Post-transcriptional gene regulation in developing oocytes of *C. elegans*

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Summary

Proper oocyte maturation and oocyte-to-embryo transition (OET) are critical processes during animal development. In metazoans, transcription halts during oocyte maturation and remains silenced until the activation of the embryonic genome. During this period, maternally deposited mRNAs and proteins regulate development, and gene expression is mainly regulated at post-transcriptional level. After fertilization, there is a shift in control of developmental processes from maternally provided gene products to those synthesized by the embryo itself. This shift is called oocyte-to-embryo transition (OET) and is accompanied by embryonic genome activation (EGA). In several organisms this transition is also called maternal-to-zygotic transition (MZT). As during oogenesis gene expression is mostly regulated by post-transcriptional means, RNA binding proteins (RBPs) and other RNA regulatory proteins play major roles. Here we used the nematode, *C. elegans* as an experimental model to study how certain RBPs regulate specific mRNAs during oogenesis and OET.

In *C. elegans*, the RBPs OMA-1, OMA-2 and LIN-41 function in the germline during oocyte maturation and OET. OMA-1 and OMA-2 are functional homologs and are henceforth referred to as OMA. LIN-41 and OMA proteins bind to a large number of mRNAs in the developing oocytes and have been shown to regulate their expression at the post-transcriptional level. While LIN-41 and OMA proteins bind mRNAs via different RNA elements, some mRNA targets are regulated by both LIN-41 and OMA. LIN-41 also regulates mRNAs independently without participation of OMA proteins. However, how LIN-41 and OMA proteins regulate their target mRNAs is not fully understood.

In order to study how LIN-41 and OMA proteins regulate mRNAs, their protein partners were previously identified by immunoprecipitation followed by mass-spectrometry. Here we performed a functional screen using two reporter strains where GFP is regulated by 3′ UTRs containing binding sites for LIN-41 (LIN-41 response elements, LRE) or OMA (OMA binding sites, OBS). We call these reporters LRE and OBS reporter respectively. The LRE reporter is regulated by LIN-41 via direct binding without the involvement of OMA proteins. However, the OBS reporter is regulated by both OMA and LIN-41 proteins. Thus, LIN-41 regulates mRNAs by via indirect binding.

By knocking down the candidate protein partners, their role in LIN-41 and OMA mediated mRNA regulation was assessed. Our screen did not identify any specific complex or pathway involved in the regulation of the LRE reporter. We found that proteins belonging to the CCR4-NOT complex and the mTORC1-4EBP pathway regulate the OBS reporter. The CCR4-NOT complex appears to have a minor role in repression of the OBS reporter via deadenylation. Our results indicate that the OBS reporter is mainly repressed via recruitment of eIF4E binding protein (4EBP), IFET-1. OMA and LIN-41 also associate with AAK-1 and ATX-2, which are known to have mTORC1 inhibitory activity. The OBS reporter is activated upon knockdown of *aak-1* and *atx-2*. In addition, inactivation of mTORC1 signaling prevented translation of OBS reporter even in the absence of OMA. These observations suggest that active mTORC1 signaling is required for derepression of the OBS reporter.

Generally, mTORC1 complex is known to function as a global regulator of cap-dependent translation. We therefore checked if translation of other spatially regulated mRNAs in the *C. elegans* germline is affected by mTORC1 activity. We found that activation of mTORC1 does not have a prominent effect in translation of other mRNAs that were tested. Taken together, our results suggest that OMA and LIN-41 proteins recruit 4EBP and mTORC1 inhibitors to specific mRNAs to regulate their expression.

Abbreviations

3′ UTR	3 prime untranslated region
4EBP	eIF4E binding protein
4EHP	eIF4E homology protein
5′ UTR	5 prime untranslated region
A	
Ago2	Argonaute 2
C	
C. Elegans	*Caenorhabditis elegans*
cDNA	Complementary DNA
D	
D. melanogaster	*Drosophila melanogaster*
D. rerio	*Danio rerio*
DIC	Differential interference contrast
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
dsRNA	Double stranded RNA
DTC	Distal tip cell
E	
E. Coli	*Escherichia coli*
eIF4E	Eukaryotic initiation factor 4E
eIF4G	Eukaryotic initiation factor 4G
G	
gDNA	Genomic DNA
GFP	Green fluorescent protein
I	
IP	

	Immunoprecipitation
IPTG	Isopropyl β-D-1 thiogalactopyranoside
L	
LB	Lysogeny broth
LRE	LIN-41 response element
M	
M. musculus	*Mus musculus*
MAPK	Mitogen-activated protein kinase
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
mTORC 1/2	Mammalian target of rapamycin complex 1/2
MZT	Maternal-to-zygotic transition
N	
NGM	Nematode growth media
O	
OBS	OMA binding site
OET	Oocyte-to-embryo transition
ORF	Open reading frame
P	
P cells	Precursor cells
PABP	Poly(A) binding protein
PI3K	Phosphoinositide 3-kinase
qPCR	Quantitative polymerase chain reaction
R	
RBP	RNA binding proteins
RISC	RNA induced silencing complex
RNA	Ribonucleic acid

RNAi	RNA interference
RNP	Ribonucleoprotein particle
RT enzyme	Reverse transcriptase enzyme

S

S. purpuratus	*Strongylocentrotus purpuratus*
siRNA	Small interference RNA

1. Introduction

1.1 Oogenesis – Formation of the female gamete

The process of oocyte maturation is crucial to form fully functional oocytes that can be fertilized to give rise to embryos that are competent to produce healthy new individuals (Leung, 2004). Meiotic division of precursor germ cells and accumulation of cytoplasm are two major events that occur during oogenesis. During meiosis, two rounds of cell division will produce a haploid egg containing one copy of each chromosome. Concurrently, a large volume of cytoplasm will be accumulated. The cytoplasm contains components that are crucial for early embryonic development (Maine, 2013). Furthermore, an important characteristic of oocyte maturation is growth. Proper oocyte growth ensures that all cellular organelles and factors that are essential for early embryonic development are accurately inherited from the mother (Spike et al., 2014). These factors include ribosomes, endoplasmic reticulum, golgi apparatus, mitochondria, maternal mRNAs, and gene products that are essential for early embryonic development.

1.1.1 Oocyte maturation

One of the final steps in the development of an oocyte is the process of maturation and it must occur prior to ovulation and fertilization of the gamete (Jamnongjit & Hammes, 2005). The programs underlying the maturation process are nuclear maturation, epigenetic maturation, and cytoplasmic maturation. Meiosis occurs exclusively in gametes to reduce the number of chromosomes from a diploid (2N) to a haploid (N) number and is initiated during early development (Leung, 2004). In almost all animals, oocytes arrest at prophase I of the first meiotic division. The prophase arrest will be released when the oocyte has undergone extensive growth, allowing the resumption and completion of meiosis prior to fertilization. This process is referred to as nuclear maturation and is dependent on appropriate stimulatory signals that must be tightly regulated to ensure that only fully grown oocytes of the highest quality mature (Detwiler et al., 2001; Spike et al., 2014). During nuclear maturation, epigenetic changes results in chromatin modifications that regulate gene expression patterns (Leung, 2004). Cytoplasmic maturation involves accumulation of cellular organelles, mRNA and proteins that are essential for fertilization and early embryonic development. The cytoplasmic and/or membrane changes during this process allows fertilization to occur (Detwiler et al., 2001).

1.2 Oocyte-to-embryo transition (OET)

In all animals, transcription halts during oocyte maturation and remains silenced until the zygotic genome is activated after fertilization. During the progression of meiotic prophase, chromosomes become condensed resulting in a transcriptionally silent genome. Therefore, during late oogenesis and early embryogenesis, post-transcriptional control of pre-existing mRNAs is the principle method for gene regulation (Lee & Schedl, 2006). Before the embryonic genome is activated, early development is controlled by the maternally deposited factors (Tadros & Lipshitz, 2009). Direction of the first mitotic division and determination of initial cell fates are some of these developmental processes (Tadros & Lipshitz, 2009). The shift in control from maternally provided gene products to those synthesized by the zygote itself is called oocyte to embryo transition (OET) in different organisms (Tadros & Lipshitz, 2009). There are two processes that together compose the maternal-to-zygotic transition. First, a group of maternal mRNAs are eliminated, second, embryonic genome activation (EGA) takes place (Tadros & Lipshitz, 2009).

The processes that encompass OET are conserved in various animals. Egg activation, which is necessary for the initiation of embryogenesis, is triggered by fertilization in echinoderms (sea urchin), nematode worms and vertebrates. However, in *D. melanogaster*, egg activation occurs prior to and independently of fertilization, and involves a combination of other factors like osmotic and mechanical stimulation. Upon egg activation, the early mitotic cycles vary in length between different species. The duration of the mitotic cycle is 8 minutes in flies, 15 minutes in zebra fish and 12 hours in mice **(Figure 1.1)**. The timing and duration of OET also varies between species. The largest fraction of maternal mRNAs is degraded by the two-cell stage in mice and four-cell stage in *C. elegans*. Finally, the process of zygotic genome activation takes place at different cleavage cycles and at different times after fertilization in various organisms. In *D. melanogaster* minor and major transcriptional waves are initiated at cleavage cycle 8 and 14 respectively. In *C. elegans*, the minor wave is initiated at the second cleavage cycle and the major wave around 6-7th cleavage cycle **(Figure 1.1)** (Tadros & Lipshitz, 2009). In this project the nematode *C. elegans* is used as an experimental model to study post-transcriptional gene regulation during OET.

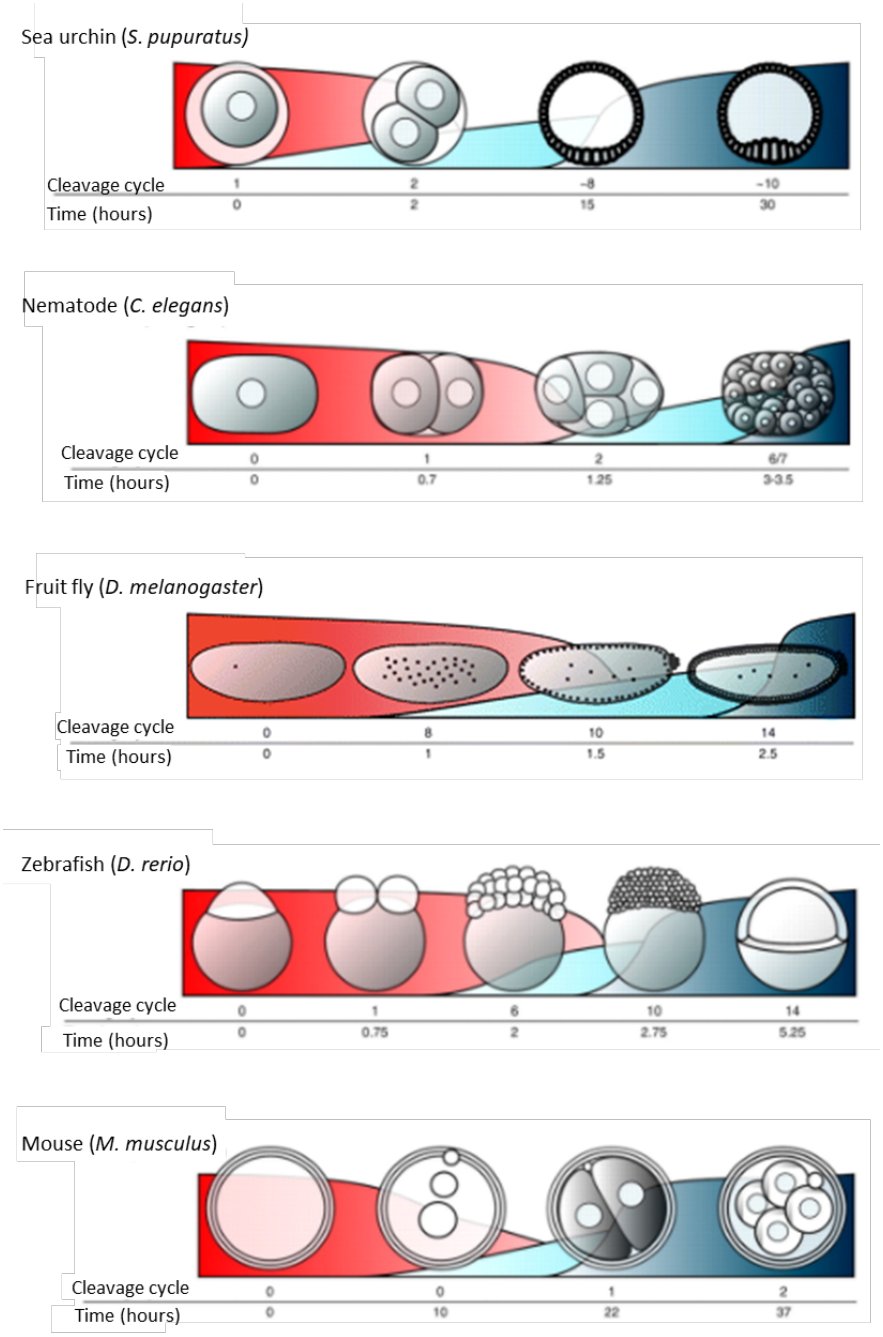


Figure 1.1 An overview of the oocyte-to-embryo transition (OET)

Important developmental stages in *S. purpuratus*, *C. elegans, D. melanogaster, D. rerio and M. musculus* are shown above the corresponding cleavage cycle and time after fertilization. The red curve represents maternal transcripts that are destabilized and degraded during the progression of embryonic development. The light and dark blue curves represent the minor and major transcriptional waves respectively, as EGA occurs in successive waves. [Figure adapted from: (Tadros & Lipshitz, 2009)]

1.3 *Caenorhabditis elegans*

1.3.1 *C. elegans* as a model organism

The nematode *Caenorhabditis elegans* is a free-living organism that lives in soil. It was introduced as a model organism by Sydney Brenner in 1963 and later showed great potential for molecular biology research. The lifecycle of a *C. elegans* consists of an initial embryonic stage, 4 larval stages (L1-L4) and adulthood. Moreover, rough environmental conditions such as nutrient limitation and unfavorable temperature can urge a *C. elegans* to enter the so-called dauer stage. The dauer stage will generate a change in how the worm behaves, its metabolism, growth and reproduction in a manner that is fit to improve the chances of survival in the newly encountered environment (Fielenbach & Antebi, 2008).

