUCUAGACUAA**CCC**UGUAAGCU	108 ± 14	1.1 ± 0.2	2	1
OBM4	UUUUUCUUAUUCUAGACUAAUAUUG**CCC**GCU	102 ± 6	2.6 ± 0.3	1	2
Triple1	UUUUUCU**CCC**UCUAGAC**CCCCCC**UGUAAGCU	457 ± 20	2.2 ± 0.2	1	0
Triple2	UUUUUCU**CCC**UCUAGACUAA**CCC**UG**CCC**GCU	460 ± 64	1.3 ± 0.1	1	0
Quadruple	UUUUUCU**CCC**UCUAGAC**CCCCCC**UG**CCC**GCU	760 ± 8	1.6 ± 0.3	0	0

Our data show that the binding of OMA-1 to this sequence of RNA depends on the presence of UA(A/U) motifs. Plotting the apparent binding affinities against the number of UA(A/U) motifs show that the binding affinity improves with increasing number of UA(A/U) motifs **(Figure 2.6)**, as expected.

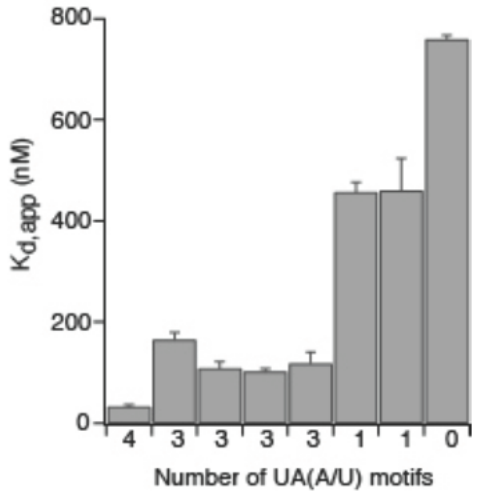


Figure 2.6 The binding affinity of OMA-1 to frag6 sequence increases with the number of UA(A/U) motifs

The plot of $K_{d,app}$ values against the corresponding number of UA(A/U) motifs show that the more the number of these motifs in the RNA sequence, the better the binding affinity for OMA 1.

glp-1 is a regulatory target of OMA-1

To test if OMA-1 and OMA-2 contribute to the regulation of *glp-1* mRNA in oocytes, we determined the effect of knocking down OMA-1/2 on the expression of a single copy integrated green fluorescent protein reporter under the control of the *glp-1* 3′ UTR (Farley and Ryder, 2012). When the reporter strain was treated with control food, GFP fluorescence was observed in the distal germline and in

embryos, but was not observed in the proximal germline or in oocytes, as has been previously reported (Farley and Ryder, 2012). When the reporter strain was treated with *oma-1, oma-2* RNAi food, we observed a strong increase in GFP fluorescence in the oocytes (88%, n = 27), **(Figure 2.7)**. We assessed knockdown effectiveness by verifying that embryos were not present, that reduced number of eggs were layed, that oocytes were larger, and that there were greater numbers of oocytes stacked in the gonad arm, hallmarks of the *oma-1, oma-2* phenotype. The data demonstrate that OMA-1 and OMA-2 are required to repress GLP-1 expression in the oocytes.

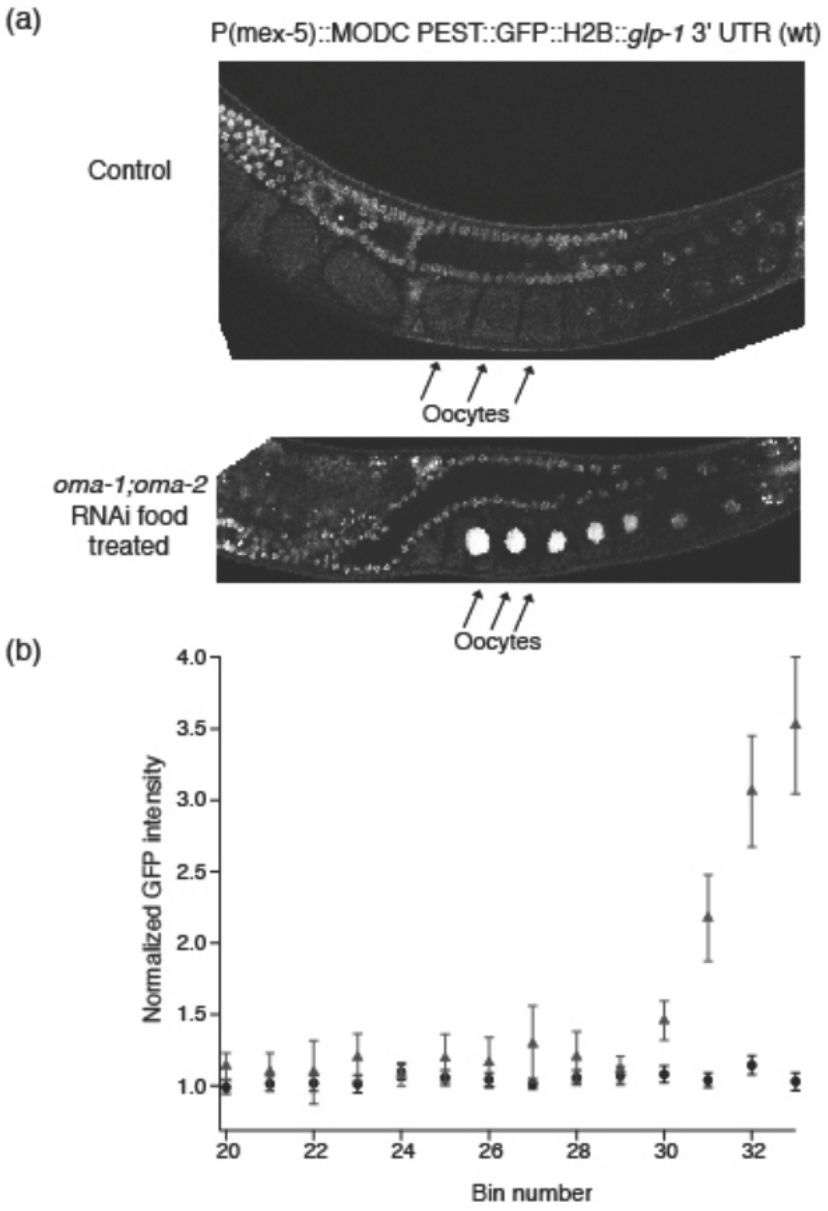


Figure 2.7 OMA-1 and OMA-2 contribute to repression of *glp-1* in the oocytes

(a) Fluorescence images of single copy integrated strains that express GFP under the control of the wild type *glp 1* 3′ UTR sequence. Top: worm grown on control RNAi food. Bottom: worm grown on *oma 1;oma 2* RNAi food. Under *oma 1;oma 2* knockdown conditions, *glp 1* is de repressed in the oocytes. (b) Quantification of confocal images of the reporter strains under the same conditions described above. GFP intensity as normalized to the average intensity across the wild type oocytes is plotted against the bin number. The intensities for *oma 1;oma 2* RNAi food are denoted with triangles and the intensities for the control food are denoted with circles.

Conclusion

In this study we demonstrated that the motif recognized by OMA-1 is different from those recognized by the related proteins TTP, POS-1, and MEX-5. From the *in vitro* selection, we showed that OMA-1 binds to UA(A/U) repeat sequences.

The relatively low information content of the OBM suggests that 1) many transcripts are regulated by OMA-1 or 2) additional factors may influence selection of its mRNA targets. In this study we show that multiple OBMs are required to achieve a high apparent binding affinity to mRNAs. It is possible that multiple OBMs are required to achieve regulation.

There are 28 OBMs in the 3′UTR of *glp-1*. Interestingly, analyzing the 3′ UTR of the putative mRNA targets *zif-1 and mei-1* revealed that there are 27 OBMs in the *zif-1* 3′-UTR and 9 OBMs in the *mei-1* 3′UTR. These OBMs are densely clustered in the *zif-1* 3′-UTR but more scattered in the *mei-1* 3′UTR. These OBMs could be the sites of regulation by OMA-1 in these mRNA targets.

glp-1 regulation by OMA-1

Our data also shows that OMA-1 regulates the translation of *glp-1* in oocytes. Many RNA-binding proteins have been shown to regulate *glp-1* mRNA post-transcriptionally. During oogenesis, PUF-5, PUF-6 and PUF-7 repress *glp-1* in early stage oocytes (Lublin and Evans, 2007). It was previously suggested that

OMA-1 and OMA-2 might repress *glp-1* in late stage oocytes as these proteins are abundant RNA-binding proteins in the maturing oocytes (Lublin and Evans, 2007). Here we have shown that OMA-1 and OMA-2 do in fact repress *glp-1* during oogenesis and oocyte to embryo transition.

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CHAPTER III: EFFICIENT GENERATION OF TRANSGENIC REPORTER STRAINS AND ANALYSIS OF EXPRESSION PATTERNS IN *CAENORHABDITIS ELEGANS* USING LIBRARY MOSSCI

Abstract

Background: In *C. elegans*, germline development and early embryogenesis rely on post-transcriptional regulation of maternally transcribed mRNAs. In many cases, the 3'UTR is sufficient to govern the expression patterns of these transcripts. Several RNA-binding proteins are required to regulate maternal mRNAs through the 3´UTR. Despite intensive efforts to map RNA-binding protein-mRNA interactions in vivo, the biological impact of most binding events remains unknown. Reporter studies using single copy integrated transgenes are essential to evaluate the functional consequences of interactions between RNA-binding proteins and their associated mRNAs.

Results: In this report, we present an efficient method of generating reporter strains with improved throughput by using a library variant of MosSCI transgenesis. Furthermore, using RNA interference, we identify the suite of RBPs that control the expression pattern of five different maternal mRNAs.

Conclusions: The results provide a generalizable and efficient strategy to assess the functional relevance of protein-RNA interactions in vivo, and reveal new regulatory connections between key RNA-binding proteins and their maternal mRNA targets.

Background and Significance

Spatial and temporal regulation of gene expression is crucial to the differentiation of tissues and organs. Cell fate specification, axis formation, cleavage and cell division rely upon regulated expression of important gene products at the right place at the right time. Changes in gene expression patterns can be regulated at the level of transcription, splicing, nuclear export, localization, translation or stability of mRNAs and proteins (Lasko, 2003; Melton, 1987; Moore, 2005; Thompson and Wickens, 2007; Wickens et al., 2000).

In early embryogenesis, there is little or no active transcription in the zygotic nucleus (Batchelder et al., 1999; Leatherman and Jongens, 2003; Newport and Kirschner, 1982; Tadros and Lipshitz, 2009); hence post-transcriptional regulation of maternal mRNAs by RNA-binding proteins plays a critical role in several systems (Colegrove-Otero et al., 2005; Farley and Ryder, 2008; Spirin, 1966). There are many examples that illustrate this point. In *Drosophila melanogaster*, repression of *hunchback* mRNA by Nanos and Pumilio, and repression of *caudal* mRNA by Bicoid, is required for anterior-posterior axis formation (Dean et al., 2002). The spatial pattern of translation and repression is mediated by elements present in the 3′ untranslated regions (3'UTRs) of target transcripts. In *Xenopus laevis*, *cyclinB1* mRNA must be kept translationally repressed in immature oocytes (Barkoff et al., 1998; de Moor and Richter, 1999). The RNA binding protein CPEB acts through the 3'UTR binding elements to mediate this repression until oocyte maturation. In *Caenorhabditis*

elegans, sexual fate of gametogenesis relies on post-transcriptional regulation of *fem-3* mRNA. Repression of *fem-3* by FBF-1 and FBF-2 RNA-binding proteins is required for the switch from spermatogenesis to oogenesis to occur after the L4 to adult molt in the hermaphroditic worm (Zhang et al., 1997). These examples highlight the key conserved role of post-transcriptional regulation of maternal transcripts during metazoan development.

C. elegans germline development is an excellent model system to study post-transcriptional regulation (Brenner, 1974). Development can be monitored in real time by light microscopy because the animal is transparent. Gene regulation can also be visualized in live nematodes using fluorescent reporter proteins (Chalfie et al., 1994), During oogenesis, transcription ceases when the oocytes enter prophase arrest. Transcription is not activated until the four-cell stage embryo, and then only in the somatic blastomeres (Blackwell and Walker, 2006; Seydoux et al., 1996; Seydoux and Fire, 1994; Walker et al., 2007). Therefore, maternal mRNAs transcribed by the germ cell nuclei must be controlled in the germline, in oocytes, and early embryos to regulate complex events of meiosis, oogenesis and early cell divisions in the embryo (Ahringer, 1997; Farley and Ryder, 2008; Seydoux, 1996; Stitzel and Seydoux, 2007). Specific RNA-binding proteins (RBPs) regulate the timing and localization of maternal mRNA translation and this regulation is conferred through specific elements in 3′UTR of maternal mRNAs (de Moor et al., 2005; Evans and Hunter, 2005).

The importance of the 3′UTR to regulation of maternal transcripts in the *C. elegans* germline is well established (Ahringer et al., 1992; Marin and Evans, 2003; Merritt et al., 2008; Mootz et al., 2004; Wickens et al., 2002; Zhang et al., 1997). Seydoux and coworkers showed that the 3'UTR is sufficient to govern the expression patterns of most maternal transcripts in the germline and early embryos (Merritt et al., 2008). Reporter transgenes expressing GFP under the control of a pan-germline promoter and a gene-specific 3'UTR recapitulated the expression pattern of the endogenous protein in 24 out of 30 of transgenes. In contrast, patterned expression was not observed in the reporter strains containing a gene-specific promoter and an unregulated 3'UTR

There are a number of RNA-binding proteins required for regulation of maternal mRNAs in the germline and early embryos. Examples include GLD-1, PUF-5/6/7, FBF-1/2, POS-1, OMA-1, MEX-3, MEX-5/6 (Detwiler et al., 2001; Farley and Ryder, 2012; Francis et al., 1995b; Huang et al., 2002; Jones et al., 1996; Kaymak and Ryder, 2013; Lublin and Evans, 2007; Marin and Evans, 2003; Pagano et al., 2009; Ryder et al., 2004; Schubert et al., 2000; Shimada et al., 2002; Spike et al., 2014b; Tabara et al., 1999; Wickens et al., 2002; Zhang et al., 1997). Published studies have identified candidate regulatory targets and/or identified RNA sequence motifs recognized by each of these proteins (Bernstein et al., 2005a; Farley et al., 2008; Farley and Ryder, 2012; Kaymak and Ryder, 2013; Pagano et al., 2009; 2007; Ryder et al., 2004; Spike et al., 2014b). For example, POS-1 and GLD-1 regulate the expression of *glp-1* mRNA in the

posterior of the early embryo through association with motifs in the 3´UTR (Farley and Ryder, 2012). Similarly, MEX-3 regulates *nos-2* translation in somatic cells of the early embryo through direct association with motifs present in the 3´UTR (Pagano et al., 2009). FBF regulates expression of *cki-2*, *fem*-3, and *gld-1* translation through direct 3´UTR interactions as well (Kalchhauser et al., 2011; Wright et al., 2010). However, the complete network of RNA interactions has not been established for most of these proteins, nor is it clear whether binding leads to regulation in all cases. Transgenic reporter studies are required to evaluate the biological consequence of binding.