C. elegans is a good model organism for many reasons. A large number of offsprings are produced in each generation and the population consists mostly of hermaphrodites and a few males. With each adult hermaphrodite having only 959 somatic cells, and male having only 1031 somatic cells, these are simple animals. Furthermore, *C. elegans* is a transparent animal with the average size of 1 mm, allowing easy visualization by light microscopy. It takes 4 days to reach adulthood and can live up to 2-3 weeks. Also, it is easy to maintain and breed in normal laboratory conditions. *C. elegans* is considered to be a great tool for genetic studies and it was the first multi-cellular organism to have its entire genome sequenced. Also, it has an invariant cell lineage, making it possible to identify all of the embryonic cells in each individual and compare them across individuals (Zacharias & Murray, 2016).

1.3.2 Germline organization of *C. elegans*

C. elegans hermaphrodites provide many advantages to study oocyte development, meiotic maturation, fertilization and OET. *C. elegans* hermaphrodites produce sperm during the L3/L4 larval stage. The sperm are stored in the spermatheca. After the late L4 stage, the germ cells entering meiosis generate oocytes. This process is continuous for the remainder of the reproductive life of the hermaphrodite (Robertson & Lin, 2015). The gonad of a hermaphrodite consists of two tube-like arms, in which cells in the distal region continuously divide mitotically to provide new supplies of germ cells **(Figure 1.2 A)**. As the cells move from the distal region

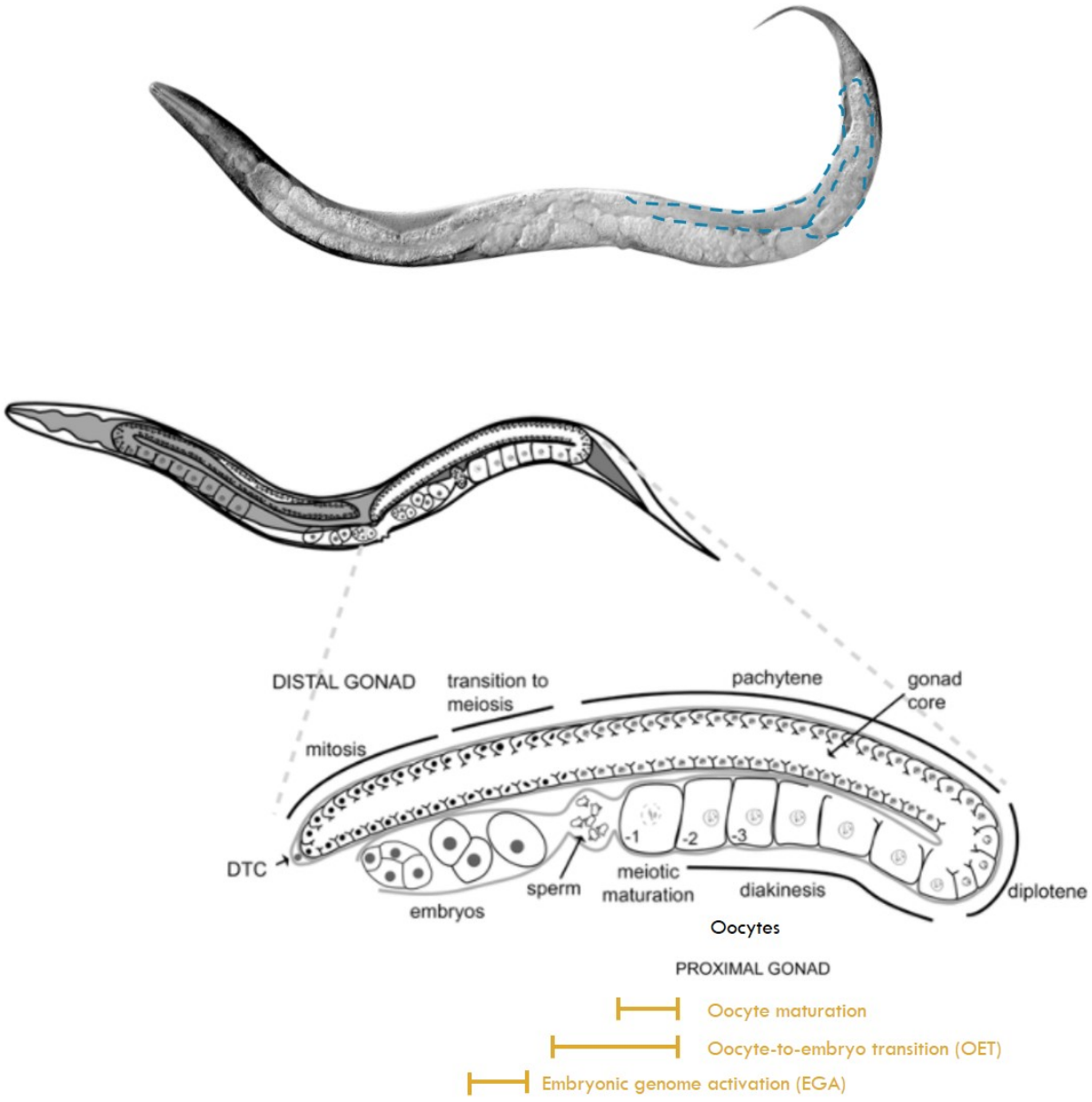


Figure 1.2 *C. elegans* hermaphrodite

A) Image of an adult hermaphrodite imaged with DIC microscopy. One arm of the worm gonad is marked with blue dotted lines [Image source: Ian D. Chin Sang, Utrecht University]. **B)** Schematic depicting a *C. elegans* adult hermaphrodite with magnified view of one gonad arm. The distal end of the gonad has a single stem cell known as distal tip cell (DTC) which gives rise to all germ cells. The figure depicts the maturation process of germ cells. The germ cells divide in the distal part transitioning to meiosis in the medial part and develop into oocytes in the proximal part of the gonad. At this stage resumption of prophase I will occur. Under the schematic, bars and arrow indicate where in the germline different processes like oocyte maturation, OET and EGA occur. [Figure adapted from (Huelgas-Morales et al., 2016)]

of the gonad, they advance through the stages of meiosis, during which oocytes will grow in volume as well. After the oocytes have undergone extensive growth, they will undergo meiotic maturation prior to being fertilized. Upon fertilization embryonic development will be initiated after meiosis is completed (Robertson & Lin, 2015) **(Figure 1.2. B)**.

1.3.3 OET in *C. elegans*

Post-transcriptional regulation of gene expression during oogenesis, transcriptional repression of the zygotic genome, EGA, and clearance of maternal products, are all crucial elements of OET. A series of asymmetric divisions in the *C. elegans* embryos result in germline precursors (P cells) that are transcriptionally repressed, and larger somatic cells that undergo transcriptional activation and differentiation. Thus, to differentiate between the transcriptionally active and silent cells, effective and reversible mechanisms for transcriptional repression are crucial during *C. elegans* early development (Robertson & Lin, 2015). In *C. elegans*, transcription of the embryonic genome is not necessary until gastrulation at the 28-cell stage, but EGA happens at the 4-cell stage (Robertson & Lin, 2015). Before EGA, during oogenesis and oocyte maturation, post-transcriptional control via RNA binding proteins (RBPs), play important roles in regulation of gene expression (Lee & Schedl, 2006). RBPs are also crucial for regulating the timing of EGA.

1.4 RNA regulation

An mRNA can experience three different fates in the cytoplasm. It may give rise to a protein by associating with the translational machinery, it can be degraded by entering the mRNA decay pathway, or it can be stored in protective Ribonucleoprotein particles (RNPs) while being repressed **(Figure 1.3 A)** (Nousch & Eckmann, 2013). RNPs are complexes formed between RNA, RNA binding proteins (RBPs) and other regulatory proteins.

1.4.1 Structure of a mature mRNA

A mature mRNA consists of an open reading frame (ORF), which serves as a template for protein synthesis, and 3´and 5´untranslated regions (UTRs) that flank the ORF. The 3´and 5´ UTRs carry information about the mRNAs ability to serve as a template for protein synthesis. The, 5´cap and 3´poly(A)-tail are added to the mRNA as a part of the maturation process in the

nucleus, and also serve as non-coding parts of an mRNA (Nousch & Eckmann, 2013). The 5´cap protects the mRNA from 5´ to 3´exonucleolytic decay and functions as the docking point for the cap-binding family of proteins that mediate initiation of translation. The poly(A)-tail is a long chain of adenine nucleotides that are added during mRNA processing to increase the stability of the mRNA **(Figure 1.3 B)**. The poly(A)-tail also enable, circularization of the mRNA that is required for translation (Wells et al., 1998).

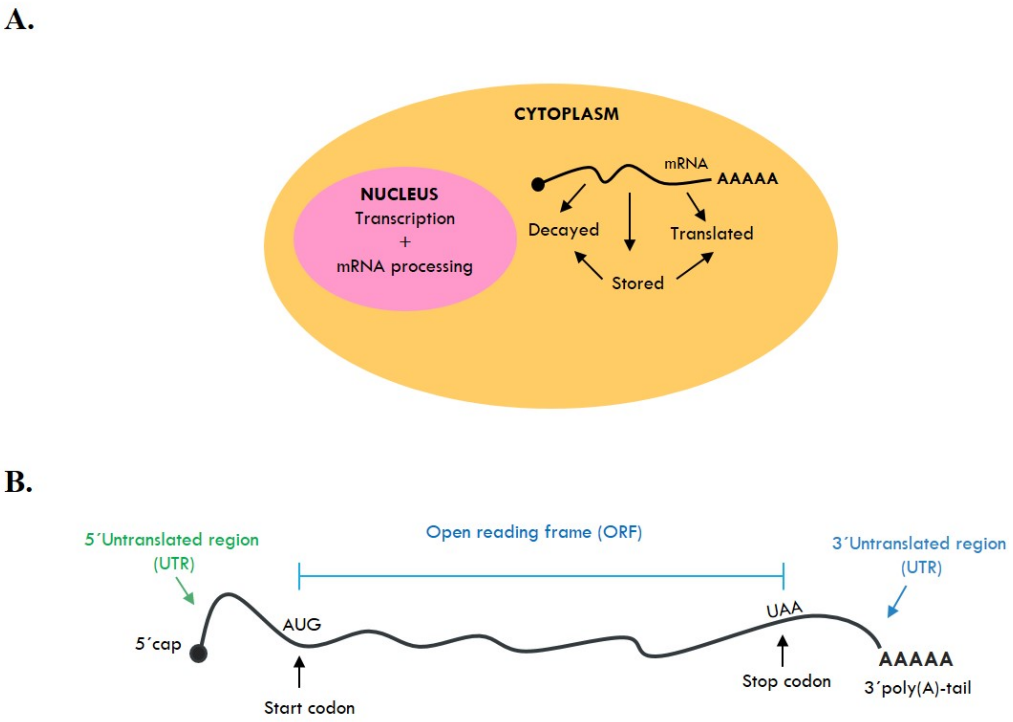


Figure 1.3 Life cycle of an mRNA

A) After mRNA is transcribed and processed in the nucleus, it can experience three different fates in the cytoplasm. It may enter the translation pool and undergo protein synthesis, or it can be degraded. It can also be stored from where it can be translated or degraded later in response to external or internal stimuli. **B)** Schematic of a mature mRNA showing 5`cap, 5`UTR, start codon, ORF, stop codon, 3`UTR and 3`poly(A)-tail.

1.4.2 Post-transcriptional regulation

Gene expression patterns that control the developmental programs in an organism are regulated at both transcriptional and post-transcriptional level (Nousch & Eckmann, 2013). In transcriptional control an input signal needs to be transmitted to the nucleus for suitable mRNA, to be made. Furthermore, after the desirable mRNA, have been synthesized they need to be

matured, quality controlled and finally exported to the cytoplasm, where they will exert their functions. However, in post-transcriptional control a pool of previously made mRNAs are localized in the cytoplasm awaiting to be translated (Nousch & Eckmann, 2013). Thus, post-transcriptional regulation provides a speed advantage. The large number of RNA regulatory proteins which are identified to play important roles in development and functions of the germline, is a reflection of the importance of post-transcriptional control.

1.4.3 RNA binding proteins

RNPs play important roles in post-transcriptional control of mRNAs during diverse cellular processes. Post-transcriptional regulation can occur at many different stages of mRNA metabolism including splicing, polyadenylation, mRNA localization and translation (Lee & Schedl, 2006). The *C. elegans* genome encodes for a large number of RBPs and around 500 genes have been identified to have one or more RNA binding domains (Lee & Schedl, 2006).

Several critical steps of early development rely greatly on precise temporal and spatial gene regulation, during which sequence-specific RBPs play crucial roles. In general, RBPs can bind to regulatory sequences located in the 5´UTR and/or 3´UTR of the mRNA to activate or repress translation. Thus, important regulatory mechanisms that are involved in embryonic development are regulated by specific elements in the 3´UTRs (Muckenthaler & Preiss, 2006). These are elements that function as a warehouse of regulatory components affecting mRNA stability, intracellular localization and translation (Szostak & Gebauer, 2013).

RBPs are able to interfere with closed-loop formation of mRNA and ribosome recruitment, which are required for active translation, by different means. By recruiting deadenylase complexes to mRNA, RBPs are able to influence the length of the 3´poly(A)-tail **(Figure 1.4)**. RBPs can also affect translation by recruiting translational inhibitors to the mRNA. Two common means to do so are by recruiting eIF4E homology proteins **(Figure 1.5 B)** (4EHP, disrupting binding of eIF4E to the mRNA cap) or by recruiting eIF4E binding protein (4EBP, disrupting biding of elF4E to eIF4G) **(Figure 1.5 C)** (Szostak & Gebauer, 2013; Teleman et al., 2005)

1.4.4 mRNA decapping

The balance between its degradation and active translation decides the cytoplasmic lifetime of an mRNA. The process of turnover is a means to control gene expression both under normal and stress conditions, and is achieved by striking a balance between stabilizing mRNAs within RNPs and mRNA clearance (Borbolis & Syntichaki, 2015). The removal of the 3´poly(A)-tail serves as the primary approach for mRNA degradation. This process is often followed by decapping, where the 5´cap structure is cleaved ahead of 5´->3´exonucleolytic digestion of mRNA. The decapping process represents an important step in turnover, as it allows for degradation of mRNA to occur **(Figure 1.4)**.

1.4.5 Deadenylation of poly(A)-tail

The length of the poly(A)-tail functions as an indicator of mRNA fate. A long poly(A)-tail promotes high rates of translation. A single Poly(A) binding protein (PABP) is able to bind about 20 adenosines (Nousch & Eckmann, 2013). The PABP is an initiation factor that specifically binds the poly(A)-tail. Upon binding to mRNA, it assists a closed-loop formation, which facilitates the recruitment of the translation machinery to the mRNA. Hence, a longer poly(A)-tail will be able to recruit more PABPs and enhance frequent translation (Nousch & Eckmann, 2013).

The poly(A)-tails of eukaryotic mRNAs are exposed to shortening in the cytoplasm in a process referred to as deadenylation. Regulatory proteins that will bind to specific sites in the mRNA decide the rate of deadenylation. Typically, these proteins are RBPs that bind to the 3´UTR of mRNA and recruit deadenylases (Temme et al., 2014). Three major deadenylation complexes have been characterized in *C. elegans*: the CCR4-NOT complex, the PAN2/PAN3 complex and PARN complex. Of these complexes, the dominating deadenylase complex in all biological systems is the CCR4-NOT complex **(Figure 1.4)** (Temme et al., 2014).

The CCR4-NOT complex is a multi-subunit deadenylase complex consisting of one core component and several accessory factors. The three proteins comprising the core module of the complex are CCR4, CCF1 and NOT1. NOT1 serves as a scaffolding protein anchoring the accessory factors to the complex, and CCR4 and CCF1 are the exonucleases (Nousch et al., 2013). Deadenylation of mRNA leads to termination of translation and is also the most efficient step in controlling mRNA decay (Decker & Parker, 1993).

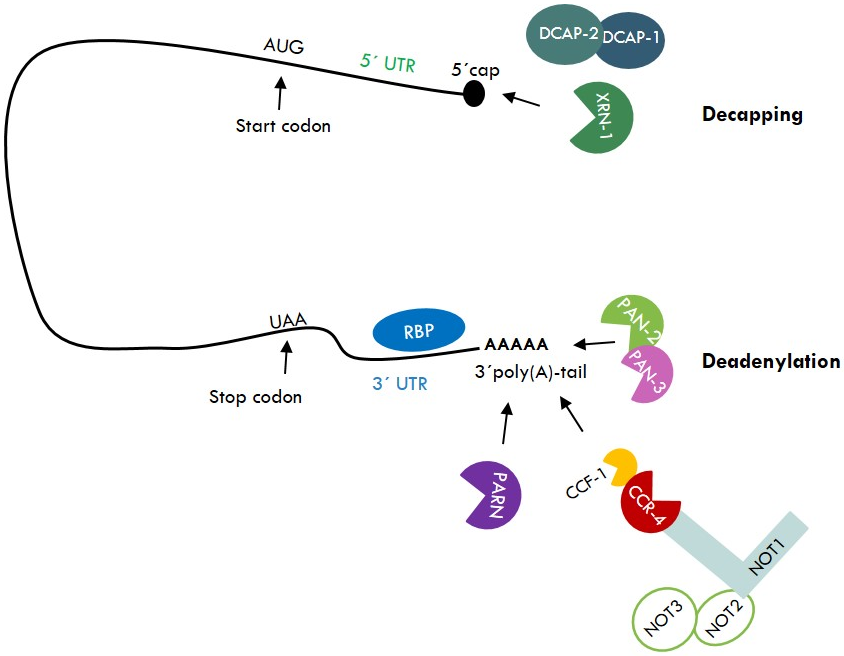


Figure 1.4 RNA degradation pathways

Schematic depicting mRNA degradation through 3` poly (A) deadenylation and 5` decapping. The CCR4-NOT complex, PAN2/PAN3 complex and PARN complex are recruited to 3` poly (A)-tail by RBPs. The DCAP-1 and DCAP-2 proteins, and XRN-1 exonuclease are recruited to the 5`cap.

1.5 Translational control

1.5.1 Specific and global regulation of translation

The level of a protein in a eukaryotic cell is determined by various translational mechanisms (Rhoads et al., 2006). There are two different types of control that occur at the translational level: global and gene specific. In global control the levels, activities or availability of translation factors are affected. This type of regulation is favored in situations of stress, like starvation or exposure to toxic agents. In these situations, global control is initiated in order to stop synthesis of new proteins and dictate an appropriate stress response (Nousch & Eckmann, 2013). Global translation is also regulated through phosphorylation of translation initiation factors. Protein kinase and phosphatase relationships that are responsible for reversible phosphorylation are mainly regulated by two major pathways. These are the Ras/MAPK

pathway which regulates the phosphorylation state of the cap binding protein eIF4E, and the PI3K/Akt/mTOR pathway which affects the phosphorylation of the ribosomal protein S6, as well as eIF4G and 4EBP (Muckenthaler & Preiss, 2006).

By contrast, gene specific control is achieved through regulatory RBPs (Hershey et al., 2012). These RBPs bind to specific elements in the mRNAs, usually present in the 5′and 3′ UTRs and influence the initiation process of translation. This type of mechanism is widely used during early development (Hershey et al., 2012).

1.5.2 The process of translation

Translation is an important part of gene expression cascade where the genetic information of an mRNA is translated into a functional protein. The process of translation is divided into three phases: initiation, elongation, and termination (Nousch & Eckmann, 2013). Each of the three steps is catalyzed by three groups of proteins respectively referred to as initiation, elongation and release factors (Rhoads et al., 2006). The initiation step involves all steps required for the assembly of the translational machinery, including the association of the 80S ribosome with the start codon. During the elongation phase, the 80S ribosome progresses along the mRNA to synthesize the polypeptide chain. Finally, the termination phase is initiated when the ribosome encounters a stop codon, which results in a newly synthesized protein, allowing the disassembly of the ribosomal machinery (Muckenthaler & Preiss, 2006).

Translation can be regulated at any of the three phases. However, the majority of the regulatory events at the translational level take place during the initiation phase. There are more than 40 initiation factors encoded in the *C. elegans* genome (Nousch & Eckmann, 2013). These initiation factors assemble into larger protein complexes accomplishing distinct functions during the initiation phase. Furthermore, two structural components of an mRNA with great importance for efficient translation are the 3′poly(A)-tail and the 5′cap-structure, as stated earlier.

1.5.3 Cap-mediated translational regulation

The length of the poly(A)-tail has a direct impact on the stability and translatability of the mRNA (Rhoads et al., 2006). The poly(A)-binding protein (PABP), which specifically binds the poly(A)-tail is an initiation factor that is required for the recruitment of mRNA to the

initiation complex. If an interaction between the 3′ poly(A)-tail and the 5′ cap occurs, translation of the mRNA is favored. Furthermore, this interaction is mediated by eukaryotic translation initiation factor eIF4G, which contains a binding site for both PABP and the cap-binding protein eIF4E, and functions as a bridge between the two proteins. Upon binding of eIF4G to eIF4E and PABP, mRNA is circularized, and together with other components of the translation machinery, ribosomes are recruited to mRNA for active translation **(Figure 1.5 A)** (Svitkin et al., 2001) .

The 4E binding protein (4EBP) competes with the eIF4G protein for interaction with eIF4E. In general, eIF4G binds to eIF4E with a higher affinity than the 4EBP. However, if 4EBP is able to bind eIF4E, eIF4G is removed from the cap-binding complex. In this way, 4EBP can act as a translational repressor, preventing the overall interaction between PABP and eIF4E, and the ribosomal recruitment to mRNA for active translation **(Figure 1.5 C)** (Richter & Lasko, 2011).

The mechanistic target of rapamycin (mTOR) regulates major cellular processes in response to growth factors, cytokines hormones and nutrients (Qin et al., 2016). It plays important roles in many pathological conditions including cancer, diabetes and neurodegeneration. mTOR is a PI3K-related kinase forming two distinct protein complexes, referred to as mTORC1 and mTORC2. In response to upstream stimuli, mTORC1 promotes translation and protein synthesis by phosphorylating S6 kinase 1 and 4EBP. Upon activation, mTORC1 phosphorylates 4EBP. The phosphorylated 4EBP will dissociate from eIF4E, allowing eIF4G to interact with eIF4E and the cap, resulting in translation initiation **(Figure 1.5 D)** (Qin et al., 2016).