There are a few different methods of generating transgenic *C. elegans* lines. The first requires introduction of DNA through microinjection into the germline (Mello and Fire, 1995; Mello et al., 1991). DNA introduced in this manner forms an extrachromosomal array that is passed to the progeny of the injected animal. This method does not generate a stably inheritable line, unless genomic integration is induced through DNA damage caused by ionizing radiation or UV exposure. A disadvantage of this method is germline silencing of transgenes due to the presence of repetitive copies of the transgene in the array (Kelly et al., 1997; Mello and Fire, 1995; Mello et al., 1991). A more recent method is biolistic transformation (Praitis et al., 2001). This results in integration of the transgene, hence stable inheritance, but the integration site is approximately random, and often there are multiple integration events, making it difficult to compare reporter expressions between strains. The most recent

advancement, termed Mos1-mediated single copy integration (MosSCI) was developed by Jorgensen and colleagues (Frøkjaer-Jensen et al., 2012; 2008). In this method, DNA is microinjected into the germline, and site specific integration is induced through site specific DNA double strand break induction followed by homologous recombination. The major advantage of this method is that a single copy integration is generated at a predetermined chromosomal location. This method has been widely adopted to generate transgenic reporter strains where comparison of reporter expression patterns under different conditions is needed. Despite these improvements, MosSCI remains a time consuming approach that requires microinjection by a skilled microscopist.

In this study, we adapted the MosSCI method to simultaneously inject a library of transgenes into the gonad. We show that integration selects individual reporters from the injected pool, yielding numerous single copy integrants of different reporters. This approach has increased the efficiency of obtaining transgenic lines through limiting the number of injections necessary. We used this approach to generate twenty-one transgenic lines, including eleven unique 3´UTR reporter strains. We then performed a candidate RNAi screen using a subset of these strains to identify RBPs that control the pattern of expression. The results provide an enhanced method to rapidly generate transgenic nematode reporter strains, and identify new regulatory connections between maternal RBPs and maternal mRNAs.

Experimental Procedures

Cloning of reporter constructs

The 3'UTR sequences were amplified from worm genomic DNA using UTR-specific primers flanked with atB2R and attB3 sequences for Gateway cloning. BP Clonase II was then used to clone the sequences into pDONRP2RP3. Multisite gateway cloning was then used to fuse this donor construct with pCM1.111 (construct carrying the *mex-5* promoter) and pBMF2.7 (construct carrying MODC PEST:GFP:H2B). LR Clonase II plus was used to integrate the fusion constructs into pCFJ150. Mutations of the *glp-1* 3'UTR reporter construct were introduced into the cloned pCFJ150 construct using Quickchange mutagenesis with Pfu Turbo.

C. elegans microinjection

MosSCI protocol (Frøkjaer-Jensen et al., 2008) was followed to generate the integrated lines with the adaptation described in this paper. A library of reporter constructs were injected at a concentration of 25ng/µl with *mos-1 transposase* (50 ng/µl), *mCherry* (2.5 ng/µl) and *peel-1* (10 ng/µl) plasmids into the gonad of EG6699 un-coordinated strain at the young adult stage. Wild-type moving, surviving worms were screened by single-worm PCR using Pfu Ultra II to identify the integrated construct.

RNAi knockdown

We knocked down *oma-1;oma-2*, *daz-1* and *pos-1* using the RNAi feeding method (Kamath, 2003). Using TA cloning, we cloned the all *oma-1* and *oma-2* open reading frames (ORFs) into the vector L4440 and transformed the clones into HT115(DE3) cells. *pos-1* and *daz-1* RNAi feeding bacterial cells were obtained from the Ahringer library. At OD_{600} = 0.4, we induced the cells for four hours by adding 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) at a final concentration of 0.4 mM. After induction, the induced RNAi cultures were concentrated 10- fold and added onto NGM plates containing 1mM IPTG and 100 µg/ml Ampicilin. Eggs obtained by bleaching adult worm strains were then plated onto RNAi plates and maintained at 25°C. The strains were imaged after 52 hours. HT115 strain bacteria transformed with the empty vector L4440 was used as our control plate.

Imaging of worm strains

Young adult worms were placed onto 2% agarose pads in 0.4 mM levamisole. DIC and GFP fluorescence images of gonad arms were taken using a 40× oil immersion objective (Zeiss Axioscope 2 plus microscope).

Results

Library MosSCI

Motif analysis predicts the presence of numerous RBP binding sites in 3'UTRs (Farley et al., 2008; Keene, 2007; Pagano et al., 2007; Wright et al., 2010). However, functional studies show that only some binding sites are capable of conferring regulatory activity in cells and animals (Evans et al., 1994; Farley and Ryder, 2012; Kalchhauser et al., 2011; Marin and Evans, 2003; Pagano et al., 2009; Wright et al., 2010). We therefore sought a way to improve the throughput of functional studies using transgenic fluorescent reporters. The improved MosSCI protocol of Jorgensen lab was used as a starting point for our experiments (Frøkjaer-Jensen et al., 2012). We reasoned that the integration step of transgenesis might select individual transgenes from a library of reporter plasmids co-injected into worms. If so, then each injection could potentially create multiple transgenic progeny, each bearing a different integrated reporter transgene. To test this hypothesis, we cloned sixteen different 3'UTRs fused to a pan-germline promoter (*mex-5*) and a destabilized GFP-histone H2B fusion (GFP::H2B::PEST domain) (Farley and Ryder, 2012; Frand et al., 2005). The identities of the 3'UTRs used in transgenic reporter constructs are listed in **Table 3.1**.

Table 3.1 List of the 3´UTRs in the reporters that were successfully integrated and their genotypes

3´ UTR strain	Strain identifier	Genotype
	EG6699	*ttTi5605 II; unc-119(ed3) III; oxEx1578*
atg-4.2	WRM10, WRM11	*sprSi10[Pmex-5::MODC PEST:GFP:H2B:: atg-4.2 3'UTR cb-unc-119(+)]* II, *unc-119(ed3)* III
cul-4	WRM12, WRM13, WRM14, WRM15	*sprSi11[Pmex-5::MODC PEST:GFP:H2B::cul-4 3'UTR cb-unc-119(+)]* II, *unc-119(ed3)* III
cwn-1	WRM16	*sprSi12[Pmex-5::MODC PEST:GFP:H2B::cwn-1 3'UTR cb-unc-119(+)]* II, *unc-119(ed3)* III
ets-4	WRM17	*sprSi13[Pmex-5::MODC PEST:GFP:H2B::ets-4 3'UTR cb-unc-119(+)]* II, *unc-119(ed3)* III
hbl-1	WRM18	*sprSi14[Pmex-5::MODC PEST:GFP:H2B::hbl-1 3'UTR cb-unc-119(+)]* II, *unc-119(ed3)* III
lin-26	WRM19, WRM20, WRM21	*sprSi15[Pmex-5::MODC PEST:GFP:H2B::lin-26 3'UTR cb-unc-119(+)]* II, *unc-119(ed3)* III
mbk-2	WRM22, WRM23	*sprSi16[Pmex-5::MODC PEST:GFP:H2B::mbk-2 3'UTR cb-unc-119(+)]* II, *unc-119(ed3)* III
mex-3	WRM24, WRM25	*sprSi17[Pmex-5::MODC PEST:GFP:H2B::mex-3 3'UTR cb-unc-119(+)]* II, *unc-119(ed3)* III
set-2	WRM26	*sprSi18[Pmex-5::MODC PEST:GFP:H2B::set-2 3'UTR cb-unc-119(+)]* II, *unc-119(ed3)* III
set-6	WRM27, WRM28, WRM29	*sprSi19[Pmex-5::MODC PEST:GFP:H2B::set-6 3'UTR cb-unc-119(+)]* II, *unc-119(ed3)* III
usp-14	WRM30	*sprSi20[Pmex-5::MODC PEST:GFP:H2B::usp-14 3'UTR cb-unc-119(+)]* II, *unc-119(ed3)* III

Equal concentrations of each of the sixteen reporter constructs were mixed together into a single mixture. 25 ng/µl of this library mixture was injected into the *C. elegans* un-coordinated strain EG6699. **Figure 3.1** shows a schematic of the injection method adapted from the MosSCI technique.

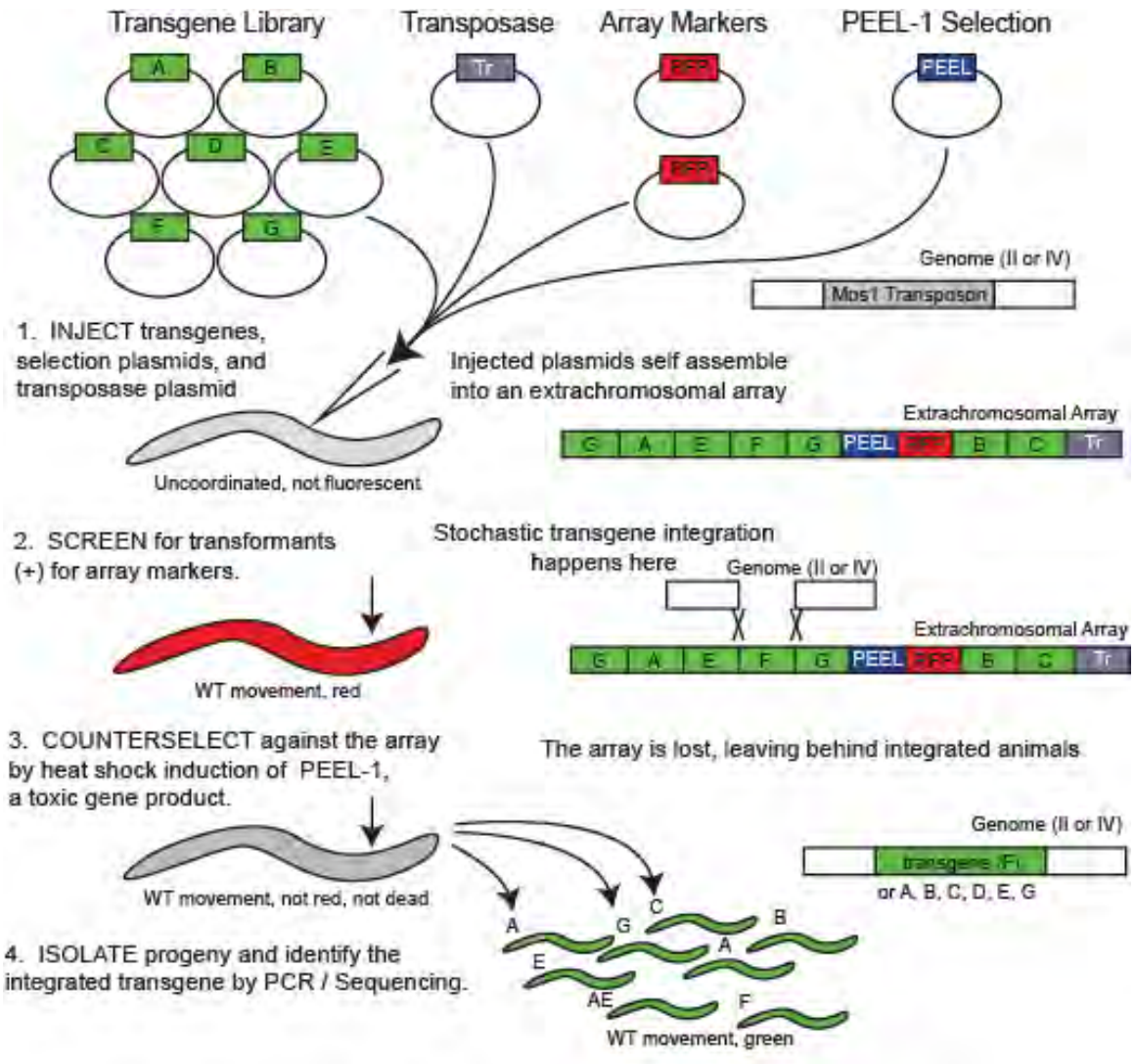

Figure 3.1 Schematic of the library MosSCI method.

A library of transgenes is mixed in equal amounts, and then microinjected into the germline of the parent strain. As with standard MosSCI, positive transformants are identified by *unc 119* rescue, which restores wild type movement, and red fluorescence in the pharynx and body wall muscle. Integrants are recovered by heat shock induction of PEEL 1, a negative selection marker which kills all worms that have not lost extrachromosomal array formed from the injected plasmid mixture.

In addition to the library of sixteen 3´UTR reporter constructs, a positive selection marker (*unc-119*), two negative selection markers, *peel-1* and *mCherry*, and a plasmid encoding the Mos1 transposase were also injected at the same time. The *peel-1* gene is under the control of a heat-shock promoter but the *mCherry* and *mos-1 transposase* genes are constitutively expressed. Once injected, the transposase generates a double strand break at a specific location in chromosome II. This break is then repaired by homologous recombination with one of the injected reporter constructs, as each of the constructs are flanked by ends homologous to the break. If construct selection is stochastic, then it is possible to get a different reporter for each successful integration event.