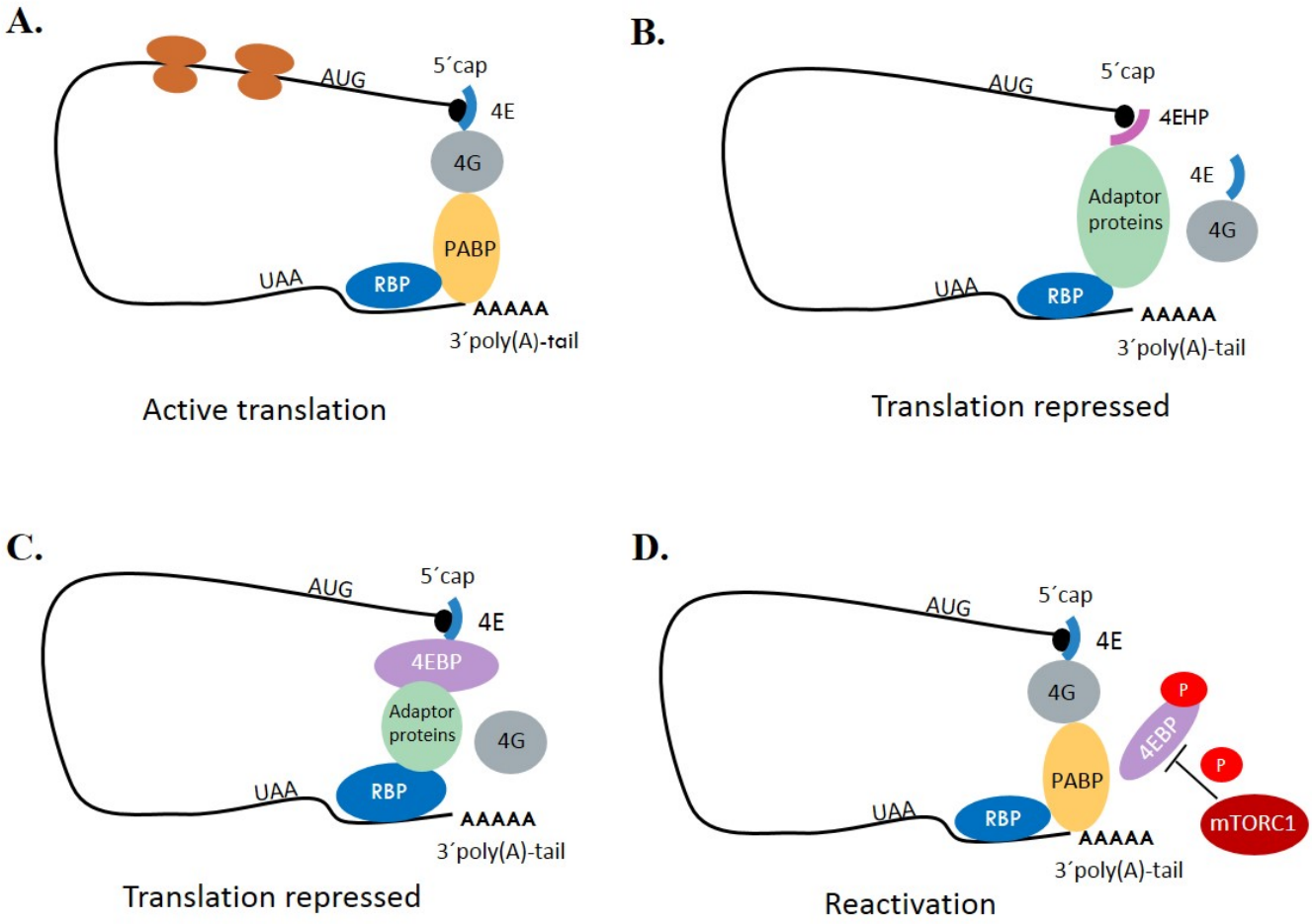


Figure 1.5 Different mechanisms by which translation is affected through 5`cap and 3`poly(A)-tail. **A)** During active translation, the initiation factor eIF4G (4G) functions as a bridge between PABP and eIF4E (4E), through which mRNA is circularized and the translational machinery is recruited to the mRNA for active translation. **B)** Translation is repressed when RBPs recruit 4EHP to the mRNA. RBPs can bind directly or indirectly via adaptor proteins. eIF4E is removed from mRNA and translation is repressed. **C)** When RBPs recruit 4EBP to the eIF4E-5`cap, eIF4G is not able to bind to eIF4E and form a bridge between the 5`cap and the 3`poly(A)-tail. eIF4G is removed from mRNA and translation is repressed. **D)** Translation can be reactivated by mTORC1 signaling. Active mTORC1 will phosphorylate 4EBP, rendering it unable to bind to eIF4E, this will allow eIF4G to bind to eIF4E and initiate translation.

1.6 Project Background

RBPs play important roles in oocyte maturation and OET. The RBPs LIN-41 and OMA-1/OMA-2, function in the developing oocytes in the *C. elegans* gonad. LIN-41 and OMA function in regulation of oocyte growth, meiotic maturation and OET. These functions of LIN-41 and OMA during oogenesis and OET have been associated with their role in mRNA regulation.

1.6.1 OMA-1 and OMA-2

The closely related proteins, OMA-1 and OMA-2, referred to as OMA, are functional homologs. In absence of OMAs, meiotic progression is blocked. The levels of OMA proteins increase in growing oocytes and reach their highest levels of expression in the -1 oocyte and the 1-cell embryo. However, following the first mitotic division, they are rapidly degraded. Oocyte lacking OMA proteins fails to properly complete its maturation process, ovulation is blocked, and the developing oocytes are accumulated in the proximal gonad. OMA-1/OMA-2 double-mutant animals are sterile as the -1 oocyte fails to complete maturation (Detwiler et al., 2001).

Both the proteins contain two CCCH-type tandem zinc finger domains through which they bind AU-rich elements (UAA/U) of their target mRNAs **(Fig 1.6 A)** (Kaymak & Ryder, 2013). OMA proteins influence turnover and translation of target mRNAs by likely recruiting regulatory proteins to the mRNAs. However, what these regulatory proteins are and how they function is not fully known.

1.6.2 LIN-41

The RBP LIN-41 is a member of the TRIM-NHL family of RBPs that are important regulators of cell proliferation and differentiation (Tocchini et al., 2014). The *C. elegans* LIN-41 is expressed in the cytoplasm of developing oocytes and is also a well-known component of the so-called heterochronic pathway in the soma. LIN-41 expressed in the *C. elegans* germline plays a critical role in the oocyte to embryo transition. In *C. elegans,* LIN-41 is expressed in the mid-pachytene stage of the germline. This expression pattern is consistent with its role in oocyte-to-embryo transition. However, its expression begin to fade during oocyte maturation and is also absent from embryos (Spike et al., 2014). In the absence of LIN-41, developing

oocytes acquire pluripotent characteristics, re-enter mitosis giving rise to cells that differentiate and form a teratoma (a tumor containing different cell types) (Tocchini et al., 2014).

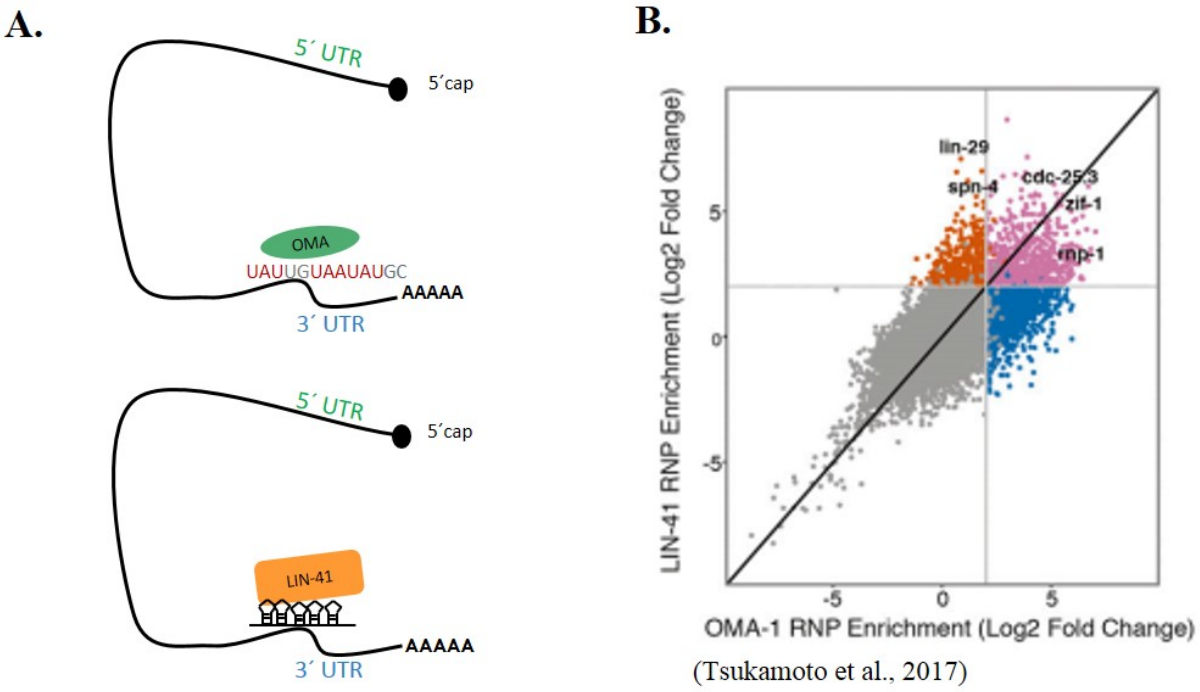
"TRIM" in TRIM-NHL refers to a tripartite motif, consisting of a RING finger domain, B-box(es), and coiled-coiled domains. The NHL repeats are named after the proteins NCL-1 HT2A and LIN-41, in which this domain was first identified. The binding specificity of LIN-41 is decided by the shape of a positively charged pocket on the surface of the NHL domain. This positively charged pocket recognizes a short stem loop (SL) element referred to as an LRE (LIN-41 response element) on a target mRNA **(Fig 1.6 A)**. Moreover, by binding to specific mRNAs, LIN-41 is able to influence their expression patterns by targeting them for either degradation or translational repression (Aeschimann et al., 2017).

It has been shown that LIN-41 has two distinct mRNA repressing activities (Aeschimann et al., 2017). Four mRNAs are known to be direct physiological targets of LIN-41 in the *C. elegans* somatic cells. Three of these targets, *mab-10, mab-3* and *dmd-3*, have LREs in their 3′ UTRs and undergo transcript degradation mediated by LIN-41. However, *lin-29A,* which has LREs in its 5′ UTR, is subjected to LIN-41 mediated translational repression (Aeschimann et al., 2017). However, the protein co-factors that execute these target specific repressing mechanism are not yet known. Furthermore, direct targets of LIN-41 in the germline have not been validated yet. Also, the mechanisms utilized by LIN-41 to repress mRNAs in the germline are not known.

1.6.3 Overlapping functions of OMA and LIN-41

RNA immunoprecipitation experiments have shown that there is an overlap between mRNAs that are bound by LIN-41 and OMA in the *C. elegans* germline (Kumari et al., 2018; Tsukamoto et al., 2017) **(**Figure **1.6 B)**. In addition, LIN-41 has been shown to regulate mRNAs that are directly bound by OMA proteins such as *cdc-25.3, zif-1 rnp-1* (Tsukamoto et al., 2017). LIN-41 can also regulate mRNAs by directly binding to them via LREs but such targets have not been validated. The underlying molecular mechanisms that the two proteins elicit on their target mRNAs remain elusive. In order to study the molecular mechanisms of LIN-41 and OMA mediated regulation; their protein partners were identified by immunoprecipitation (IP) followed by mass-spectrometry using adult stage animals (Kumari et al., 2020 Unpublished) **(Figure 1.6 C)**. In the IP experiments LIN-41 co-purified with OMA-1 and vice-versa suggesting that the two proteins work in a complex. Furthermore, several proteins were found

to be significantly enriched in the immunoprecipitated fraction for both pull-downs. Studying the role of these proteins and associated pathways in LIN-41 and OMA mediated mRNA regulation, will help us understand how the RBPs regulate specific mRNA targets.



C.

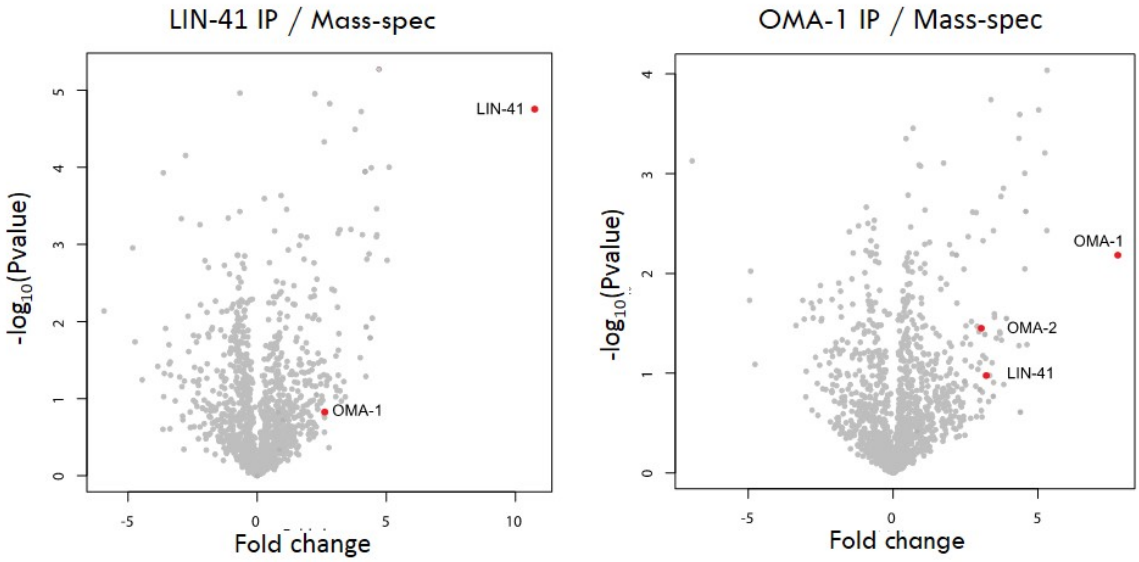


Figure 1.6 mRNA targets and protein partners of LIN-41 and OMA-1

A) Schematics depicting mRNA targets of OMA-1 and LIN-41. OMA-1 recognizes UAA/U sequence motifs (OMA binding sites) and LIN-41 recognizes short stem-loop structures named as LIN-41 response elements (LREs). **B)** Distribution of mRNAs according to their enrichments in LIN-41 and OMA-1 immunoprecipitates (IPs). mRNAs indicated in pink are highly enriched in both LIN-41 and OMA IPs [Figure taken from: (Tsukamoto et al., 2017)]. **C)** Protein partners of LIN-41 and OMA were identified by IPs and mass-spectrometry. LIN-41 is detected in OMA-1 pull-down, and vice versa, confirming their association in the *C. elegans* germline (Kumari et al., 2020 Unpublished).