Successfully integrated animals must then be distinguished from worms that still carry an extrachromosomal array. Worms that do not lose the array will have mCherry red fluorescent marker in their pharynx. These worms will also carry the *peel-1* plasmid. PEEL-1 is a toxin that will select against the transformants that are carrying an extrachromosomal array (Frøkjaer-Jensen et al., 2012; Seidel et al., 2011). When PEEL-1 expression was induced by heat shock at 34°C for two hours, worms harboring the array died.

We then selected surviving worms and screened them and their progeny by PCR in order to identify which (if any) reporter construct had been integrated into the genome. We performed single worm PCR using a homozygous population of each candidate line. **Figure 3.2** shows representative PCR data for the integrated lines obtained. Direct sequencing of the PCR products using a

primer that anneals to the GFP reporter sequence led to unambiguous identification of which reporter construct had been integrated.

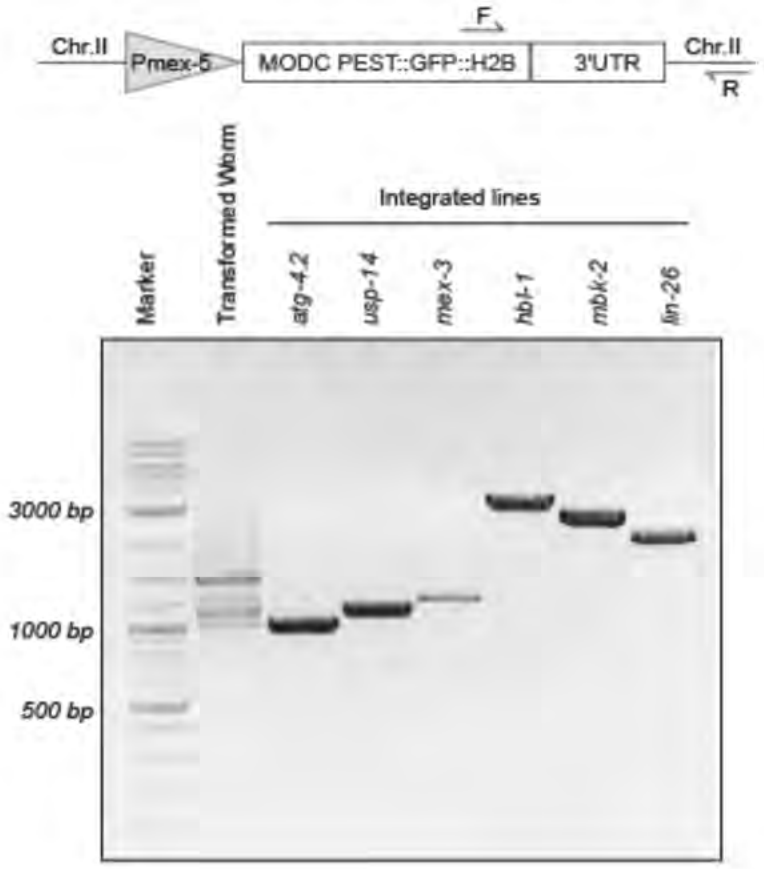

Figure 3.2 Design of a single-worm based primer system to detect and characterize integrated lines.

The first lane shows amplification from a transformed worm population, prior to counter selection with PEEL 1, which harbors multiple reporters in an extrachromosomal array. The remaining lanes show the PCR amplified products of transgenes that were integrated. We determined which UTR reporter was integrated by sequencing the PCR products. The transgene schematic is not drawn to scale.

Fifteen transgenic lines were obtained from 217 injections. In this initial set of strains, we noticed that some constructs were overrepresented. Four of the integrated lines contained an integration of P*mex-5*::GFP::H2B::PEST::*cul-4* 3'UTR. Three of the integrated lines contained P*mex-5*::GFP::H2B::PEST::*lin-26* 3'UTR. Two of the integrated lines contained P*mex-5*::GFP::H2B::PEST::*mbk-2* 3'UTR. Two more of the integrated lines contained P*mex-5*::GFP::H2B::PEST::*mex-3* 3'UTR. Additional two of the integrated lines contained P*mex-5*::GFP::H2B::PEST::*atg-4.2* 3'UTR. Of the fifteen recovered lines, we obtained seven unique reporters. To prevent recovering multiple copies of the same strain, we then reduced the size of the library to include just the remaining nine constructs from our library of sixteen. In additional 52 injections, we obtained six additional independent lines, resulting in four additional unique reporter strains. In total, we were able to generate eleven unique transgenic strains in 269 injections. These are listed in **Table 3.1**.

The rate of successful injections giving wild-type moving transformants was higher than the rate of integration steps. In the total of 269 attempted injections, 93 gave rise to wild-type moving transformant progeny. Of these, 21 contained a single copy of a transgenic construct. As such, we estimate our successful injection rate to be 35%, the integration rate to be approximately 23%, and the unique strain recovery rate (per successful injection for our relatively small library of sixteen reporters) to be ~11%. While overall success is still limited by successful injection rate (governed by the ability of the injector), the

overall rate of recovery of unique reporters represents a large improvement over previous benchmarks (Frøkjaer-Jensen et al., 2012; 2008).

Expression patterns of GFP in the integrants

Having established new lines, we then used direct fluorescence imaging of the germline to determine the expression patterns of the transgenic reporter strains. Three out of the twenty-one lines we generated did not show GFP fluorescence, presumably due to germline transgene silencing (Kelly et al., 1997). The eighteen remaining strains prepared by library MosSCI showed GFP expression in the germline and/or embryos. As expected, the pattern of expression varied with the identity of the 3'UTR. The expression patterns are summarized in **Figure 3.3**.

Pan germline expression: Some 3´UTR reporters showed pan-germline expression, including *ets-4, usp-14*, *hbl-1*, *lin-26* and *cwn-1*. Reporter expression was observed in the distal region of the germline, in mitotic progenitor cells, as well as in the syncytial region, in the germline bend, in oocytes, and in embryos. We note that the *hbl-1* reporter expression was faint in all regions of the germline. In four of the five reporters (*usp-14*, *lin-26, hbl-1*, and *cwn-1*), no expression was observed in sperm, consistent with the findings of Seydoux and co-workers that suggests sperm expression is governed via transcriptional regulation at the promoter, rather than post-transcriptionally through 3´UTR level

(Merritt et al., 2008). In direct contrast, *ets-4* reporter expression remained strong in sperm (n=12/23), suggesting that at least some 3´UTRs can direct retention of sperm specific expression.

The set-2 pattern: We also studied the pattern of a *set-2* 3'UTR that was integrated using standard reporter MosSCI, rather than the library approach presented here. *set*-2 3'UTR showed faint GFP expression in the distal end followed by an increased expression in the syncytial region, which then decreased around the recellularization region and oocytes. As with *ets-4*, the GFP expression remained strong in sperm (n=11/15), providing a second example of a 3´UTR that can direct expression of a reporter in male gametes.

Oocyte repression: Other 3´UTR reporters, such as *atg-4.2*, *cul-4, him-14* and *set-6,* and *mbk-2* showed strong expression in the syncytial region of the gonad and in embryos but little or no expression in oocytes. *atg-4.2* and *cul-4* showed GFP expression in the distal mitotic zone followed by increased expression in the syncytial region, and decreased expression around the recellularization region and in early oocytes. Weak expression in oocytes appeared to increase as the oocytes neared the spermatheca. In contrast, *set-6* and *him-14* 3´UTR reporters showed no increase in oocyte expression in maturing oocytes. Interestingly, *set-6* reporter also showed GFP expression in

sperm (n=10/17). Only the *mbk-2* reporter showed a complete lack of expression in oocytes.

Oocyte-and embryo-specific expression: The *mex-3* 3'UTR reporter is unique in that it showed strong GFP expression in the oocytes, with expression peaking in the most mature oocytes. Little or no expression was observed in the distal germline or in the syncytial region. Expression was also observed in the anterior cells of early embryos, but not in the posterior, consistent with the patterned expression of endogenous MEX-3 (Draper et al., 1996).

Out of the eleven 3'UTR reporter strains we studied, endogenous protein expression patterns are known for LIN-26, MEX-3, MBK-2 and SET-2. Antibody staining experiments showed that SET-2 and MEX-3 endogenous patterns match our reported patterns. MEX-3 is seen in the oocytes and anterior cells of two and four cell stage embryos matching our GFP reporter pattern (Bowerman et al., 1997; Draper et al., 1996). SET-2 is observed strongly in the mid-pachytene region of the germline but also in pharynx, neurons and intestines (Xu and Strome, 2001). We do not expect to observe somatic expression with our reporters, which include a germline specific promoter. No sperm expression was reported. For MBK-2, antibody staining was reported at the cortex of developing oocytes and in cytoplasm of embryos; however, we have not seen reporter expression in the oocytes of the *mbk-2* 3'UTR reporter strain (Stitzel et al., 2007).

These differences could be due to transcriptional regulation by the endogenous promoter used, or due to post-translational regulation. LIN-26, on the other hand, is endogenously expressed in the somatic gonad and hypodermal cells of embryos and larvae of all stages; however, germline expression pattern was not reported (Labouesse et al., 1996). Endogenous protein expression patterns have not been published for ATG-4.2, CUL-4, HIM-14, ETS-4 SET-6, and USP-14.

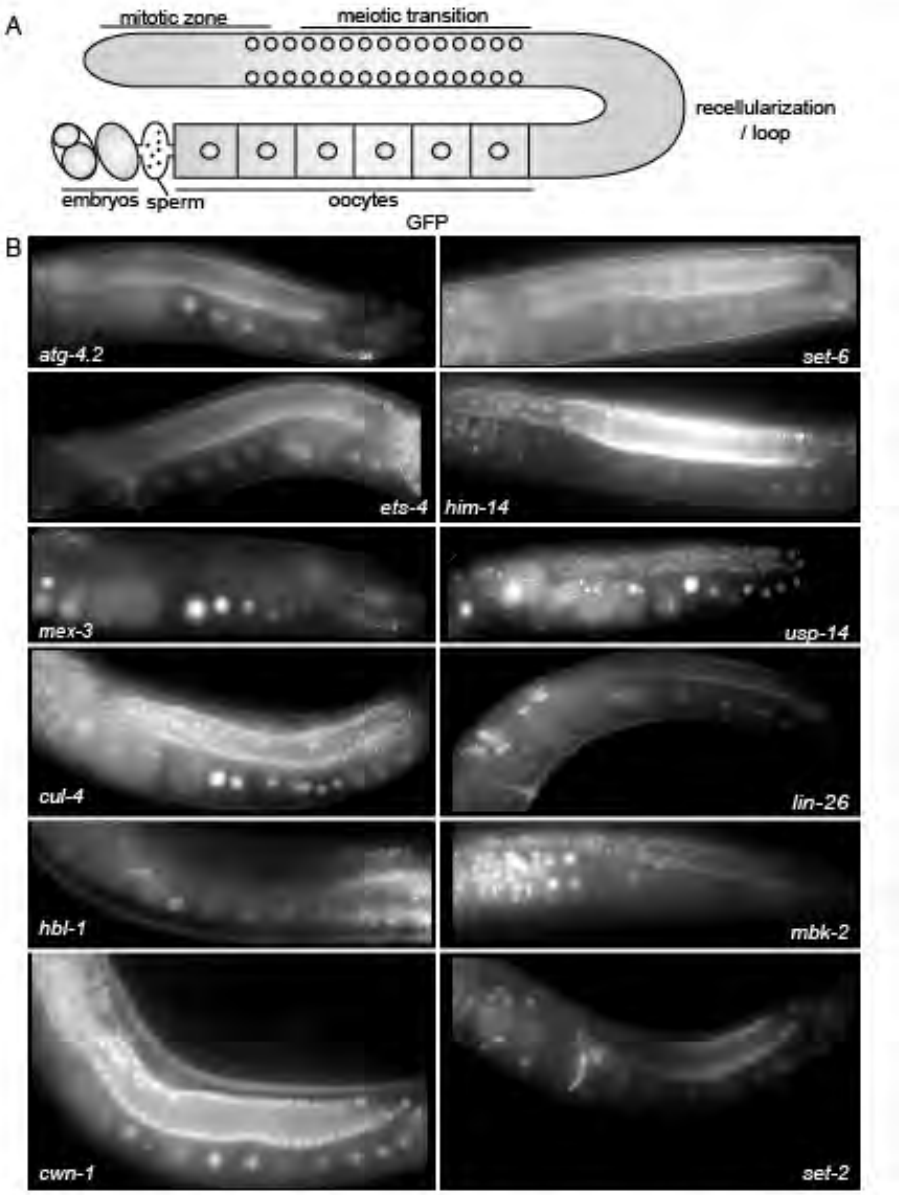
A
mitotic zone
meiotic transition
recellularization
/ loop
embryos
sperm
oocytes
GFP
B
atg-4.2
set-6
ets-4
him-14
mex-3
usp-14
cul-4
lin-26
hbl-1
mbk-2
cwn-1
set-2
C
mitotic zone
transition
loop
oocytes
sperm
embryos
n
atg-4.2 34
hbl-1 18
mex-3 26
usp-14 17
lin-26 24
mbk-2 27
him-14 20
cwn-1 16
set-6 17
ets-4 23
cul-4 31
set-2 14

Figure 3.3 GFP expression patterns of integrated 3´UTR strains

A: Schematic of the *C. elegans* germline. The syncytial region of nuclei in the distal arm of the gonad, the oocytes, sperm, and embryos in the uterus are shown. B: Representative images of single copy integrated reporter strains that express GFP under the control of different 3'UTRs. C: A table summarizing the GFP expression patterns of the reporter strains in different parts of the germline and embryos. Gray bars denote expression. The number of animals imaged is indicated to the right.

Targeted RNAi screening of transgenic reporter strains

We wished to identify RNA-binding proteins that directly or indirectly control the expression pattern of the new 3´UTR reporter strains. We chose a subset of reporter strains that have distinct patterns of GFP expression to study further by RNAi knockdown studies. The strains we chose to investigate carry the *atg-4.2* 3'UTR, *cul-4* 3'UTR, *set-2* 3´UTR, *set-6* 3'UTR, *mex-3* 3'UTR, or *ets-4* 3'UTR. In addition to their interesting patterns of expression, these 3'UTRs also contain binding motifs for RBPs with important roles in germline development and early embryogenesis. We wanted to identify which RNA-binding proteins contribute to the varying patterns of GFP expressions in the reporter strains. We looked for expression pattern changes under *oma-1;oma-2* RNAi, *daz-1* RNAi *pos-1* RNAi, and control treatments. We chose to knockdown these transcripts because they encode germline expressed RNA-binding proteins that have an easy to score phenotype. *oma-1;oma-2* RNAi, and *daz-1* RNAi lead to sterility and *pos-1* RNAi leads to embryonic lethality (Detwiler et al., 2001; Karashima et al., 2000; Tabara et al., 1999).

OMA-1 and OMA-2 are tandem zinc-finger RNA-binding proteins redundantly required for oocyte maturation. The phenotype of *oma-1;oma-2* RNAi knockdown is more than 90% penetrant when performed by the feeding method. Knockdown of *oma-1* and *oma-2* by RNAi leads to oocytes with increased size, a greater number of oocytes in the gonad arm, and sterility

(Detwiler et al., 2001; R. Lin, 2003). Knockdown in *atg-4.2* 3'UTR, *ets-4* 3'UTR, *cul-4* 3'UTR, *set-6* 3'UTR, *mex-3* 3'UTR led to a strong increase in the expression of GFP in oocytes. In contrast, knockdown had no effect on the *set-2* 3'UTR reporter **(Figure 3.4)**. The results suggest that OMA-1 and OMA-2 repress expression of *atg-4.2*, *ets-4*, *cul-4*, and *set-6* in oocytes. It is not clear why or how the *set-2* retains oocyte repression in oocytes. We suspect it is likely to be repressed by a different pathway.

DAZ-1 is an RNA-binding protein required for oogenesis (Otori et al., 2006). Knockdown of DAZ-1 results in absence of oocytes and sterility. The *daz-1* RNAi-induced phenotype is 70-80% penetrant by the feeding method. Worms cultured under *daz-1* RNAi conditions contain an abundance of non-cellularized nuclei around the germline bend, where oocytes normally form. This then leads to an absence of oocytes in the proximal region of the gonad arm. *daz-1* RNAi was performed in strains carrying the *atg-4.2* 3'UTR, *ets-4* 3'UTR, *cul-4* 3'UTR, *set-6* 3'UTR, *mex-3* 3'UTR and *set-2* 3'UTR reporters. We observed a change only in the reporter strain containing the *set-2* 3'UTR. This strain does not express GFP around the loop region under wild-type conditions but when treated with *daz-1* RNAi there was a strong increase in GFP expression in the recellularization/loop region **(Figure 3.4)**. The results suggest that *set-2*, in contrast to *atg-4.2, ets-4, cul-4, set-6, and mex-3*, is regulated by DAZ-1, directly or indirectly.

POS-1 is another tandem zinc-finger RNA-binding protein that is required for the development of the posterior in the embryos. RNAi knockdown of this protein leads to embryonic lethality. The phenotype of *pos-1* RNAi knockdown was about 80% penetrant. *pos-1* RNAi knockdown did not show a change in the reporter expression in the germline and oocytes for any of the strains tested. In contrast, *pos-1* knockdown has been previously shown to lead to expression of a *glp-1* 3'UTR reporter in all cells of an early embryo. The data suggest that POS-1 does not regulate many genes that harbor a putative POS-1 binding site, as has been previously suggested (Farley and Ryder, 2012).

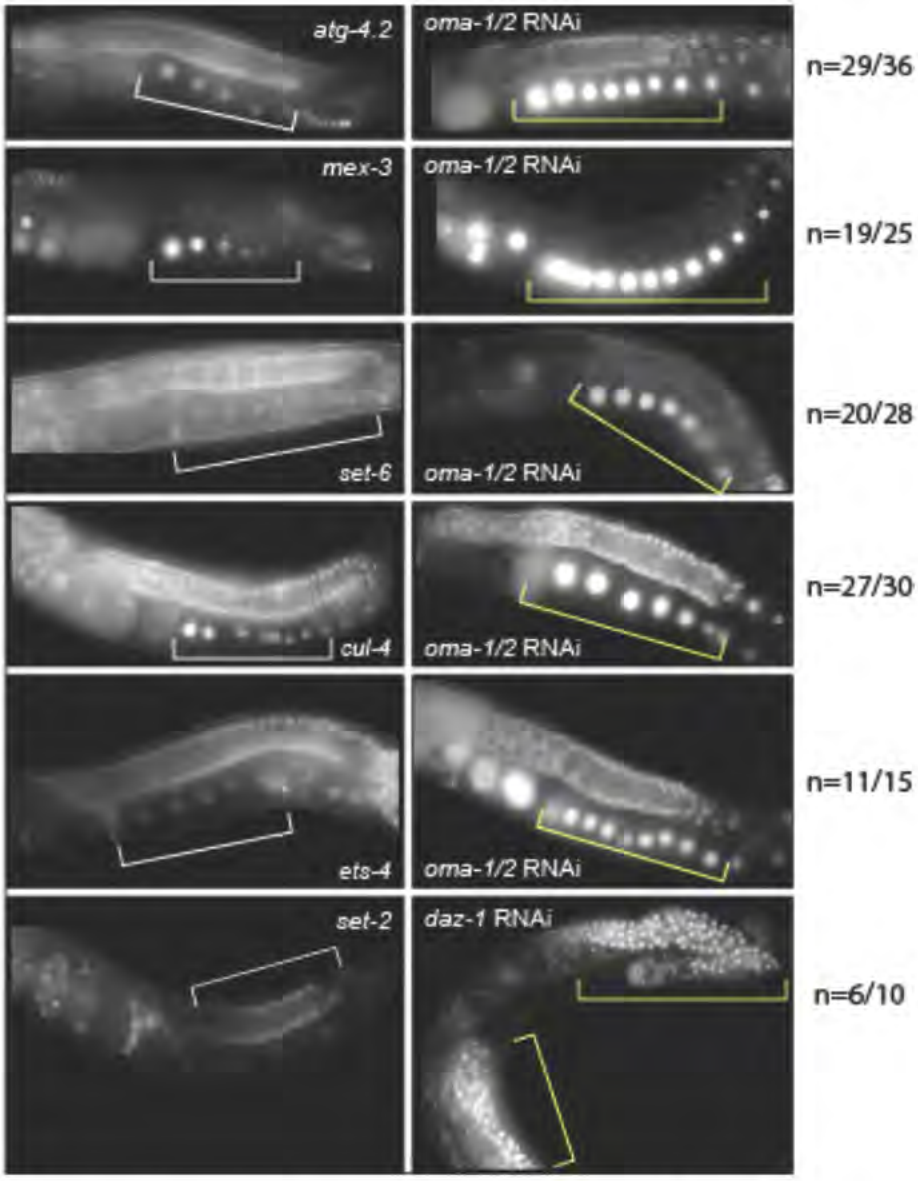

Figure 3.4 Targeted RNAi screen against OMA-1/2, DAZ-1 and POS-1

RNAi reveals that OMA 1 and OMA 2 repress the expression of *atg 4.2*, *mex 3*, *set 6*, *ets 4* and *cul 4* reporters in oocytes, while DAZ 1 regulates repression of *set 2* in the syncytial and loop regions of the germline. The left panel shows the expression pattern of the reporter fusion strain and the right panel shows the expression patterns of the worms cultured with bacteria that express double stranded RNA targeting the *oma 1*, *oma 2*, or *daz 1* mRNAs. Changes in the pattern of reporter expression are annotated by comparing the white bars (left panel) to the yellow bars (right panel). The penetrance of the change in expression is indicated to the right of the images.

Conclusions

In this study, we have shown that the rate of generating transgenic strains can be improved using an adaptation to the MosSCI technique. Injecting a library of transgenic constructs reduced the total time consumed to make nineteen independent lines by three- to four-fold in our hands. This was achieved through stochastic integration of transgenic constructs for every successful injection. It is not yet clear if increasing the library size further will further improve the success rate. We used this approach to make new UTR reporter lines, revealing for the first time that specific UTRs can drive reporter expression in sperm. We also used new strains in a targeted RNAi screen which revealed new regulatory connections between RNA-binding proteins and mRNAs.

Regulation by OMA-1/2

There are different ways OMA-1/2 could mediate repression of the 3´UTR reporters developed in our study. OMA-1/2 could be directly binding and repressing translation or indirectly regulating transgene expression through antagonistic interactions with other proteins.

OMA-1 and OMA-2 repressed protein expression of most of the 3´UTR reporter transgenes we studied. This supports the hypothesis that OMA-1 might be a general repressor of translation during oocyte development and maturation (Kaymak and Ryder, 2013). The mRNAs that were regulated by OMA-1/2 encode

proteins that influence a diverse array of biological phenomena, like ATG-4.2, ETS-4, CUL-4, SET-6 and MEX-3. *atg-4.2* encodes a homolog of human autophagic cysteine protease that does not have an obvious RNAi phenotype (Wu et al., 2012). ETS-4 is a transcription factor that regulates aging (Thyagarajan et al., 2010). CUL-4 is a cullin ubiquitin ligase that prevents re-replication of DNA (Zhong et al., 2003). *set-6* is predicted to encode an H3K9 methyltransferase that regulates transcription (Andersen and Horvitz, 2007). *mex-3* encodes a KH-domain RNA-binding protein that specifies the anterior of the embryo (Draper et al., 1996). As oocytes develop in the gonad arm, there is no autophagy, transcription, embryonic cell-fate determination or bulk DNA replication going on. This can be a reason why the mRNAs are kept in a silent state through OMA-1/2 acting as the major regulator or one of the intercommunicating regulators.

By contrast, *oma-1, oma-2* RNAi did not repress the translation of the *set-2* 3´UTR reporter transgene. *set-2* is a histone methyltransferase that can be involved in modifiying histones during chromatin remodeling which is required for the tight regulation of gene expression in sperm development (Simonet et al., 2007). Intriguingly, DAZ-1 appears to regulate translation of the *set-2* 3'UTR. DAZ-1 is required for meiotic progression and formation of oocytes in the germline of *C. elegans* (Karashima et al., 2000). The RNA-binding specificity of DAZ-1 is not known, but its mammalian homolog DAZL (DAZ-like) binds stretches of polyU sequences with G or C bases distributed throughout

((G/CUn)n) (Venables et al., 2001). DAZ-1 represses *set-2* 3'UTR at the recellularization/loop region of the germline. *set-2* is a methyltransferase that is required for proper germline development (Simonet et al., 2007). It is not yet clear why this UTR is repressed by DAZ-1, but not OMA-1/2. More work is needed to understand why some transcripts are repressed by OMA-1/2 in oocytes, yet others are repressed by DAZ-1.

Sperm retention driven by the set-2, ets-4 and set-6 3'UTR

The Seydoux lab previously reported that promoters are necessary and sufficient for sperm expression for sperm-expressed reporter transgenes, while the 3´UTR sequence is dispensible for expression in sperm (Merritt et al., 2008). Here we show an exception to this finding where the 3´UTR of *set-2*, *ets-4* and *set-6* drives strong GFP expression in the sperm. Understanding how and why this 3´UTR enables expression in sperm may lead to new insights in sperm specific gene expression patterns. Moreover, we propose that incorporation of the *set-2* or *ets-4* 3´UTR into a transgenic construct could provide a useful tool to enable studying the effect of driving expression of specific gene products in sperm. One way we propose these 3´UTR's allow transgene expression in sperm is that translation may be enabled in sperm due to the absence of a repressor acting on these UTRs at this specific location.

Acknowledgements

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CHAPTER IV: DISCUSSION

In the research described in this dissertation, my aim was to understand how OMA-1/2 bind and regulate their cognate mRNA targets at the oocyte-to-embryo transition. Knowing the site where OMA-1/2 bind on their targets is the first step in identifying direct mRNA targets regulated by OMA-1/2. Therefore, I first set out to determine the RNA-binding sequence specificity of OMA-1 and showed that it binds UA(A/U) elements with high affinity. This sequence is similar to the binding sequence of TTP, which is UAUUUAUU (Lai et al., 1999), yet the binding affinity of OMA-1 to this sequence is about 50 fold weaker. Similarly, OMA-1 binds weakly to POS-1 and MEX-5 motifs, revealing that its specificity is different from paralogs expressed in *C. elegans*.

OMA-1 Sequence Specificity

It is likely that differences in primary sequence and structure account for the variance in RNA recognition properties. The NMR structure of the zinc finger domain of TIS11d, a mammalian TZF protein, showed that each finger folds into a similar conformation that binds to UAUU. The RNA binding specificity was proposed to come from hydrogen bonding of the protein backbone to the Watson-Crick edges of the bases. In addition, side chains of conserved aromatic amino acids lead to stacking interactions with the RNA bases which are essential for RNA recognition (Hudson et al., 2004). It was reported that an amino acid in

each finger, termed the “discriminator” residue, accounts for the difference specificity between TTP and MEX-5. In TTP, the discriminator residue is a glutamate in both fingers. In the NMR structure, the side chain carboxylate accepts a hydrogen bond from the N6-exocylic amine of an adenosine in the motif UAUU. In MEX-5, which binds to RNA with relaxed specificity, the corresponding amino acid is a lysine in finger 1 and an arginine in finger 2, predicted to form non-specific backbone ionic interactions at the expense of the base specific hydrogen bonds found in Tis11D. Mutagenesis experiments confirm the importance of each amino acid to binding specificity (Kaymak et al., 2010; Pagano et al., 2007). POS-1 has small hydrophobic residues at the corresponding positions and binds to RNA with different specificity compared to that of Tis11D and MEX-5 (PRE = $UAU_{2\ 3}RDN_{1\ 3}G$). It is not clear how the discriminator residues contribute to POS-1 RNA recognition. OMA-1 and OMA-2 have a basic residue in finger 1 and small hydrophobic residue in finger 2. Hence, a hybrid specificity between POS-1 and MEX-5 was expected (Pagano et al., 2007). In line with this expectation, we showed that the RNA binding sequence specificity of OMA-1 is neither as relaxed as that of MEX-5 nor as specific as the POS-1 recognition element. The motif observed (UA(A/U)) bears some similarity to the 5′-portion of the PRE. More work, including structure determination of the OMA-1, POS-1, and MEX-5 RNA-bound complexes is required to fully assess this hypothesis.