1.7 Project objectives

The focus of this project is to understand how LIN-41 and OMA proteins regulate mRNA targets. We wanted to investigate if the two proteins work together or deploy independent regulatory mechanisms to regulate common mRNA targets. In order to do so, we had the following objectives:

1. Screen the identified protein partners of LIN-41 and OMA for their role in mRNA regulation by using reporter strains, wherein GFP mRNA expression is regulated by LIN-41 and/or OMA.

2. Determine how the identified protein partners participate in mRNA regulation.

2. Materials and Methods

The recipes for all the buffers and media used in different methods are available in the appendix section.

2.1 Maintenance of *C. elegans*

The *C. elegans* were grown on agar plates containing 2% nematode growth media (NGM). The animals are fed with *E. coli* strain OP50, which was seeded onto the NGM plates (Brenner, 1974). This bacterial strain has limited growth on the NGM plates and allows for easier observation and better mating of the animals. The animals were maintained at 20 °C, unless specified otherwise in an experiment.

2.1.1 Strains

In this project the N2 Bristol strain was used as a reference for wild type. Other reporter strains and their genotypes are mentioned in **Table 2.1**. The different reporter strains for studying mRNA regulation were designed in the same way. In the strains, a GFP fusion protein (PEST-H2B-GFP) is expressed under the control of a specific germline promoter (*mex-5*) and a specific 3′UTR. For OBSs, we used *cdc-25.3* 3`UTR and for LREs we used a fragment of *mab-10* 3′ UTR containing 6 LREs. H2B ensures that GFP is concentrated in the nucleus of the cells, which makes it easier to detect. PEST motif mediated turnover of the fusion protein, allows better detection of changes in gene expression.

Table 2.1. Names, strain ID, and genotypes of strains used in this project.

Name	Strain ID in Ciosk lab	Genotype
OBS	1915	rrrSi452[Pmex-5::PEST::GFP::H2B::cdc-25.3 3'UTR; unc-119(+)] II; unc-119(ed3) III.
LRE	1894	rrrSi447[Pmex-5::GFP::H2B::PEST::unc-54+mab-10 (500bp) 3'UTR; unc-119(+)] II; unc-119(ed3) III
gld-1	673	unc-119 (ed3) III; rrrSi107 [mex-5 pro::PEST:GFP-H2B::gld-1 3′UTR; unc-119(+)]II.
glp-1	688	rrrSi117 [mex-5 pro::GFP-H2B::glp-1 3′UTR wt; unc-119(+)]II
pal-1	690	rrrSi119 [mex-5 pro:: GFP:H2B::pal-1 3′UTR wt; unc-119(+)]II

2.1.2 Synchronization of *C. elegans*

Many experiments required the animals to be synchronized at the same stage of their life cycle. To do so, a bleaching solution consisting of sodium hypochlorite and potassium hydroxide was added to a tube containing adult animals (recipe in appendix). The bleaching solution dissolved the adults while the eggs remain intact. When the eggs were left on a shaker platform overnight, they hatched and remained arrested at L1 stage in absence of food. These L1s were then plated on NGM2% plates or RNAi plates as required in specific experiments.

Procedure:

1. M9 buffer was used to wash off and collect animals from plate. Plates were tilted to gather the liquid in a 15 ml falcon tube.

2. Falcon tube was centrifuged at 1500 g for 1 minute. The animals were collected at the bottom of the tube.

3. Supernatant was carefully removed with suction.

4. 5 ml of bleaching solution was added, and tube was left on rocking platform for 7 minutes.

5. After the animals had dissolved, embryos were collected by centrifuging at 1500 g for 1 minute.

6. Bleaching solution was removed.

7. Eggs were washed with M9 buffer three times.

8. Eggs were left on rocking platform overnight. During this time eggs had hatched and remained arrested at L1 stage. The rocking movement made sure the L1s had enough oxygen.

9. Hatched L1s were centrifuged at 1500 g for 1 minute and supernatant was removed.

10. Finally, 300 µl of M9 was added and animals were plated.

2.2 RNA interference

The technique of RNA interference (RNAi) was first developed in *C. elegans* and has now become a widely used method for genetic manipulation. RNAi is a rapid and simple approach to determine loss-of-function phenotypes of specific genes (Conte et al., 2015). The technique takes advantage of the cell´s ability to protect itself from exogeneous double stranded RNA (dsRNA). When dsRNA is introduced into the cell cytoplasm, it is cleaved by the Dicer complex into small interfering RNA (siRNA). The siRNA will then be separated into single strands and integrated into the RNA-induced silencing complex (RISC complex). After integration into the RISC complex, the siRNA will serve as a template to recognize complementary mRNA strands. Finally, when the target mRNA is base paired with siRNA, it will be cleaved by the catalytic subunit of RISC called Argonaute 2 (Ago2). As a result, the target mRNA will not be able to serve as a template for translation **(Figure 2.1)** (Mello & Conte, 2004).

dsRNA can be introduced into *C. elegans* through microinjection, feeding or soaking. In microinjection, *in vitro* preparations of dsRNA are injected directly into the body of the animal by using a needle. During the process of soaking, dsRNA is introduced by passively soaking the worms in solution containing dsRNA. In this project, RNAi was induced in worms by feeding bacteria expressing dsRNA (Conte et al., 2015).

Procedure:

1. NGM plates were made according to standard instructions. Ampicillin, tetracycline and IPTG were added before plating (see appendix for concentration). Plates were left to dry. These plates are called RNAi plates.

2. The desired bacterial strain for introduction of dsRNA were taken from -80 °C and stroked on selection plates. Bacteria was grown at 37 °C for 24 hours.

3. The next day, colonies were selected for inoculation in liquid lysogeny broth (LB) media. The selected colonies were inoculated at 37 °C for 24 hours under shaking conditions.

4. IPTG at a final concentration of 1 mM was added the next morning to induce expression for one hour.

5. RNAi plates were seeded with 100 µl of the induced bacterial culture.

6. Synchronized L1s were placed on RNAi plates. Animals were observed at desired developmental stages for specific phenotypes. For OMA knockdowns, a double RNAi with both OMA-1 and OMA-2 sequences were used.

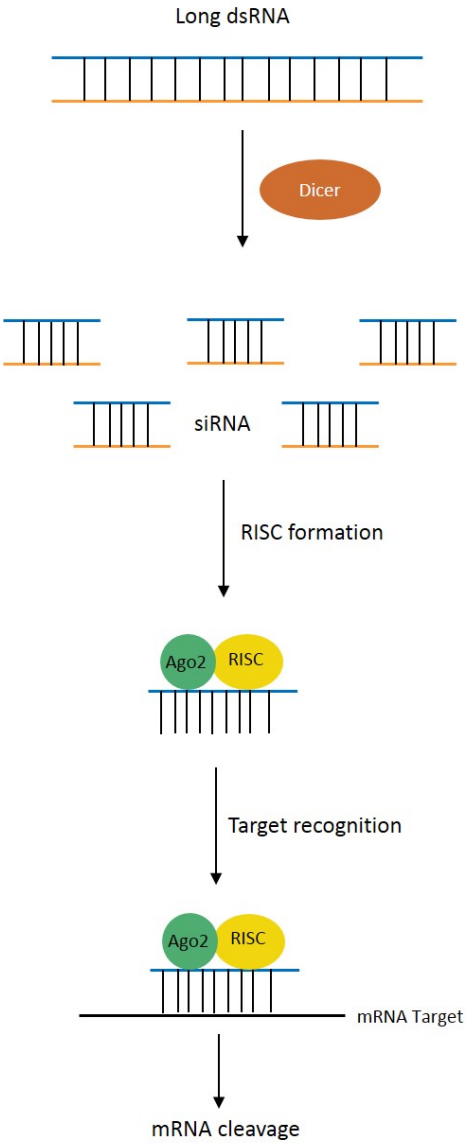


Figure 2.1 Schematic depicting the process of RNAi

When dsRNA is introduced into a system, it will be cleaved by the enzyme Dicer. Cleavage of dsRNA by Dicer will produce multiple smaller fragments of dsRNA, called small interference RNA (siRNA) duplex. One strand of siRNA will be incorporated into the RISC complex. siRNA will serve as a guide and bring the RISC complex to target mRNA by base pairing. Upon recognition of target mRNA, it is cleaved by Ago2.

2.3 Rapamycin treatment

Procedure:

1. Animals were synchronized according to the synchronization protocol.

2. RNAi was performed according to the RNAi protocol using synchronized animals from the previous step.

3. After 24 hours of regular RNAi treatment, between L1 to L4 stage, L4 staged animals were transferred to RNAi plates containing rapamycin. The transfer was done by picking. Rapamycin was dissolved in DMSO at 50 mg/ml and added to agar plates at a final concentration of 100 μM (Robida-Stubbs et al., 2012). Control plates contained same amount of DMSO. Animals were later observed at young adult stage.

2.4 Controlled Starvation

Procedure:

1. Animals were synchronized according to the synchronization protocol.

2. RNAi was performed according to the RNAi protocol using synchronized animals as described above.

3. After 24 hours of regular RNAi treatment, between L1 and L4 stage, L4 staged animals were transferred to plates containing lesser amounts of food (OP50 bacteria). The seeding arrangements used in this experiment for each RNAi condition are listed in **Table 2.2**. The transfer was done by picking. Animals were later observed at young adult stage

Table 2.2. Amounts of food used in starvation experiment for each RNAi condition.

RNAi condition	**Amount of food (stationary phase bacteria)**
Empty vector (EV)	5 μl, 0.5 μl, 0.05 μl
oma-1/2	5 μl, 0.5 μl, 0.05 μl

2.5 Reverse transcription quantitative PCR (RT-qPCR)

2.5.1 Isolation of total RNA using TRIzol reagent

In order to measure the expression levels of different genes, total RNA must be isolated. When isolating RNA with TRIzol, the reagent is used to separate total RNA from other molecules. TRIzol will inhibit RNase activity, while disrupting and dissolving other cell components. Next, chloroform is used to separate the aqueous phase from the organic phase, thus the RNA is separated from proteins. After total RNA is isolated, a DNase reaction containing DNase and gDNA wipeout buffer is made to digest genomic DNA from the reaction. Thereafter, heat is applied to inactive DNase. At the final stage of total RNA isolation, 1 volume of isopropanol is used to precipitate RNA. Later, 80% ethanol is used to wash the isolated RNA pellet. Then the RNA pellet is dried and dissolved in water and the concentration is measured using a spectrophotometer.

Procedure:

1. Animals were collected with M9 buffer into 1,5 ml Eppendorf tubes and centrifuged at 1500 rpm for 1 minute. Supernatant was removed.

2. 250 µl of TRIzol was added to the Eppendorf tubes containing the animals.

3. Worms were Freeze-cracked to break cell membranes. Eppendorf tubes containing samples were put into liquid nitrogen and thawed at 37 °C. These steps were repeated 10 times for effective lysis.

4. Chloroform was added to separate the aqueous phase from organic phase. 1/5 volume of chloroform of the amount of TRIzol was added to the tubes and mixed well.

5. Tubes were centrifuge at 12 000 rpm for 10 minutes at 4 °C.

6. The upper aqueous phase containing RNA was removed and 1 volume isopropanol was added to precipitate RNA.

7. Samples were frozen at -20 °C overnight for effective precipitation.

8. The next day the samples were centrifuged at 20 000 rpm for 20 minutes at 4 °C and RNA was collected at the bottom of the tube. Isopropanol was poured off without losing pellet.

9. 80% ethanol was added. Samples were centrifuged at 20 000 rpm for 5 minutes and supernatant was poured off without losing pellet and left to dry.

10. Sample was dissolved in 90 µl nuclease-free water.

11. Made DNase reaction as follows:

Component	**Volume**	**Final concentration**
10X gDNA wipeout buffer	8 µl	1 X
Template RNA	90 µl	
DNase	2 µl	2 units
Total Volume	**100 µl**	

12. Incubated for 30 minutes at 37 °C.

13. 1 volume of isopropanol was added to precipitate RNA. Steps 7-9 were repeated.