How UA(A/U) elements help selection of mRNAs by OMA-1 for regulation is not fully understood. We observed that the abundance of UA(A/U) elements is not sufficient to determine mRNA targets regulated by OMA-1. Hence, how OMA-1 recognizes a sequence and consequently result in a functional outcome; such as repression, is not clear. The sequence specificity of OMA-1 has low information content. This suggests that OMA-1 regulates multiple transcripts or additional factors are required for selection of its mRNA targets among a complex pool of RNA sequences. In this dissertation I show that OMA-1's apparent binding affinity cooperatively increases as the number of OBMs (OMA-1 binding motifs) increases, suggesting that multiple OBMs are required to achieve a high apparent binding affinity to mRNAs. Possibly, multiple OBMs are required to achieve regulation as well. Consistent with this hypothesis, in Chapter II, I showed that OMA-1 and OMA-2 mediate *glp*-1 repression in the oocytes. There are 28 OBMs the 3′-UTR of *glp-1* and mutation of sequences in the 3′-UTR of *glp-1* corresponding to OBM1, OBM3, and a double mutation of OBM1 and OBM3 in previous studies did not lead to activation of the glp-1 reporter in oocytes (Farley and Ryder, 2012). Perhaps OBMs function with some redundancy to ensure *glp-1* repression.

OMA-1 Target Selectivity

We do not understand how OMA-1 selects mRNAs from a pool of RNA sequences it can interact with for a functional regulation. We do not also know whether the targets of OMA-1 identified thus far are regulated directly or indirectly by OMA-1. In the case of *glp-1* mRNA, we were not able to map a site that is necessary and sufficient for OMA-1 mediated repression in the oocytes. Other known targets of OMA-1 did not also provide a site through which a regulation is conferred. Identifying additional mRNA targets that are regulated by OMA-1 and analyzing the context of the OBMs in an effort to identify sites necessary and sufficient for regulation may provide more insight into RNA-recognition properties of OMA-1.

It is possible that there might be a longer consensus sequence recognized by OMA-1 or OMA-1 might be acting through multiple UA(A/U) elements to achieve target selectivity. Understanding the context of OBMs in mRNAs that are associated with OMA-1 *in vivo* can provide additional information on the role of OMA-1's sequence specificity. High-throughput sequencing of the crosslinked fragments (HITS-CLIP) (Licatalosi et al., 2008) or Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) (Hafner et al., 2010) can identify mRNAs associated with OMA-1 in mature oocytes and one-cell embryos, where OMA-1 is expressed. Motifs that are in

common among the mRNAs that are enriched in immunoprecipitations can then be studied to investigate OMA-1 binding sites. When a similar approach was used to see whether a motif longer than the OBM was enriched in OMA-1 interacting mRNAs, no such motif was identified (Spike et al., 2014b). The OBMs, however, were slightly enriched in mRNAs that significantly interact with OMA-1, when compared to *C. elegans* 3´UTRs with similar lengths (Spike et al., 2014b). This study was based on mRNAs enriched in RNA-immunoprecipitation experiments without crosslinking. Performing this analysis upon crosslinking will be valuable because in a crosslinking and immunoprecipitation experiment, ultraviolet irradiation will be used to form covalent crosslinks between protein-RNA complexes that are in direct contact in intact cells. The cross-linked complexes can then be enriched by antibody purification under stringent conditions (Hafner et al., 2010; Ule et al., 2006). PAR-CLIP provides an increase in the efficiency of crosslinking. In this method, 4-thiouridine (4-SU) is incorporated into transcripts (Hafner et al., 2010). It was shown that 4-SU containing transcripts crosslinked more efficiently upon UV 365 nm irradiation compared to the conventional 254 nm irradiation. As long as the modification of uridines does not interfere with OMA-1 binding to its target transcripts, RNA recovery can be improved using PAR-CLIP. Moreover, crosslinked sites show a T to C transition after sequencing (Hafner et al., 2010). Therefore, analyzing mutations in the recovered transcript can identify the position of crosslinking. That is, clusters of sequence reads that show a high frequency of T to C

mutations represent the crosslinking sites. The stringent purification conditions and knowledge of the crosslinking sites can narrow the list of mRNA targets that are directly in contact with OMA-1.

In addition to providing information on where OMA-1 can bind to in mRNA transcripts, these methods will also identify *in vivo* mRNA targets. Future work on investigating regulation of these targets will shine light on the function of OMA-1 and OMA-2 in oocyte maturation. The mRNA targets of OMA-1 identified so far are known to function in diverse developmental pathways. Targets I have identified throughout the work described in this thesis are: *glp-1*, *atg-4.2*, *ets-4*, *cul-4*, *set-6*, and *mex-3*. Others have also identified mRNAs that show a strong OMA-1/2 dependent de-repression of reporter expression oocytes. These mRNAs are: *nos-2*, *zif-1*, *mom-2*, *cdc-25.3*, *rnp-1*, and *rnf-5* (Guven-Ozkan et al., 2010; Jadhav et al., 2008; Oldenbroek et al., 2012; Spike et al., 2014b). **Table 4.1** summarizes the full-range of various functions of OMA-1/2 targets in the germline and embryos.

Table 4.1 List of targets of OMA-1 and their known functions.

Gene	Known Function	References
glp-1	Notch homolog required for germline mitotic to meiosis switch and anterior formation in embryos	(Austin and Kimble, 1987)
atg-4.2	Autophagic cysteine protease homolog	(Wu et al., 2012)
cul-4	Cullin ubiquitin ligase that prevents DNA re-replication	(Zhong et al., 2003)
ets-4	Transcription factor participating in regulation of aging	(Thyagarajan et al., 2010)
mex-3	KH-domain RBP required for anterior cell-fate specification	(Draper et al., 1996)
set-6	Methyltransferase involved in regulation of transcription	(Andersen and Horvitz, 2007)
mom-2	Wnt pathway ligand required for endodermal cell fate specification	(Rocheleau et al., 1997)
nos-2	Nanos homolog required for primordial germ cell development	(Subramaniam and Seydoux, 1999)
rnp-1	RBP required for regulating the switch from spermatogenesis to oogenesis.	(Maeda et al., 2001)
rnf-5	E3 ubiquitin ligase required for migration of cells at distal end of germline	(Didier et al., 2003)
cdc-25.3	Phosphatase controlling oocyte growth	Ashcroft et al., 1998
zif-1	E3 ubiquitin ligase involved in maternal protein degradation pathways	(DeRenzo and Seydoux, 2004)

These mRNA targets are not directly related to the oocyte maturation defect phenotypes observed in *oma-1;oma-2* (RNAi) worms. Their repression might be a general function of OMA-1/2 as a translational repressor. As oocytes develop in the gonad arm, there is no ongoing autophagy, transcription, embryonic cell-fate determination, maternal protein degradation or bulk DNA replication. This can be a reason why some of these mRNAs (*glp-1*, *atg-4.2*, *ets-4*, *cul-4*, *set-6*, *mex-3*, *nos-2*, *zif-1*) are kept in a silent state through OMA-1/2 acting as the major regulator or one of the intercommunicating regulators. However, the phenotype observed in the absence of OMA-1/2 is not intuitively

described by the repression of the mRNA targets of OMA-1/2 that are identified so far.

Model for OMA-1/2 Mediated RNA Regulation

There are multiple ways OMA-1/2 could repress expression from the 3'UTR reporters developed in our study. OMA-1/2 could be directly binding and repressing translation. Consistent with this hypothesis, each of the UTRs contain multiple UA(A/U) motifs recognized by OMA-1 **(Table 4.2)**.

Table 4.2 List of target mRNAs of OMA-1 and the number of UA(A/U) elements in their 3´UTRs

Gene	Number of UA(A/U) elements
glp-1	28
atg-4.2	9
cul-4	10
ets-4	48
mex-3	35
set-6	28
mom-2	20
nos-2	19
rnp-1	14
rnf-5	18
cdc-25.3	12
zif-1	27

OMA-1/2 could also be indirectly regulating transgene expression through competitive or cooperative interactions between multiple regulatory proteins. As shown in Spike et al., there are multiple proteins that associate with OMA-1/2 suggesting that OMA-1/2 are functioning as ribonucleoprotein (RNP) complexes (Spike et al., 2014b; 2014a). The context of OMA-1/2 RNP could affect their regulatory activity. Indeed, regulation of one of the targets of OMA-1/2, *cdc-25.3*, was studied in detail and it was shown that OMA-1 acts antagonistically with a TRIM-NHL protein, LIN-41, to repress *cdc-25.3* in oocytes. *cdc-25.3* encodes for a tyrosine phosphatase that participates in activating oocyte maturation by activating a cyclin dependent kinase, CDK-1 (Kumagai and Dunphy, 1991). Therefore, repression of *cdc-25.3* by OMA-1/2 in oocytes might seem

contradictory to the role of OMA-1 and OMA-2 in promoting oocyte maturation. An antagonistic model between OMA-1 and LIN-41 explained this discrepancy (Spike et al., 2014a). LIN-41 also regulates *cdc-25.3* and prevents precocious meiotic maturation by repressing *cdc-25.3* in immature oocytes. In maturing oocytes, however, LIN-41 must be degraded for proper maturation. This is where OMA-1/2 mediated regulation promotes oocyte meiotic maturation. One possibility is that OMA-1/2 participate in degradation of LIN-41 by activating CDK-1, a factor that is required for elimination of LIN-41 in mature oocytes (Spike et al., 2014a). This can be achieved either by repressing negative target mRNA regulators of CDK-1 or by directly inhibiting LIN-41. This would suggest an interplay between OMA-1 and other proteins to select specific targets for regulation. Detailed analysis of other mRNA targets of OMA-1/2 can provide additional models for the role of translational regulation by OMA-1/2 in oocyte maturation. Another mRNA target of OMA-1/2, *rnp-1*, is involved in proper oogenesis by regulating sperm-to-oocyte switch. Its repression in developing oocytes is not directly related to regulation of oocyte maturation. A similar mechanistic analysis can point to a biological relevance of this target to the oocyte maturation defective phenotype.

A list of OMA-1 interacting proteins has been identified using mass spectrometry. Most abundantly interacting proteins were involved in translational regulation mechanisms. The list contained translational repressors such as IFET-1, an eIF4E-binding protein (Sengupta et al., 2013), translational activators, such

as GLD-2, a poly(A) polymerase subunit (Wang et al., 2002) and other RBPs, such as OMA-2, PUF-5, POS-1, GLD-1 and MEX-3 (Detwiler et al., 2001; Draper et al., 1996; Jones et al., 1996; Lublin and Evans, 2007; Tabara et al., 1999). As discussed in Chapter I, translational repression mechanisms through eIF4E-BP's is a conserved mechanism seen in oogenesis of multiple species. It is likely that *C. elegans* may also employ a similar mechanistic approach in oocyte repression. IFET-1 was shown to be crucial to germline development and was proposed to act as a general translational repressor (Sengupta et al., 2013). IFET-1 was also shown to contribute to repression of targets OMA-1, such as *mom-2* and *zif-1* (Guven-Ozkan et al., 2010; Oldenbroek et al., 2013), further supporting a model of 4E-BP mediated translational repression mechanism by OMA-1 (Spike et al., 2014b). Ribosome profiling in the presence and absence of OMA-1/2 can also highlight the role of these proteins in translational repression by showing the percentage of mRNAs that are repressed via the OMA proteins. Translational activation of maternal mRNAs upon fertilization by poly(A) polymerases is also a commonly seen mechanism of activation mRNAs required for cell-fate specification events in early embryos. In several cases, mRNAs are kept in a stable, deadenylated state in oocytes but are then activated by polyadenylation at the correct developmental time (Jacobson and Favreau, 1983; Mangus et al., 2003). There is no evidence of translational activation of mRNAs by OMA-1 yet; but GLD-2, along with its RNA-binding partner RNP-8, was shown to be a wide-range regulator of oogenesis (Kim et al., 2010). Since OMA-1 is

involved in coordinating oocyte maturation with the cellular events occurring at the oocyte-to-embryo transition, it is possible that OMA-1 may act with GLD-2 to selectively activate translation of mRNAs by changing poly(A) tail length of mRNAs required for completion of maturation or early embryogenesis.

Interaction of OMA-1 with other RBPs that are required for oogenesis and early embryogenesis suggests yet another model of regulation. The findings presented in this thesis suggest that OMA-1 can be acting through clusters of overlapping binding sites. *glp-1* mRNA repression by OMA-1 is supportive of this hypothesis. As shown previously, regulation of *glp-1* is spatially and temporally regulated (Marin and Evans, 2003; Ogura et al., 2003; Lublin and Evans, 2007; Farley and Ryder, 2012). *glp-1* gain of function mutation leads to a tumorous germline due to excessive proliferation of mitotic germ cells (Berry et al., 1997). To prevent ectopic expression of GLP-1, the mRNA is tightly regulated by multiple RNA-binding proteins such as GLD-1, POS-1, PUF-5/6/7 and OMA-1/2 (Farley and Ryder, 2012; Lublin and Evans, 2007; Marin and Evans, 2003; Ogura et al., 2003). In the germline, GLD-1 represses *glp-1* in the syncytial region, PUF-5/6/7 take over around the loop region. Regulation is then handed over to OMA-1 and OMA-2. OMA-1 and OMA-2 repress *glp-1* in late stage oocytes where the other RNA-binding proteins are not present. At the oocyte to embryo transition, OMA-1 is marked for degradation by phosphorylation. This leads to a rapid degradation of OMA-1 at one-cell stage embryo. Thus, as OMA-1 is degraded, it might hand-off the regulation of *glp-1* to embryonic RNA-binding factors. It was

shown that in the embryos, this regulation is via a conserved cluster of overlapping binding sites through which POS-1 and GLD-1 compete for binding. This is plausible as the POS-1 and GLD-1 binding sites that are overlapping with OBMs will be accessible upon OMA-1 and OMA-2 degradation. Interestingly, all novel targets of OMA-1 I identified, except *ets-4*, also have *glp-1*-like cluster of overlapping binding sites in their 3´UTRs (**Figure 4.1)**.

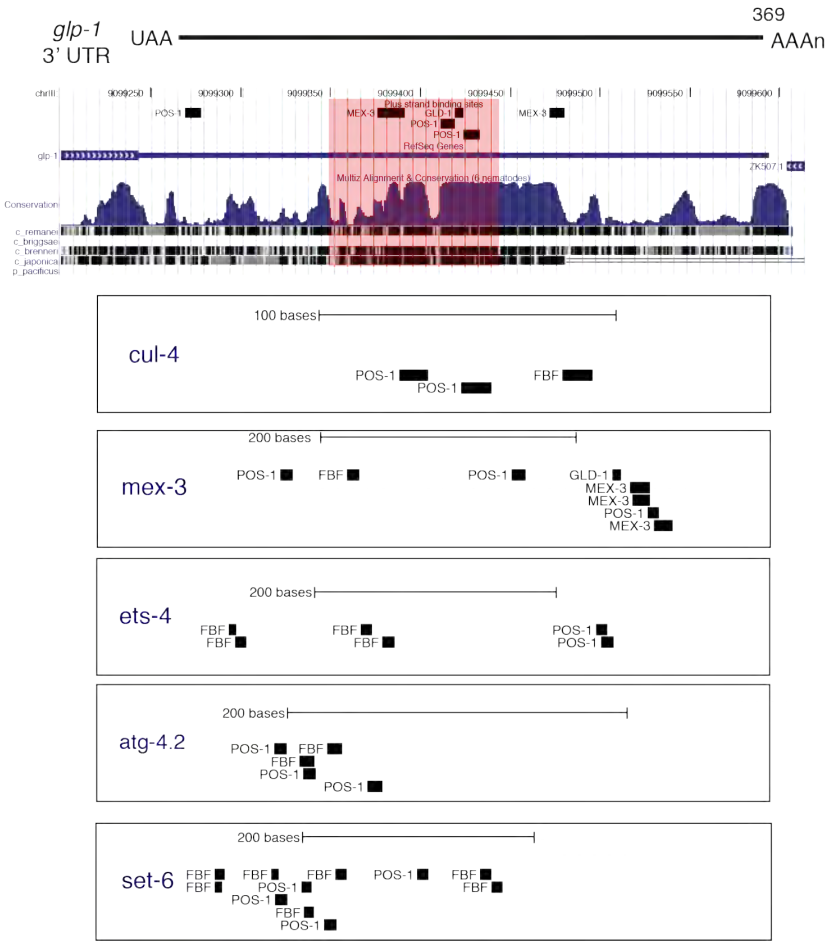

Figure 4.1 Cluster of predicted binding sites for RNA-binding proteins in the targets of OMA-1

glp 1 3´UTR contains a dense cluster of predicted binding sites for FBF, GLD 1, POS 1, MEX 3. Top panel shows the *glp 1* 3´UTR as annotated in the UCSC genome browser. The region highlighted in pink denotes the cluster of predicted RBP binding sites (black bars). Below the image of the *glp 1* 3´UTR are the clusters of binding sites present in the 3´UTR's of the mRNA targets that are repressed by OMA 1/2. All targets, except *ets 4*, show a densely populated cluster. The images are exported from UCSC genome browser created by the Genome Bioinformatics Group of UC Santa Cruz.

Dissecting functionally related targets of OMA-1/2 in oocytes

Using an RNA-centric approach, biotinylated capture oligos can be used to select mRNA targets that show OMA-1 mediated regulation. Similar to the interactome capture assay developed by the Hentze lab (Castello et al., 2012; Marraffini et al., 2013), crosslinking of protein complexes interacting with the specific 3´UTRs prior to immunoprecipitation will allow identification of proteins associated with the transcripts by mass spectrometry. Subsequently, investigating overlapping sets of regulated mRNA targets for functionally related groups of proteins might provide more information on molecular functions of OMA proteins. Such analysis will also help us understand the molecular mechanisms behind OMA-1 gene regulation. For example, if IFET is present as a co-purifying protein for a group of transcripts repressed by OMA-1, it might point to a repression of translational machinery. However, if for another group of transcripts associated with OMA-1 co-purifies with GLD-2, a translational activator, those mRNAs will be candidates for activation during oocyte-to-embryo transition.

To conclude, OMA-1 and OMA-2 likely prevent premature expression of mRNAs involved in embryonic cell fate pattering events prior to fertilization. The relatively relaxed RNA-binding specificity of OMA-1 suggests that it binds to many mRNAs. As such, OMA-1 could be a general repressor of mRNA

translation in oocytes. Alternatively, OMA-1 directed regulation could require additional factors that alter or enhance its RNA binding specificity. In that case, competitive or cooperative interactions between OMA-1 and other proteins that bind overlapping binding sites regulate target mRNAs. Future work will distinguish between these possibilities, and define the mechanism of OMA-1 mediated repression.

Function of OMA-1/2 in Embryos

OMA-1 and OMA-2 are also abundant in one-cell embryos but their function in embryos has not been studied in detail. A model for the role of OMA-1/2 in embryos suggest these proteins act as transcriptional repressors by sequestering TAF-4, an essential component of transcription machinery, in the cytoplasm (Guven-Ozkan et al., 2008). However, high-throughput sequencing experiments, such as global transcription activity profiling, to determine the percentage of OMA-1 mediated transcriptional repression have not been performed yet. There is also evidence that OMA-1/2 may act as translational repressors in embryos as well. *mei-1*, a katanin subunit, is repressed in embryos for proper mitotic spindle assembly (Clark-Maguire and Mains, 1994b; 1994a; Li et al., 2009). In one-cell embryos, OMA-1 and OMA-2 may directly be involved in repressing *mei-1*. It is intriguing that OMA-1 might have different roles in oocytes or embryos and might regulate different targets in different cellular contexts. It is possible to isolated oocytes and one-cell embryos separately to assess different

functions of OMA-1 in different developmental environments during oocyte-to-embryo transition. For enrichment of one-cell embryos Piano and Rajewsky developed a method that enriches for one-cell embryos expressing OMA-1 (Stoeckius et al., 2009). A GFP reporter strain harboring the endogenous promoter of *oma-1* fused to the *oma-1* coding sequence which is fused to GFP (P(*oma-1*)::*oma-1*::GFP:: *oma-1* 3´UTR) is available and OMA-1 encoded by the strain is functional. Stoeckius et al. used this strain to collect precisely one-cell staged embryos using fluorescence activated cell sorting (eFACS) as highest level of GFP seen in mature oocytes and one-cell embryos. They have analyzed, by flow cytometry, mixed staged embryos extracted from adult hermaphrodites of the *OMA-1::GFP* strain. A population of embryos expressing high GFP signals was selected for sorting in FACS. This yielded 70% enrichment in one-cell staged embryos. RIP-SEQ experiments can then be applied to these embryos to characterize the DNA and/or mRNA targets.

A disadvantage of studies that identify mRNAs that associate with RNA-binding proteins is that they do not show which targets are direct targets that are regulated by OMA-1. They can only show where OMA-1 can bind to in the mRNA but not necessarily regulate. To identify and validate functionally regulated mRNAs, reporter studies are crucial. In Chapter III, I discuss in detail how we improved the technology to generate reporter strains and how this technology, combined with the ease of performing RNAi studies in *C. elegans*, led to identification of five new regulatory targets of OMA-1. Other interacting partners

were also identified along with an interesting sperm expression pattern. These patterns are discussed in detail in Chapter III.

Library MosSCI

In Chapter III, we showed that adapting the MosSCI method for generating single copy integrated transgenic strains to a library format increased our rate in generating reporter strains. The success rate of transgenesis is limited by the number of successful injections and by the extent of transgene integration. The rate of successful injections will vary between different injectors. The recent development of a microfluidic device to automate the injection procedure could help improve the number of successful injections (Gilleland et al., 2010). In this work, we used the direct insertion method of MosSCI. The extent of integration can be improved through the use of different promoters driving Mos1 transposase expression. For example, use of the *eft-3* promoter has been shown to increase the rate of transformation presumably by increasing the extent of Mos1 transposon excision (Frøkjaer-Jensen et al., 2012). With this improvement, fewer injections may be sufficient to generate a number of strains after random integrations at the heat-shock step.

Obtaining transgenic strains at an increased rate will be advantageous in multiple ways. Library injection may be adapted to CRISPR-based approaches to make targeted mutations (Friedland et al., 2013; Jinek et al., 2012; H. Kim et al., 2014). In an endogenous genomic locus of interest, a set of randomized

insertions/deletions can be introduced through injection of a library of guide RNAs targeted for that locus. Using multiple CRISPR guides per injection can help ensure a mutation in the gene of interest, as has recently been shown in zebrafish (Gagnon et al., 2014).

In this study, we used library MosSCI to make 3'UTR reporters but this method could easily be adapted to make different promoter reporters or protein fusions to help define other aspects of regulatory biology, including transcription regulation and protein modification. A mutagenesis or deletion library analysis would help identify key cis-regulatory elements that control transcription regulation patterns critical to somatic differentiation in later stages of embryogenesis, after zygotic gene activation. Library MosSCI can also be used to rapidly generate mutants within a single UTR of interest and screen mutant strains to help map functional elements in a regulatory region of a UTR of interest. Another potential application of this technology could derive from systematically analyzing protein variants. Transgenic strains can be used to rescue a mutant phenotype by overexpressing a wild-type copy of the mutant. In such a case, injecting a library of overlapping fragments of the gene simultaneously could help identify the fragment that is minimally sufficient for rescue.

Concluding Remarks

The ability to generate transgenic strains in high yield will enable improved functional mapping of regulatory interaction networks between maternal mRNAs and RNA-binding proteins. Methods like CLIP, RIP-SEQ and PAR-CLIP identify interacting partners *in vivo* but may identify interactions that have no regulatory consequence. There are instances where an RNA-binding protein can play an active role in regulating a transcript through a binding site. In this case, the target site is necessary and sufficient for regulation. In other cases, the effect of an RNA-binding protein might be indirect or context dependent. *In vivo* studies with reporter strains carrying regulatory elements is necessary to distinguish between interactions of RNA-binding proteins that have a relevance to the regulation of an mRNA or not. As we have done in this work, the study of transgenic reporter strains carrying different *C. elegans* 3´UTRs can be done by RNAi screening. High-throughput RNAi screens could identify additional RNA-binding proteins that regulate these reporter transgenes. Once regulatory partners are identified, the necessity and sufficiency of target sites can be tested using library MosSCI to identify binding sites that are functionally important. Ultimately, the utility of large data sets that yield high resolution contact maps will be defined by their predictive power in functional studies. In order to keep pace, new technology to improve the output of functional studies in live animals is needed. My work here

demonstrates a simple strategy to improve the throughput of *C. elegans* single copy transgene strain production, a key first step towards this goal. Using this strategy, we can expand on making more reporter strains carrying 3´UTRs bearing clusters of binding sites and identify more novel targets of OMA-1 regulated by OMA-1. Alternatively, we can expand on generating strains carrying mutations in OMA-1-binding motifs of various RNA targets to identify functionally relevant binding sites. Ultimately, identifying the full range of direct targets of OMA-1/2 and understanding how they are regulated will illuminate mechanisms of regulation during oocyte-to-embryo transition.

When we started working on understanding the roles of OMA-1 and OMA-1 in regulating oocyte to embryo transition, their roles were not well-defined. It was known that these proteins are redundantly required for oocyte maturation (Detwiler et al., 2001); however, the molecular mechanisms behind regulation of oocyte maturation was poorly understood. *nos-2*, *mei-1* and *zif-1* mRNAs were proposed to be regulated by OMA-1 and OMA-2 in developing oocytes (Jadhav et al., 2008; Li et al., 2009; Guven-Ozkan et al., 2010). This pointed towards the importance of the role of these proteins as post-transcriptional regulators in oocytes. We therefore set out to identify more mRNA targets of OMA-1 and OMA-2 and provide a mechanistic overview of how these proteins regulate oocyte maturation. As of today, us and others have identified and validated 12 mRNA targets regulated by OMA-1 and OMA-2 (Kaymak and Ryder, 2013, Spike et al., 2014b). These targets were involved in diverse biological pathways hinting

to a role of OMA-1 and OMA-2 as general repressors during oocyte-to-embryo transition. In addition to novel mRNA targets, OMA-1 interacting proteins were also identified (Spike et al., 2014b). The identification of eIF4E-BP's as translational repressor proteins interacting with OMA-1 provides a model whereby OMA-1 interacts with other proteins to achieve its target specificity and lead to a translational repressor. Expanding on how the novel targets of OMA-1 are regulated mechanistically can now help us shine light on how OMA-1 and OMA-2 contribute to proper oocyte maturation and timely transitioning into and embryo.

CHAPTER V: APPENDICES

Appendix A: Library MosSCI Mutagenesis

Significant background and results

We wondered if we could use library MosSCI to screen for mutants within a single UTR of interest. We chose to study the *C. elegans* Notch receptor homolog, *glp-1*. *glp-1* mRNA is present throughout the germline and embryos but the protein is expressed only at the distal end of the germline where it regulates the mitosis to meiosis switch and the anterior cells of the four-cell embryo where it specifies mesodermal cell fates (Austin and Kimble, 1987; Crittenden et al., 1994; Evans et al., 1994).

We are interested in *glp-1* because at least five RNA-binding proteins (GLD-1, POS-1, MEX-3, PUF-5/6/7, OMA-1/2) that repress the *glp-1* mRNA are known (Farley and Ryder, 2012; Lublin and Evans, 2007; Marin and Evans, 2003; Ogura et al., 2003; Pagano et al., 2009; Ryder et al., 2004). The region of the UTR sequence that is sufficient for regulation has been mapped. When in vitro transcribed, capped and polyadenylated mRNAs encoding ß-galactosidase and containing deletions of the *glp-1* 3'UTR were injected into worm gonads, a region of the UTR termed the spatial control region (SCR) was found to be necessary to confer the endogenous GLP-1 pattern of expression in the LacZ reporter (Evans et al., 1994). To determine which region or regions of the SCR were sufficient for regulation, fragments of the SCR were added to an unregulated 3'UTR (*unc-54*) and the reporters were injected in the same LacZ

reporter design. A 34-nucleotide sub-region of this region was found to be sufficient to generate the *glp-1* translation pattern. This region contained repression and de-repression elements found by mutational analysis in the LacZ reporters (Marin and Evans, 2003). The binding sites within this region that are sufficient for the repression have also been mapped. For example, POS-1 and GLD-1 repress *glp-1* 3'UTR through a conserved site of overlapping binding sites (Farley and Ryder, 2012). We wanted to examine this conserved site that has clusters of binding sites for the proteins repressing *glp-1* mRNA to identify a mutation in a binding site that is sufficient to change the reporter expression pattern.