14. The pellet was dissolved in 25 µl nuclease-free water.

15. RNA concentration was measured.

2.5.2 cDNA synthesis

To make a stable template from the isolated RNA, the RNA must be converted to complementary DNA (cDNA). cDNA is made using the enzyme reverse transcriptase. This protocol is based on the FIREScript kit from Solis BioDyne.

Procedure:

1. The following reaction was mixed:

Component	**Volume**	**Amount/ Final concentration**
Template RNA	Variable	500 ng
Oligo (dT) primer or random hexamer primers	1 µl	5 µM

Nuclease-free water	Up to 16 µl	
Total	**16 µl**	

2. Template RNA and primer mix were incubated at 65 °C for 5 minutes and placed immediately on ice. Heat was applied to denature any secondary RNA structures.

3. After a short spin, the following components were added into the mix containing template RNA and primers. Another similar reaction was made. Except nuclease-free water was added instead of the RT enzyme. This reaction served as a RT- control reaction.

Component	Volume	Final concentration
10X RT Reaction Buffer with DTT	2 µl	1X
dNTP mix (20 mM of each)	0,5 µl	500 µM
FIREScript RT	1 µl	10 U
RiboGrip RNase inhibitor (40 U/µl)	0,5 µl	0,5 U
Total	**20 µl**	

4. The following programme was followed for cDNA synthesis:

Step	Temperature	Time
Reverse transcription	42 °C	30 minutes
Enzyme inactivation	85 °C	5 minutes

5. Proceeded directly with RT-qPCR.

2.5.3 qPCR

qPCR was performed using a thermal cycler, LightCycler 96, using HOT FIREPol SolisGreen qPCR mix. SolisGreen is a fluorescent dye which emits fluorescence when bound to double stranded DNA. It is widely used for detection and analysis of DNA, due to its ability to increase its brightness (> 1,000-fold) when bound to double stranded DNA (Dragan et al., 2012). SolisGreen is used for quantitative purposes as the amount of fluorescence can be measured after every PCR cycle. This gives an indication of the amount of DNA that was originally present in the sample and the amount that is being amplified in every cycle (Dragan et al., 2012).

During the process of qPCR, template DNA is amplified for several cycles. In every cycle, the number of DNA is doubled, resulting in an exponential amplification of the targets until saturation. When a sample contains more targets, the fluorescence will be detected in earlier cycles. The cycle in which the fluorescence is detected is called the quantitation cycle (Cq). A low Cq value, means higher initial copy numbers of the target.

In qPCR experiments, a reference gene with stable expression is used as a control to normalize expression of target genes. In the qPCR, actin was used as a reference gene. To separate double stranded DNA, the thermal cycler begins at 95 °C. To further allow the binding of primers to newly available single stranded DNA, the temperature is reduced to 60 °C. Finally, polymerization occurs at 72 °C, which is the most optimal temperature for primer extension as the processivity of DNA polymerases is high.

Four independent biological replicates were used in the experiment, and the average of the replicates was used. Each well contained 10 µl reaction mixture containing the following:

Component	**Volume**	**Stock concentration**
cDNA	1 µl	
Primer mix	1 µl	10 µM
HOT FIREPol SolisGreen qPCR mix	2 µl	5 X
H_2O	6 µl	
Total	**10 µl**	

The following primers were used in the experiment:

Primer	**Primer**	**Sequence (5´ to 3´)**
GFP	Forward	CTTGTTGAATTAGATGGTGATGTT
GFP	Reverse	ACAAGTGTTGGCCATGGA
Actin	Forward	GTTGCCCAGAGGCTATGTTC
Actin	Reverse	CAAGAGCGGTGATTTCCTTC

2.6 Microscopy

The Zeiss Axiomager Z1 microscope was used for all microscopy images taken in this project. The microscope was used for taking DIC (Differential interference Contrast) and fluorescent images. The magnitude of the objectives used for the micrographs is 40 X. Oil is used on the coverslip as the immersion medium.

A 2% agarose solution was made and heated up to 70 °C and placed as a thin film on the microscope slide on which animals were mounted on. Two microscope slides were carefully pressed against each other to make a thin agarose film **(Figure 2.2)**. A levamisole solution (10 mM) was used to keep the worms motionless during imaging, and a coverslip was placed on top.

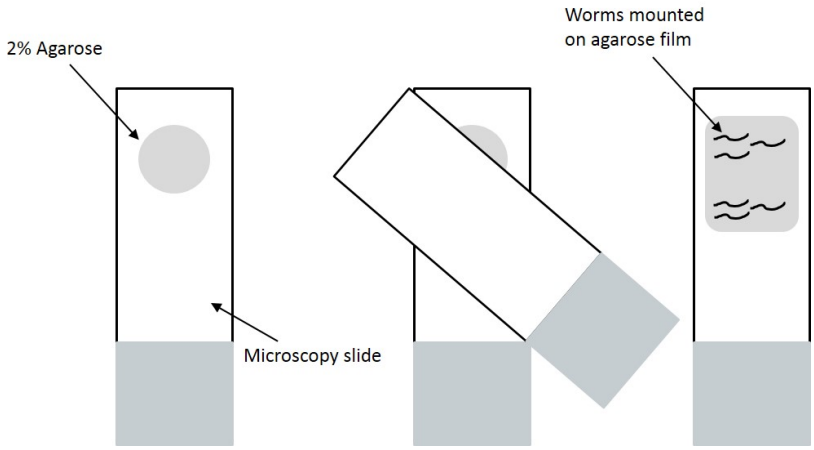


Figure 2.2 Schematic depicting sample preparation for live microscopy

A molten 2% agarose solution was put on a microscope slide. Another slide was carefully put on top to make a thin film of agarose. When the agarose had solidified, the top slide was carefully removed. Finally, the live worms were mounted on the slide in a drop of 10 mM Levimisole in H_2O and a coverslip was placed on top to image.

3. Results

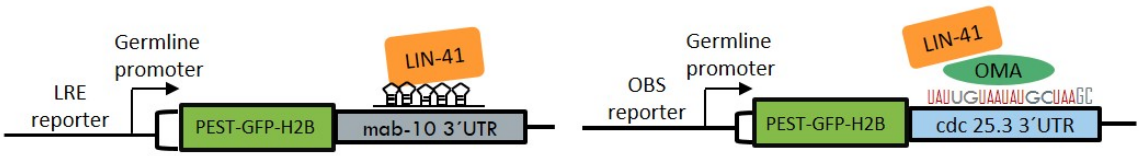
3.1 A functional screen using GFP reporters identifies protein partners involved in LIN-41/OMA mediated mRNA regulation

To understand the molecular mechanism of LIN-41/OMA mediated mRNA regulation, it is important to identify their protein partners. In order to identify the protein partners LIN-41 and OMA-1 were immunoprecipitated (IP) from adult stage animals and the proteins were identified by mass spectrometry (Kumari et al., 2020 Unpublished). A number of proteins were found to be significantly enriched in the pull-downs. LIN-41 and OMA proteins are known to co-regulated several mRNAs (Tsukamoto et al., 2017). In such cases, LIN-41 regulates mRNAs that are directly bound by OMA via OBSs. It is now known if LIN-41 regulates mRNAs by directly binding to OMA or to associated proteins. Consistent with that, we found several proteins present in both LIN-41 and OMA-1 pulldowns.

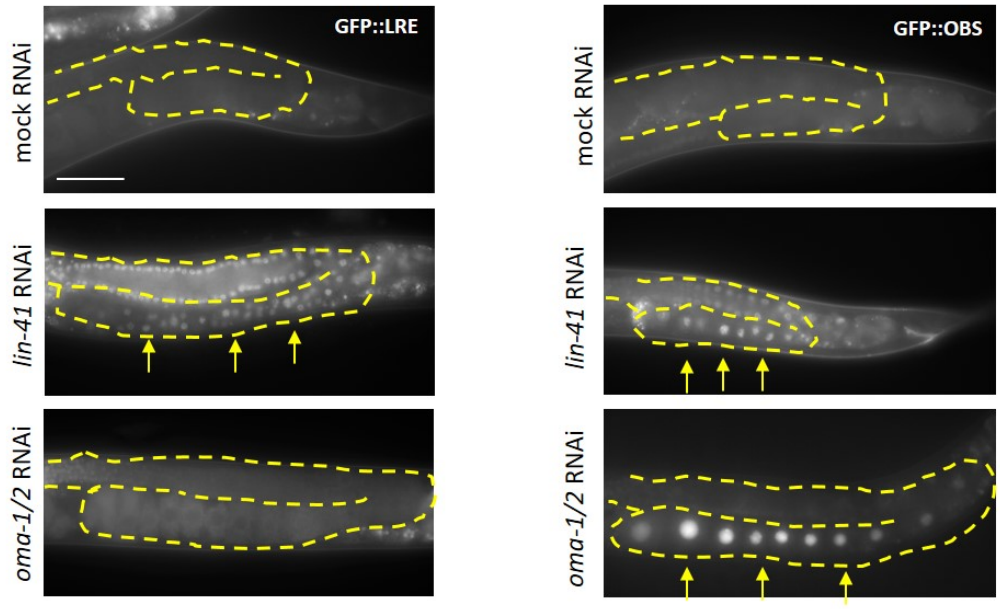
To check which of these protein partners participate in LIN-41/OMA mediated mRNA regulation, we designed a functional screen. In the screen, we used two distinct reporter lines, where in GFP expression is controlled by LREs or OBSs in the 3′UTR **(Figure 3.1 A).** Henceforth, we called these reporters LRE or OBS reporters respectively. Both of these reporters contain a germline promoter from *mex-5*, which is fused to and drives a GFP fusion protein with PEST and the histone H2B. Here PEST is responsible serves as a protein degradation signal and is responsible for a quick turnover of the protein, while H2B translocates the protein to the nucleus, making it easier to detect and quantify protein expression. In order to test the putative roles of identified protein partners, we knocked them down by RNAi in the two reporter lines.

In control RNAi, both LRE and OBS reporters are not expressed in the developing oocytes **(Figure 3.1 B)**. Knockdown of *lin-41* in the LRE reporter resulted in GFP expression in the germline tumor in the distal part of the gonad (germline tumor is a *lin-41* phenotype). However, upon knockdown of *oma*, the LRE reporter was not expressed in the oocytes **(Figure 3.1 B)**. By contrast, the OBS reporter was derepressed in the oocytes upon knockdown of both *lin-41* and *oma*. Taken together, this suggests that the OBS reporter is regulated by both LIN-41 and OMA, whereas the LRE reporter is regulated only by LIN-41.

A.



B.



C.

F16B12.6	
C06E1.9	RNA binding activity, histone-glutamine methyltransferase activity, rRNA methyltransferase activity
PHAT-4	Involved in pharyngeal and gland morphogenesis
PUF-5	RNA binding – involved in positive regulation of embryonic development

D.

ALG-1	Argonaute – RNA binding and nuclease activity	
NFYB-1	Nuclear transcription factor – contributes to RNA polymerase II sequence specific DNA-binding activity	
GEI-4	Involved in negative regulation of Ras protein signal transduction and vulval development	
MTR-4	RNA helicase activity	
NRDE-2	Involved in RNA interference and chromatin silencing	
XND-1	X chromosome NonDisjunction factor	
UBR-1	E3 component of ubiquitin protein ligase	
SCD-1	Suppressor of dauer formation	
SRG-22	Transmembrane signalling receptor activity	
AAK-1 ++	AMP-Activated Kinase	
PQN-39	Prion-like-(Q/N-rich)-domain-bearing protein	mTOR
T16H12.4	General transcription factor	
DKF-2	Protein kinase D3	
LGL-1	Rab GTPase and syntaxin binding	
ATX-2 ++	Translational regulator – component of mTOR pathway, upstream of daf-15 and rheb-1	
IFET-1 +++	4E BP, mRNA binding activity – involved in negative regulation of translation	mTOR
NOT-1 ++	Poly (A) removal	mTOR
NOT-2	Poly (A) removal	CCR4-NOT
CCR-4	Poly (A) - specific ribonuclease activity	CCR4-NOT
CPB-3	mRNA 3´UTR binding activity, translation regulator activity	CCR4-NOT
CCF-1	Poly (A) – specific ribonuclease activity	CCR4-NOT

Figure 3.1 Functional screen to test role of different protein partners in mRNA regulation

A) Schematics depicting the GFP reporters used in the screen. Left: PEST-GFP-H2B fusion protein driven by a germline promoter under the control of a 3'UTR containing LREs. Right: PEST-GFP-H2B fusion protein driven by a germline promoter under the control of a 3'UTR containing OBSs. LIN-41 associates with this 3'UTR either by binding to OMA or other associated proteins. **B)** Micrographs of *C. elegans* young adults treated with *lin-41*, *oma* or mock RNAi, showing LRE and OBS reporter expression in oocyte nuclei (yellow arrows indicate gonad regions where the reporter is expressed). Yellow dotted line demarcates one arm of the *C. elegans* gonad. Scale bar = 50 µm. At least 30 worms were tested for each condition. **C)** List of protein partners, upon knockdown of which the LRE reporter was derepressed **D)** List of protein partners, upon knockdown of which the OBS reporter was derepressed.