We prepared a library of forty germline GFP reporters containing single nucleotide substitutions of the *glp-1* 3'UTR. We selected a contiguous forty-nucleotide region containing well-characterized binding sites for GLD-1 and POS-1. Every single nucleotide in this stretch was mutated. Adenosines were mutated to cytidines, and thymidines were mutated to guanosines, or vice versa. Through ~70 injections, we recovered strains that had incorporated six of the forty different mutations. These strains are listed in **Table 5.1**.

Table 5.1 List of *glp-1* 3´UTR reporter strains with introduced mutations

UTR library	Independent lines
glpA	t198g
glpB	c191a
glpC	t169g
glpD	t162g
glpE	g176t
glpF	g200t

We compared the GFP expression patterns of the reporter strains bearing a different point mutation in the *glp-1* 3'UTR to each other and to the wild-type reporter carrying no mutations in the UTR **(Figure 5.1)**. There were no apparent expression differences observed between the recovered *glp-1* mutant reporters and the wild-type GFP reporter, suggesting that the mutations do not disrupt a functional regulatory element. By contrast, mutations that target GLD-1 or POS-1 binding sites—previously generated by single reporter mosSCI—led to large changes in the germline and embryo (Farley and Ryder, 2012). We conclude that library mosSCI can be used to rapidly generate mutant strains to help map

functional elements in a regulatory region, but suggest that larger mutations may be necessary to complete an efficient scan.

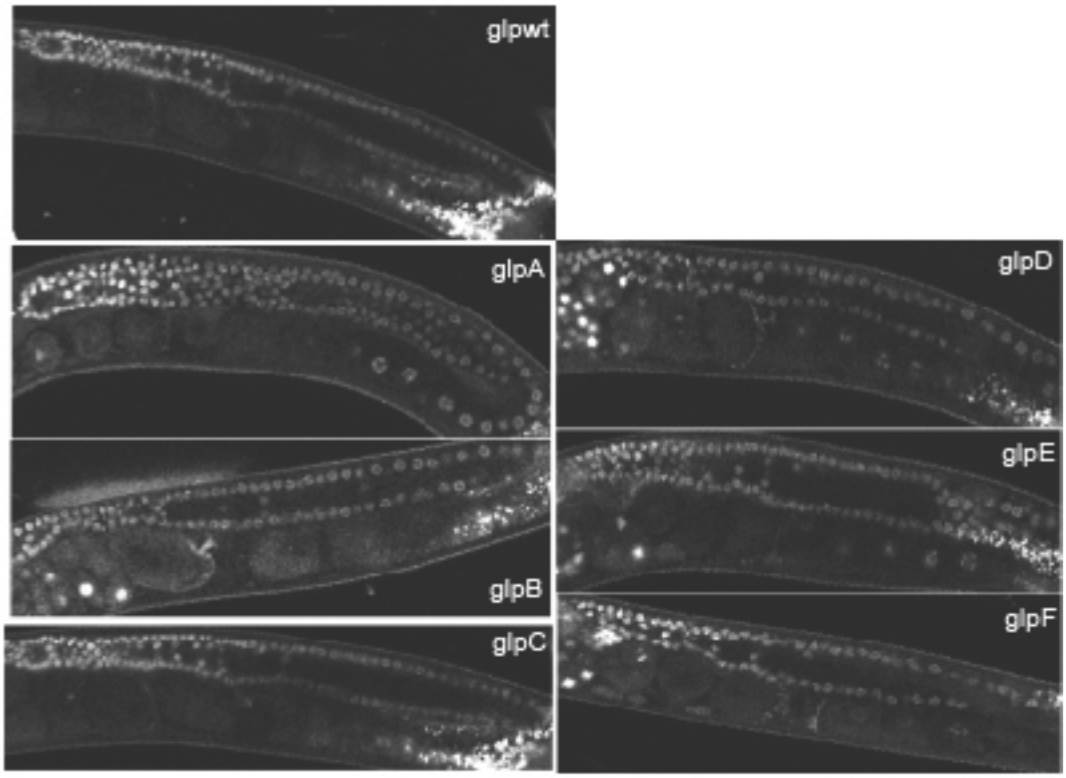

Figure 5.1 GFP expression analysis of *glp-1* mutants

Integrated strains that contain single point mutations in the *glp 1* 3´UTR did not show any difference in GFP expression patterns.

Experimental procedure

Library MosSCI was done as described in detail in Chapter III.

Single point mutations were inserted into *glp-1* 3´UTR using standard Quickchange protocol.

Appendix B: Analysis of Cluster of Binding Sites in *neg-1* 3´UTR

Significant background and results

The work described in this appendix appeared as part of the publication by Ahmed Elewa (Elewa, A., Shiriyama, M., **Kaymak, E**, Harrison, P.F., Powell, D.R., Du, Z., Chute, C.D., Woolf, H., Yi, D., Ishidate, T., Srivnivasan, J., Bao, Z. Beilharz, T.H., Ryder, S.P., Mello, C.C. (2015) POS-1 promotes endo-mesoderm development by inhibiting the cytoplasmic deadenylation of *neg-1* mRNA. *Dev. Cell*). Ahmed in the Mello lab characterized the *neg-1* gene (Elewa et al., 2015). *neg-1* was identified in a genetic screen as a suppressor of *pos-1*. *pos-1* null mutants show a gutless phenotype and *neg-1* was found to suppress the gutless phenotype and result in a properly differentiated endodermal and pharyngeal tissue in embryos (Elewa et al., 2015).

Analysis of the *neg-1* 3´UTR showed that there is a cluster of overlapping binding sites for MEX-5, MEX-3 and POS-1 (named the RBP cluster) **(Figure 5.2A)**. RBP cluster is similar to the cluster we observed in the *glp-1* 3´UTR. I was involved in determining the contribution of these binding sites to the binding affinity of POS-1, MEX-5 and MEX-3. For this purpose, I first compared the binding of POS-1, MEX-5 and MEX-3 to the wild-type sequence with of the RBP cluster and a mutated version of this cluster. The mutated cluster contained disrupted binding sites for POS-1, MEX-5 and MEX-3 **(Figure 5.2B, C)**. Upstream the RBP cluster there is a polyU sequence which is the predicted binding site for MEX-5. This region is named M5B. I did a fluorescence

polarization assay to determine the binding affinity of MEX-5 to M5B. As compared to the RBP cluster, MEX-5 bound weakly to M5B **(Figure 5.2C, right panel)**.

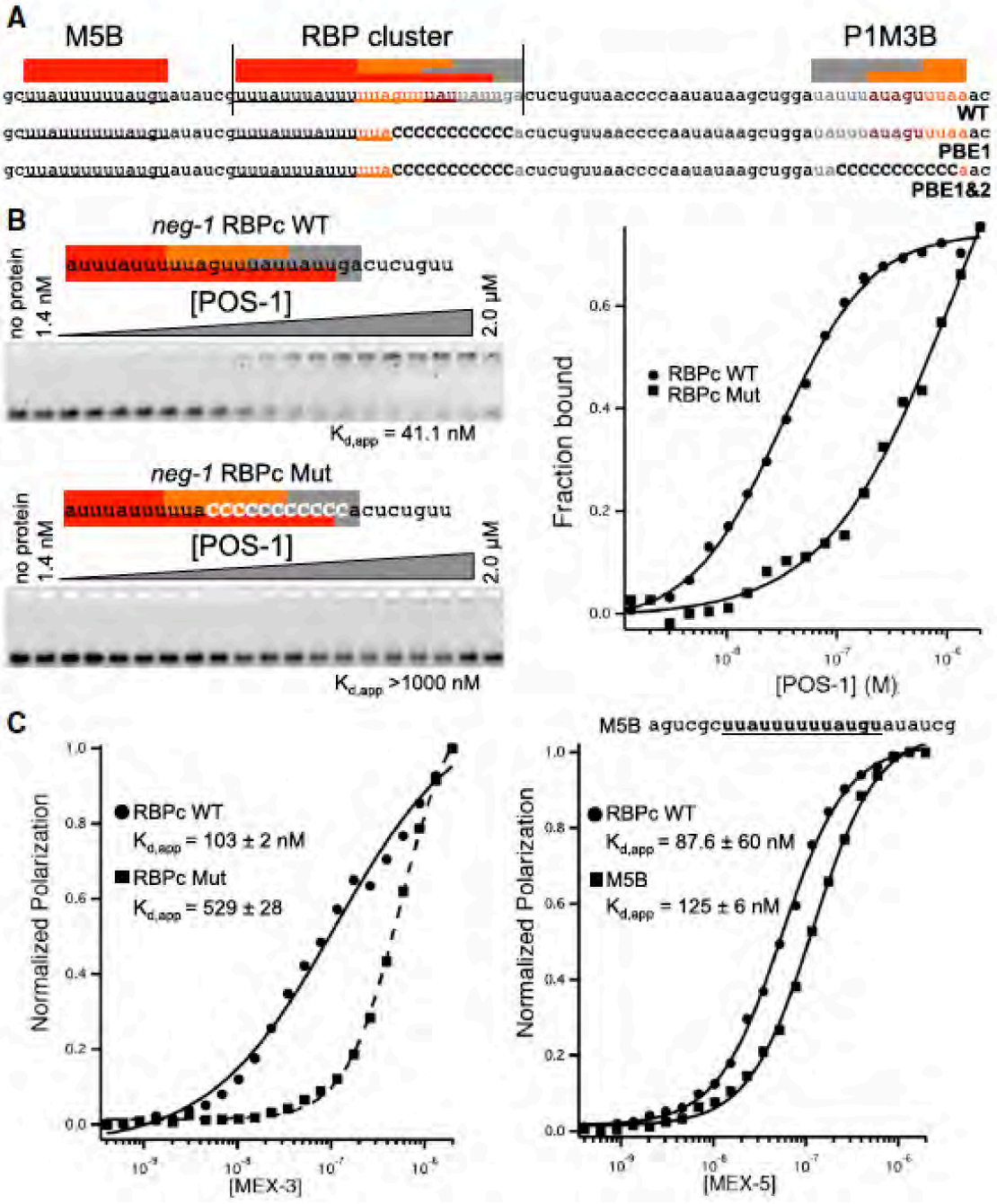
A
M5B
RBP cluster
P1M3B
WT
PBE1
PBE1&2
B
neg-1 RBPc WT
no protein
1.4 nM
[POS-1]
2.0 µM
K d,app = 41.1 nM
neg-1 RBPc Mut
K d,app >1000 nM
Fraction bound
RBPc WT
RBPc Mut
[POS-1] (M)
C
M5B agucgcuuauuuuuuauguauaucg
Normalized Polarization
RBPc WT
K d,app = 103 ± 2 nM
RBPc Mut
K d,app = 529 ± 28
[MEX-3]
K d,app = 87.6 ± 60 nM
M5B
K d,app = 125 ± 6 nM
[MEX-5]

Figure 5.2 Electrophoretic gel shift assays and fluorescence polarization assays of POS-1, MEX-5 and MEX-3 show that all bind RBP

A. Nucleotide sequence of the *neg 1* 3´UTR showing cluster of binding sites for POS 1, MEX 5 and MEX 3.
B. EMSAs show that POS 1 binds to RBPc WT with an apparent affinity of 41 nM. Mutating the RBP sequence reduces the binding affinity dramatically.
C. Fluorescence polarization data shows that MEX 3 binds RBPc. MEX 5 binding, on the right, shows that MEX 5 also can bind RBP WT and M5B.

In vivo reporter studies with mutations in the MEX-5 and POS-1 binding sites within the RBP cluster showed that these sequences have functional relevance. Accordingly, we wanted to test whether POS-1 and MEX-5 compete each other. Competition assays showed that MEX-5 binds favorably to RBPc than POS-1 **(Figure 5.3)**.

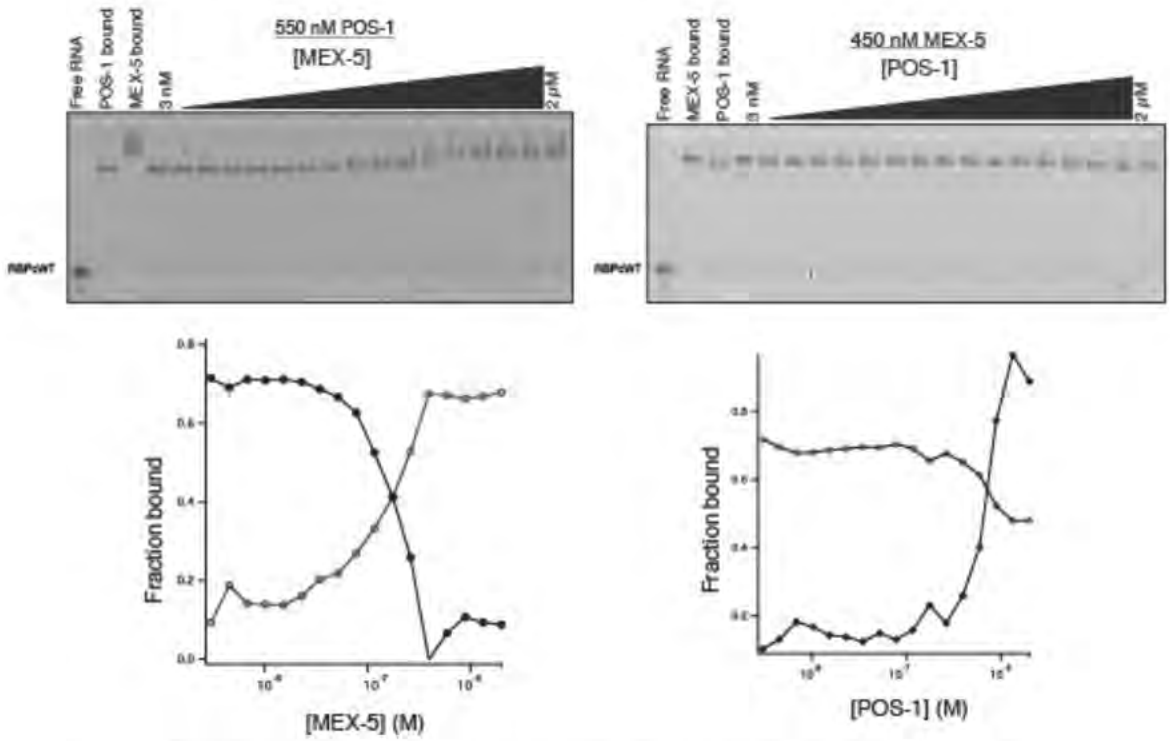

Figure 5.3 In vitro competition assay between POS-1 and MEX-5 shows that MEX-5 competes POS-1

In the top gel, MEX 5 (236 350) is titrated into a fixed concentration of POS 1 (80 180). The bottom gel shows the competition whereby POS 1 (80 180) is titrated into a fixed concentration of MEX 5 (236 350). The fixed concentration of the proteins is chosen to be a concentration at which the proteins are 70% bound. Below the gels are the quantifications of the competition experiments. The plot on the left shows the fraction of bound RNA as MEX 5 is titrated. Open circles denote MEX 5 bound to RNA and the filled circles denote POS 1 bound to RNA. The plot on the right shows the fraction of bound RNA as POS 1 is titrated. Open circles denote MEX 5 bound to RNA and the filled circles denote POS 1 bound to RNA.

Downstream the RBP cluster, there is another region which contains overlapping binding sites for POS-1 and MEX-3. This region was named P1M3B. We then tested whether these proteins can bind to this cluster. Gel shift assays showed that both proteins bind P1M3B.

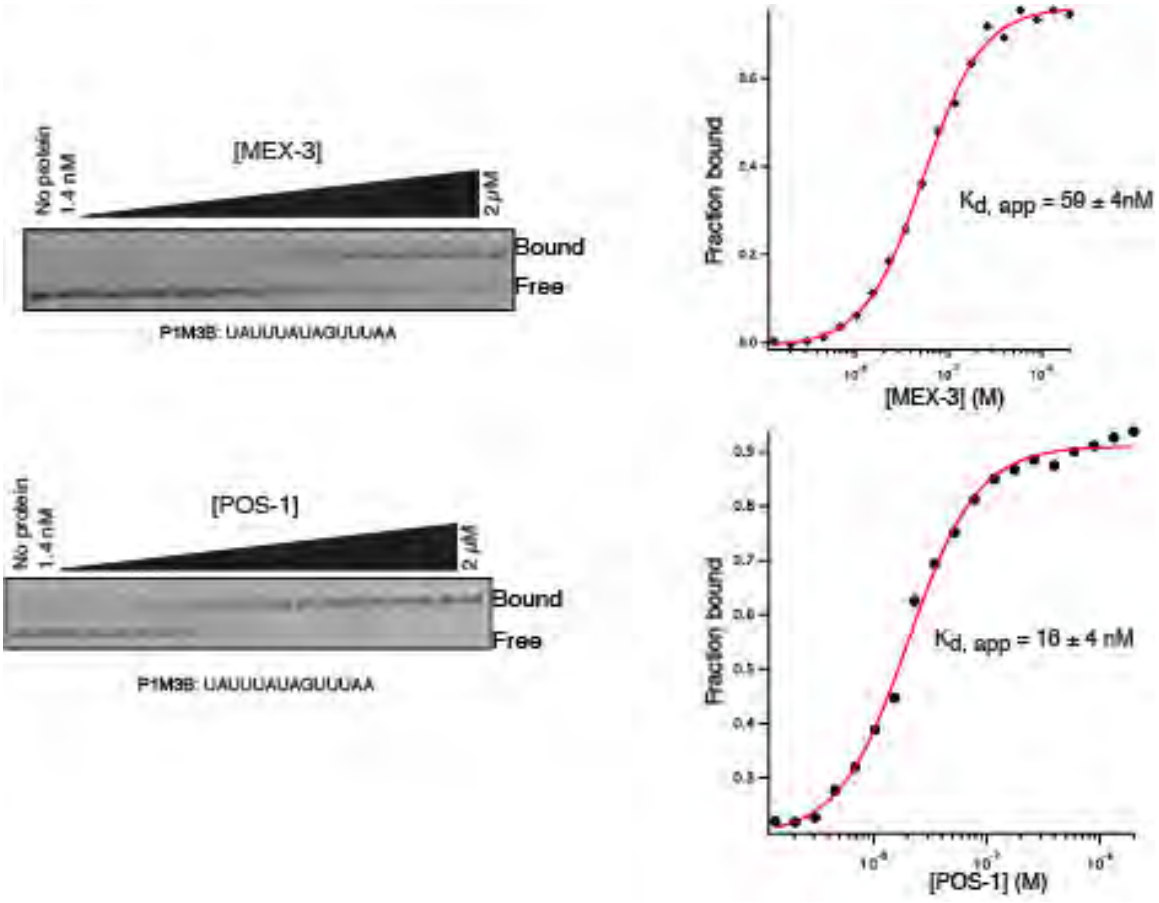

Figure 5.3 MEX-3 and POS-1 bind P1M3B in *vitro*

Electrophoretic mobility shift assays for MEX 3 and POS 1 are shown on the left. The graph of fraction bound against protein concentration is on the right showing that MEX 3 binds with a 50 nM affinity and POS 1 binds with a 16 nM affinity.

Experimental procedure:

Fluorescence anisotropy and electrophoretic mobility shift assays using purified recombinant MBP-tagged POS-1 (80-180), MEX-3 (45-205) and MEX-5 (236-350) were done as described in Farley et al. 2008, Pagano et al. 2009 and Pagano et al. 2007, respectively. All RNA oligonucleotides used in this study were chemically synthesized and fluorescently labeled at the 3´end with fluorescein amidite (FAM) by Integrated DNA Technologies (IDT). Competition assays are set up similar to the EMSA assays as described in Farley et al, 2012. 550 nM of POS-1 (80-180) or 450 nM MEX-5 (236-350) was added to the RNA equilibration buffer to get 70% RNA bound complex. Then the corresponding competing protein was titrated to the reaction mixture at varying concentrations. After 3 hours of equilibration, the reaction mixture was run on a 5% native polyacrylamide gel in 1X TB for 3 hours, at 120V. Quantifications were done by determining the pixel intensity of the RNA species bound by protein relative to the pixel intensity of total RNA species to give the fraction bound of RNA. The pixel intensities of each band were determined and background corrected by using Image Gauge (Fujifilm, Tokyo, Japan).

Appendix C: An RNA-Centered Approach to Determine Positively Interacting RNA-Binding Proteins and RNA Targets

Significant background and results

The work described in this appendix appeared as part of the publication by Alex Tamburino: Tamburino, A.M., **Kaymak, E.**, Shrestha, S., Ryder, S.P. Walhout, A.J.M. (2015) PRIMA: an RNA-centered protein-RNA interaction mapping assay (submitted).

Alex in Marian Walhout's lab has developed a technology to identify interacting RNA-binding proteins and their cognate 3´UTR targets. Since physical interaction between proteins and RNA result in a functional regulation, a secondary assay was necessary to validate the positive hit that are generated by the high-throughput assay, PRIMA (Tamburino et al., 2015) is a yeast based fluorescence assay that relies on increased translational efficiency of GFP upon a positive interaction between an RNA-binding protein and a 3´UTR sequence. Since we had generated 3´UTR reporter strains using library MosSCI, we wanted to combine PRIMA with our transgenic lines. PRIMA allowed us to prioritize the list of RNA-binding proteins we would like to test in an RNAi screen. I was involved in doing the RNAi experiments. As a proof of concept, Alex decided to use *glp-1 3´UTR* and *nos-2* 3´UTR, as these UTRs were studied extensively. The highest scoring interactions for the *glp-1* 3´UTR included the RNA-binding proteins: FBF-1/2, PUF-3, PUF-5 and POS-1. PUF-5 and POS-1 were already shown to repress *glp*-1 (Farley and Ryder, 2012; Lublin and Evans, 2007). In

contrast, PUF-3 was identified as a novel regulator of *glp-1* **(Figure 5.5A)**. Two of these, MEX-3 and POS-1, have already been shown to regulate *nos-2*. We were able to recapitulate this result *in vivo* The highest scoring interactions for the *nos-2* 3´UTR included the RNA-binding proteins: MEX-3, POS-1, HRP-1, R09B3.2, ZTF-4 and PIE-1. One of these, MEX-3, has already been shown to regulate *nos-2* (Pagano et al., 2009) . We were able to recapitulate this result *in vivo* **(Figure 5.5B).**

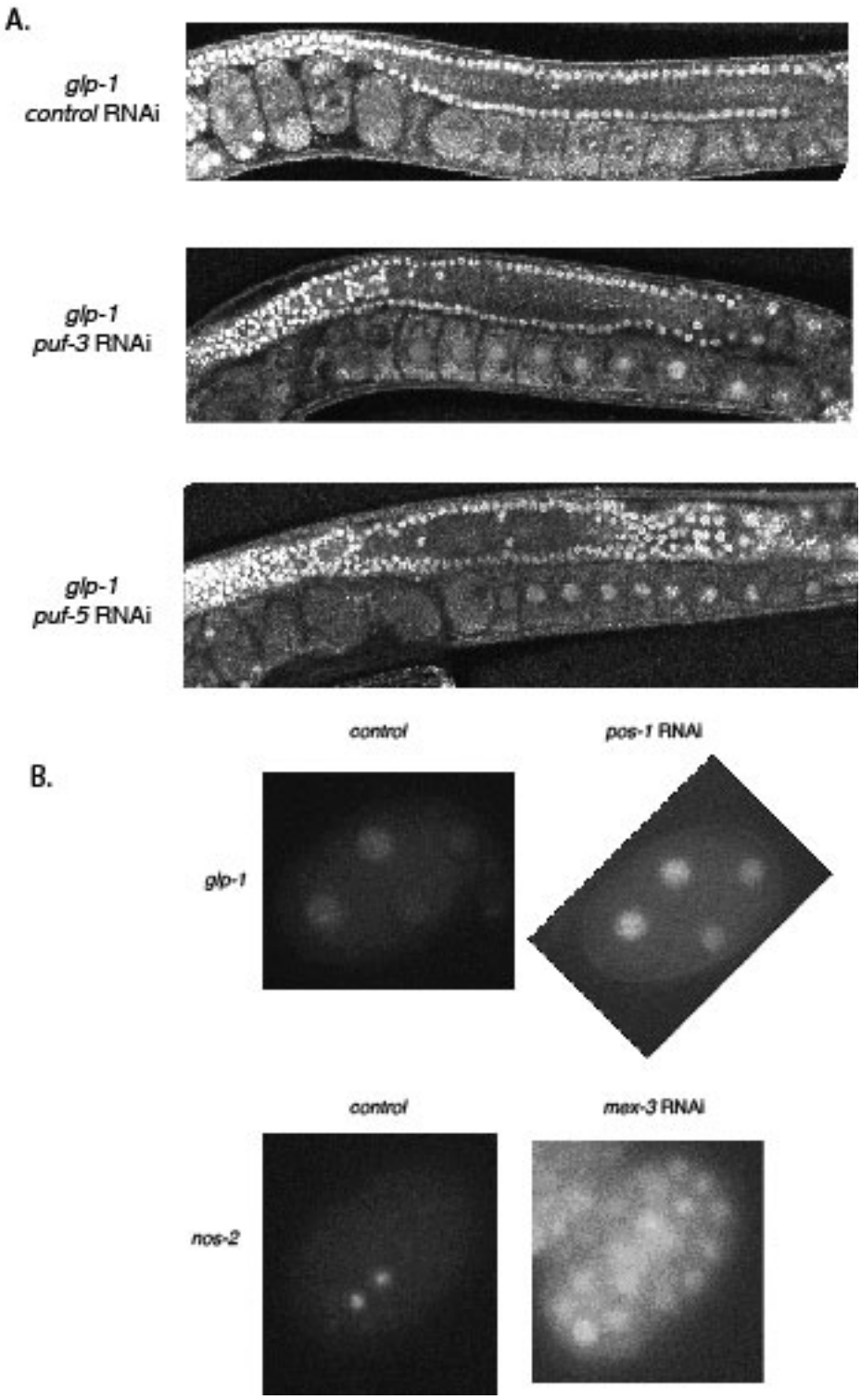
A.
glp-1
control RNAi
glp-1
puf-3 RNAi
glp-1
puf-5 RNAi
B.
control
pos-1 RNAi
glp-1
control
mex-3 RNAi
nos-2

Figure 5.5 RNAi experiments to test the in vivo regulation of *glp-1* and *nos-2* 3´UTRs

A. Using transgenic strains expressing GFP under the control of *glp 1* 3´UTR and *nos 2* 3´UTR, bound RNA binding proteins were tested for regulatory activity using RNAi knockdown. *puf 3* and *puf 5* RNAi resulted in increased expression of GFP in immature oocytes of *glp 1* UTR strain.
B. In the embryos, *pos 1* RNAi resulted in ectopic GFP expression in the cells of four cell embryo of the *glp 1* UTR strain. *mex 3* RNAi resulted in ectopic GFP expression in the cells of 28+ cell embryo of the *nos 2* UTR strain.

Experimental procedure

RNAi knockdown: The knockdowns were performed using the RNAi feeding method as described I Kamath et al., 2003. The open reading frames (ORFs) into the RNAi feeding vector construct L4440 and transformed into HT115(DE3) cells. The transformed colonies were grown to OD_{600} = 0.4 and induced with isopropyl 1-thio-β-D-galactopyranoside (IPTG) at a final concentration of 0.4 mM for 4 hours. After induction the 50 ml cultures were concentrated 10- fold and 50µl of the culture was added onto NGM plates containing 1mM IPTG and 100 µg/ml Ampicillin. After bleaching worms, eggs were plated onto these plates and kept at 25°C for 2 days before imaging. HT115 strain bacteria transformed with the empty vector L4440 was used as the control RNAi.

Imaging of worm strains: Worms were placed in 0.4 mM levamisole on to 2% agarose pads. Emryo dissections were done in M9 solution. DIC and GFP fluorescence images were taken on Zeiss Axioscope 2 plus microscope (Carl Zeiss, Jena, Germany) using an oil-immersion 40X objective. Confocal images were taken under 40X magnification using Leica DMIRE2 microscope (Leica, Wetzlar, Germany).

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