As there is evidence that LIN-41 and OMA work together at least on some targets, we knocked down all identified protein partners in both the LRE and OBS reporters. The complete list of protein partners that were tested in the screen is in **appendix 2**. The list of all the proteins that were found to regulate the expression of LRE and OBS reporters are listed in **Figure 3.1 C-D**.

The protein partners that regulate the expression of LRE reporter do not belong to any common complex or pathway. By contrast, we found that multiple protein partners, whose inhibition led to derepression of the OBS reporter belong to a specific protein complex or pathway. Among these *not-1*, *not-2*, *ccr-4* and *ccf-1,* are all components of the CCR4-NOT deadenylase complex, and *aak-1*, *atx-2,* and *ifet-1* are components of the mTORC1-4EBP pathway.

Interestingly, knockdown of no single protein affected expression of both LRE and OBS reporter. This suggests that direct targets of LIN-41 (mRNAs containing LREs) are regulated by different mechanisms as compared to indirect targets of LIN-41 (mRNAs containing OBSs). Going forward in this project we decided to focus only on the mechanisms regulating the OBS reporter.

3.2 CCR4-NOT deadenylase complex regulates mRNA with OBS in their 3´UTRs

RBPs often recruit deadenylase complexes to repress specific mRNAs (Temme et al., 2014). The CCR4-NOT complex consists of one core module and accessory factors. The proteins that comprise the core module of the deadenylase complex are CCR-4, CCF-1, and NOT-1 **(Figure 3.2 A)**. NOT-1 serves as a scaffolding protein that anchors the accessory factors to the complex, and CCR4 and CCF1 are exonucleases. In the functional screen, the knockdown of *not-1*, *not-2*, *not-3, ccr-4* and *ccf-1* resulted in a mild derepression in the developing oocytes of the OBS reporter **(Figure 3.2 B)**.

To test the scale at which deadenylation is contributing to mRNA repression, we performed RT-qPCR to quantify total and polyadenylated reporter (GFP) mRNA in control versus *LIN-41* and *oma* knockdown animals. RT-qPCR results using whole worm extracts from different RNAi experiments were not statistically significant. This is mostly due to inconsistency in RNAi efficacy and slightly varying developmental stages within the worm population. There is not much difference at total mRNA levels. However, there is around a 2-fold increase in the amount of polyadenylated reporter mRNA when LIN-41 and OMA are knocked down **(Figure 3.2 C)**. This suggests that, in the absence of OMA and LIN-41, the deadenylase complex is not recruited to the mRNA and the polyadenylated mRNA is available for translation. Taken together this slight increase in the levels of polyadenylated mRNA of the GFP reporter suggests that the CCR4-NOT complex has a rather minor role in the regulation of OMA targets (mRNAs containing OBSs in their 3´UTR).

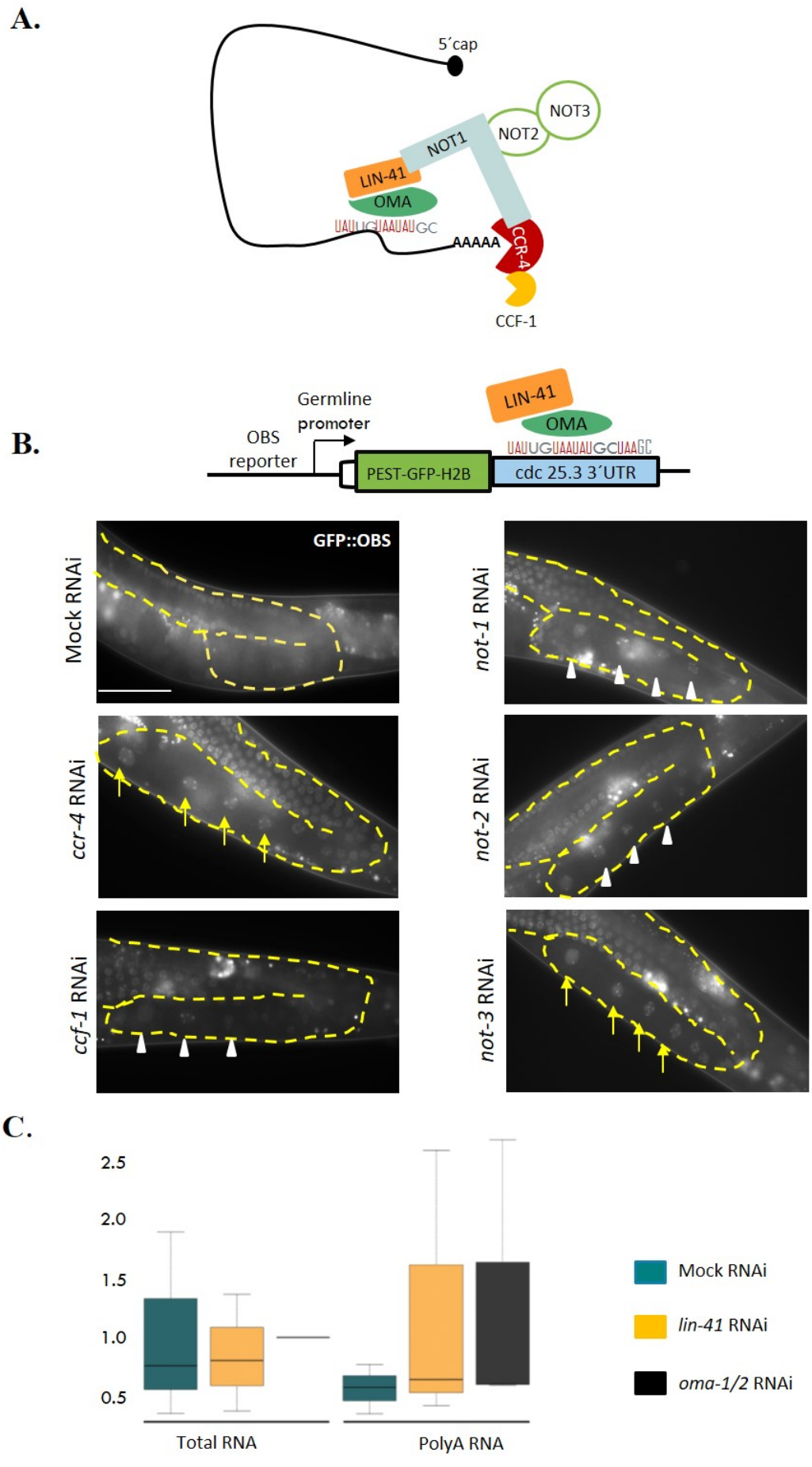

A.
5´cap
NOT3
NOT2
NOT1
LIN-41
OMA
UAUUGUAAUAUGC
AAAAA
CCR-4
CCF-1
B.
Germline
promoter
OBS
reporter
LIN-41
OMA
UAUUGUAAUAUGCUAAGC
PEST-GFP-H2B
cdc 25.3 3´UTR
GFP::OBS
Mock RNAi
not-1 RNAi
ccr-4 RNAi
not-2 RNAi
ccf-1 RNAi
not-3 RNAi
C.
2.5
2.0
1.5
1.0
0.5
Total RNA
PolyA RNA
Mock RNAi
lin-41 RNAi
oma-1/2 RNAi

Figure 3.2 OBS reporter is regulated by CCR4-NOT deadenylase complex

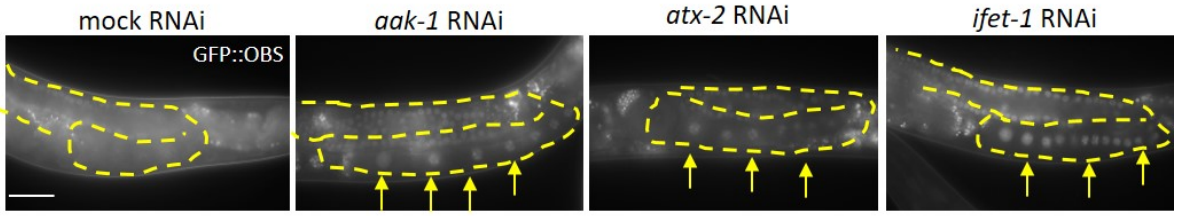
A) Schematics depicting recruitment of CCR4-NOT deadenylase complex to the OBS reporter mRNA via the RBPs. **B)** Micrographs of *C. elegans* young adults treated with *ccr-4*, *ccf-1*, *not-1*, *not-2* and mock RNAi, showing OBS reporter expression in nuclei (yellow arrows indicate gonad regions where the reporter is expressed). The yellow dotted line demarcates one arm of the *C. elegans* gonad and white arrow heads indicates weak GFP expression. At least 30 worms were tested for each condition. Scale bar = 50 µm. **C)** Box and whisker plot from RT-qPCR experiment showing an increase of polyadenylated mRNA upon knockdown of *lin-41* and *oma*. The plot represents data from four biological replicates.

3.3 mTORC1-4EBP pathway regulates mRNA with OBS in their 3´UTRs

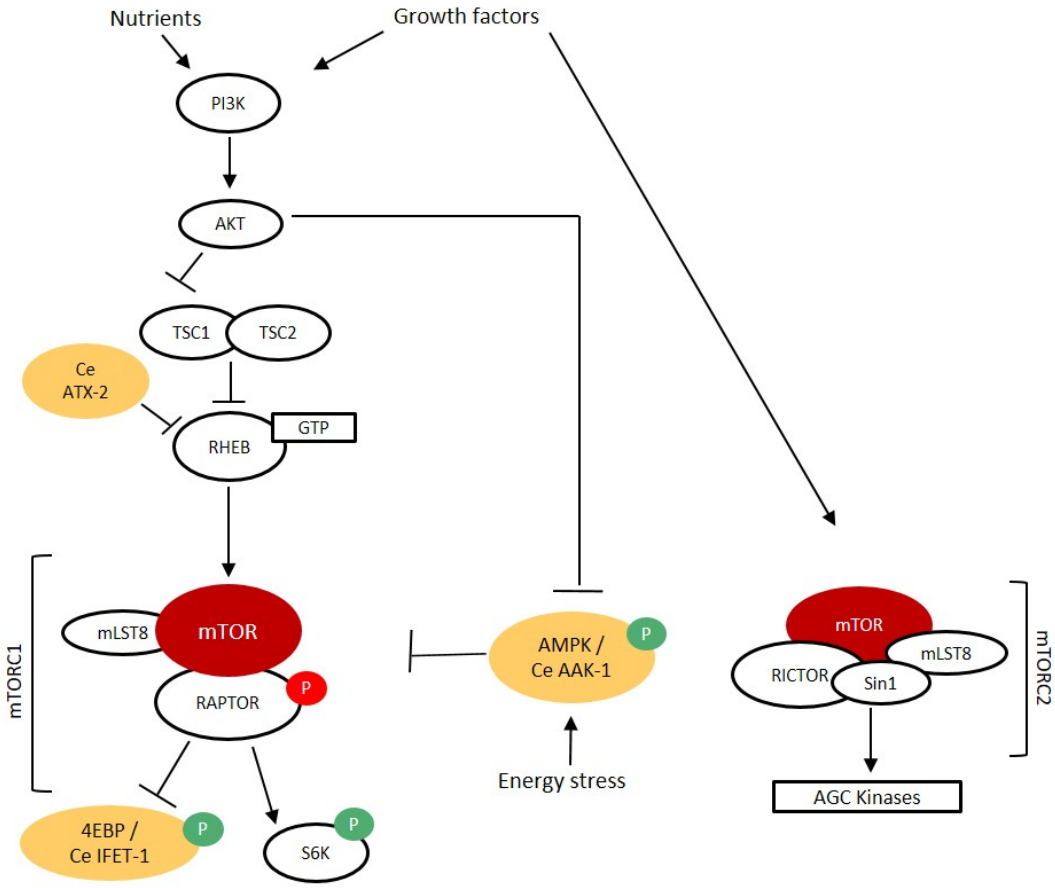
In the functional screen, the strongest derepression of the OBS reporter was observed upon knockdown of three genes, *aak-1, atx-2* and *ifet-1* **(Figure 3.3 A)**. Interestingly, these three genes belong to the mTORC1-4EBP pathway. In this pathway, AAK-1 and ATX-2 are inhibitors of mTORC1 (Bar et al., 2018), and IFET-1 which is, a 4EBP (Huggins et al., 2020), is negatively regulated by mTORC1 **(Fig 3.3 B)**. Therefore, we hypothesized that the RBPs recruit inhibitors of mTORC1 to repress mRNA translation. In the absence of RBPs or the mTOR inhibitors (ATX-2, AAK-1), mTORC1 will get access to the mRNA activating its translation.

To confirm this, we looked for other genetic means to activate/upregulate mTORC1. mTOR is a PI3K-related kinase forming two distinct protein complexes, referred to as mTORC1 and mTORC2 (Qin et al., 2016). RICT-1 functions as an adaptor protein of mTORC2. Upon knockdown of *rict-1*, there is a shift in equilibrium between the two mTOR complexes towards mTORCI. This results in the upregulation of mTORC1 activity **(Figure 3.3 C)** (Nukazuka et al., 2011). Therefore, we checked if the knockdown of *rict-1* will have the same effect on the OBS reporter as the knockdown of *aak-1* and *atx-2*. Indeed, we observed derepression of GFP in developing oocytes of *rict-1* RNAi treated animals **(Figure 3.3 C)**. Taken together, this suggests that OMA and LIN-41 likely repress the OBS reporter by recruiting IFET-1 (4EBP) to the RNP. The repressive RNP also contain mTORC1 inhibitors which may function to continue the repression by preventing inactivation of IFET-1 via mTORC1.

A.


mock RNAi
GFP::OBS
aak-1 RNAi
atx-2 RNAi
ifet-1 RNAi

B.


Nutrients
Growth factors
PI3K
AKT
TSC1
TSC2
Ce
ATX-2
RHEB
GTP
mTORC1
mLST8
mTOR
RAPTOR
P
4EBP /
Ce IFET-1
S6K
AMPK /
Ce AAK-1
Energy stress
mTOR
RICTOR
mLST8
Sin1
mTORC2
AGC Kinases

C.

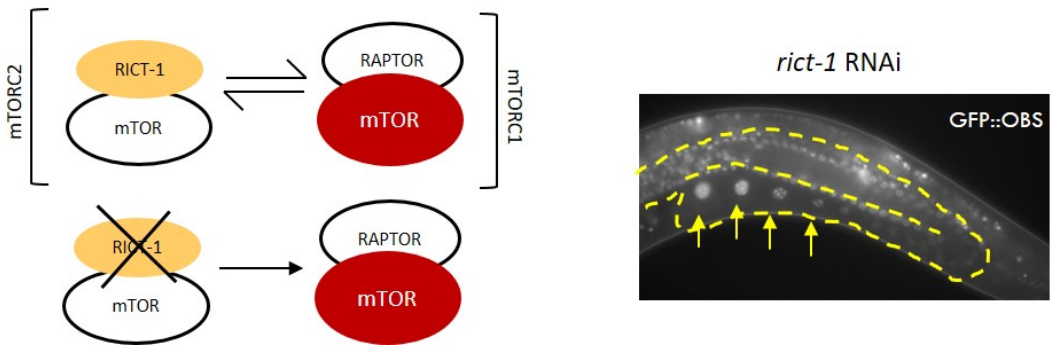

mTORC2
RICT-1
mTOR
RAPTOR
mTOR
mTORC1
RICT-1
mTOR
RAPTOR
mTOR
rict-1 RNAi
GFP::OBS

Figure 3.3 OBS reporter is regulated by 4EBP and mTORC1 inhibitors

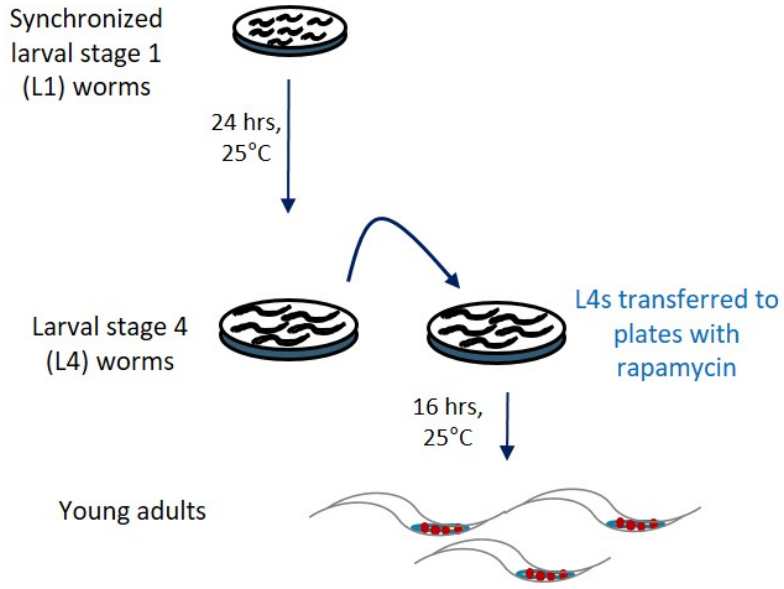
A) Micrographs of *C. elegans* young adults treated with *aak-1*, *atx-2*, *ifet-1* and mock RNAi, showing OBS reporter expression in nuclei (yellow arrows indicate gonad regions where the reporter is expressed). Yellow dotted lines demarcate one arm of the *C. elegans* gonad. At least 30 worms were tested for each condition. Scale bar = 50 µm. **B)** A simplified schematics depicting the mTORC1-4EBP pathway. The components identified in the functional screen are colored in orange. Green phosphorylation events result in activation of translation whereas red phosphorylation events result in repression of translation. **C)** Schematics depicting the shift of equilibrium from mTORC2 to mTORC1 upon knock down of *rict-1* (Nukazuka et al., 2011). Micrograph of *C. elegans* young adults treated with *rict-1* RNAi, showing OBS reporter expression in nuclei (yellow arrows indicate gonad regions where the reporter is expressed). At least 30 worms were tested for each condition. Scale bar = 50 µm.

3.4 Rapamycin treatment prevents re-activation of OBS reporter in the absence of OMA

Our results so far suggest that active mTORC1 is required for translational reactivation of the OBS reporter in the absence of repressive RNPs. Therefore, we reasoned that inhibition of mTORC1 may result in continued repression even in the absence of repressive RNPs. To further investigate the role of mTORC1 in OMA and LIN-41 mediated mRNA regulation, we inactivated mTORC1 in the absence of repressive RNPs. As it is known that rapamycin, together with its receptor, binds to the mTORC1 complex and inhibits signaling to downstream targets (Saxton & Sabatini, 2017), we used this drug to repress mTORC1 activity. In this experiment, OBS reporter animals were grown on control or *oma* RNAi plates. Synchronized L1s were grown till L4 stage, and then L4 staged animals were transferred to respective RNAi plates containing rapamycin. When the animals developed to young adult stage, we analyzed them for expression of GFP in the developing oocytes **(Fig 3.4 A)**.

In control RNAi condition, the OBS reporter was not expressed in either control-treated or rapamycin-treated animals. The existence of embryos in these animals indicated that treatment with rapamycin did not affect the development of the animals severely **(Fig 3.4 B)**. Upon *oma* RNAi treatment the OBS reporter was derepressed in control-treated animals. However, in rapamycin treated animals, *oma* knockdown resulted in much weaker derepression of GFP expression. The GFP expression in the oocytes of rapamycin-treated animals showed a varying range, from weak to none **(Fig 3.4 B)**. Taken together, these results suggest that in the absence of repressive RNPs, active mTORC1 is required for reactivation of translation.

A.


Synchronized
larval stage 1
(L1) worms
24 hrs,
25°C
Larval stage 4
(L4) worms
L4s transferred to
plates with
rapamycin
16 hrs,
25°C
Young adults

B.

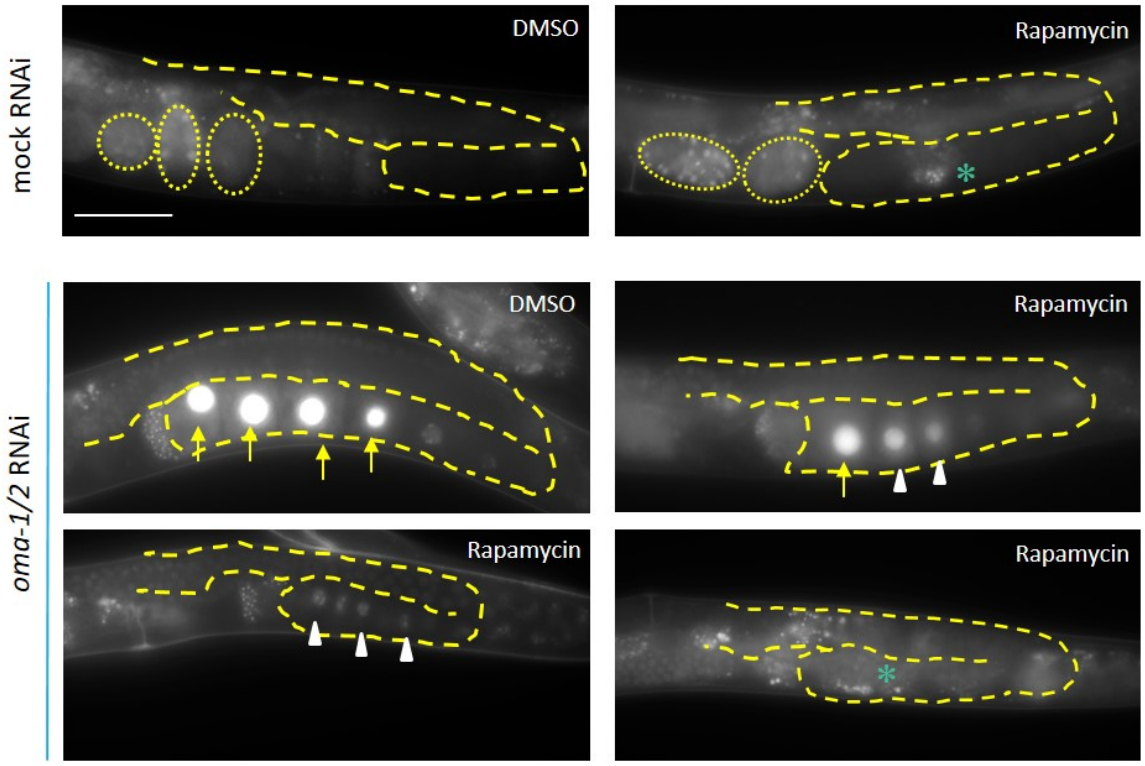

mock RNAi
DMSO
Rapamycin
oma-1/2 RNAi
DMSO
Rapamycin
Rapamycin
Rapamycin

Figure 3.4 Rapamycin treatment interferes with reactivation of translation upon knockdown of *oma*

A) Schematics depicting the protocol for rapamycin treatment. Synchronized L1s were transferred to plates containing rapamycin upon reaching L4 stage, and observed after reaching young adult stage. **B)** Micrographs of *C. elegans* young adults treated with rapamycin, showing OBS reporter expression in nuclei (yellow arrows indicate gonad regions where the reporter is expressed). Yellow dotted line demarcates one arm of the *C. elegans* gonad and white arrow heads indicate weak GFP expression. Scale bar = 50 µm. The presence of embryos in mock RNAi treated animals (indicated by yellow circles) showed that rapamycin treatment did not arrest the development of the worms. Mock treated animals showed no derepression of OBS. *oma* RNAi treated animals showed weak or no activation of OBS in oocytes after treatment with rapamycin, as compared to DMSO (control). At least 30 worms were tested for each condition. Green stars indicate auto fluorescence.

3.5 Controlled starvation prevents re-activation of OBS reporter in the absence of OMA

In addition to the pharmacological means of inactivating mTORC1 by using rapamycin, we wanted to test a physiological condition that inactivates mTORC1. We discovered *aak-1* in our original screen. AAK-1 is the AMP kinase that inactivates mTORC1. AAK-1 itself is activated by low energy levels (Kim et al., 2016). So, we decided to limit the energy levels in *C. elegans* by controlled starvation. We optimized the starvation protocol so that we provided just enough food so that the animals develop to adulthood and make viable progeny.

In this experiment, OBS reporter animals were grown on control or *oma* RNAi plates. Synchronized L1s were grown till L4 stage, and then L4 staged animals were transferred to plates containing reducing amounts of food (OP50 bacteria). Once the animals developed to young adult stage, we analyzed them for expression of GFP in the developing oocytes **(Fig. 3.5 A)**.

In control RNAi conditions, the OBS reporter is not expressed in either well-fed or starved animals. The existence of embryos indicated that nutrient deprivation did not fully interfere with the proper development of the animals **(Fig. 3.5 B).** Upon *oma* RNAi treatment the OBS reporter was derepressed in well-fed animals. However, in starved conditions, *oma* knockdowns resulted in much weaker activation of GFP expression. The GFP expression in the oocytes of starved animals showed a varying range from weak to none **(Fig. 3.5 B)**. Taken together these results suggest that conditions of limited nutrition, which generally inactivates mTORC1, lead to continued repression of OBS reporter even in absence of repressive RNPs.

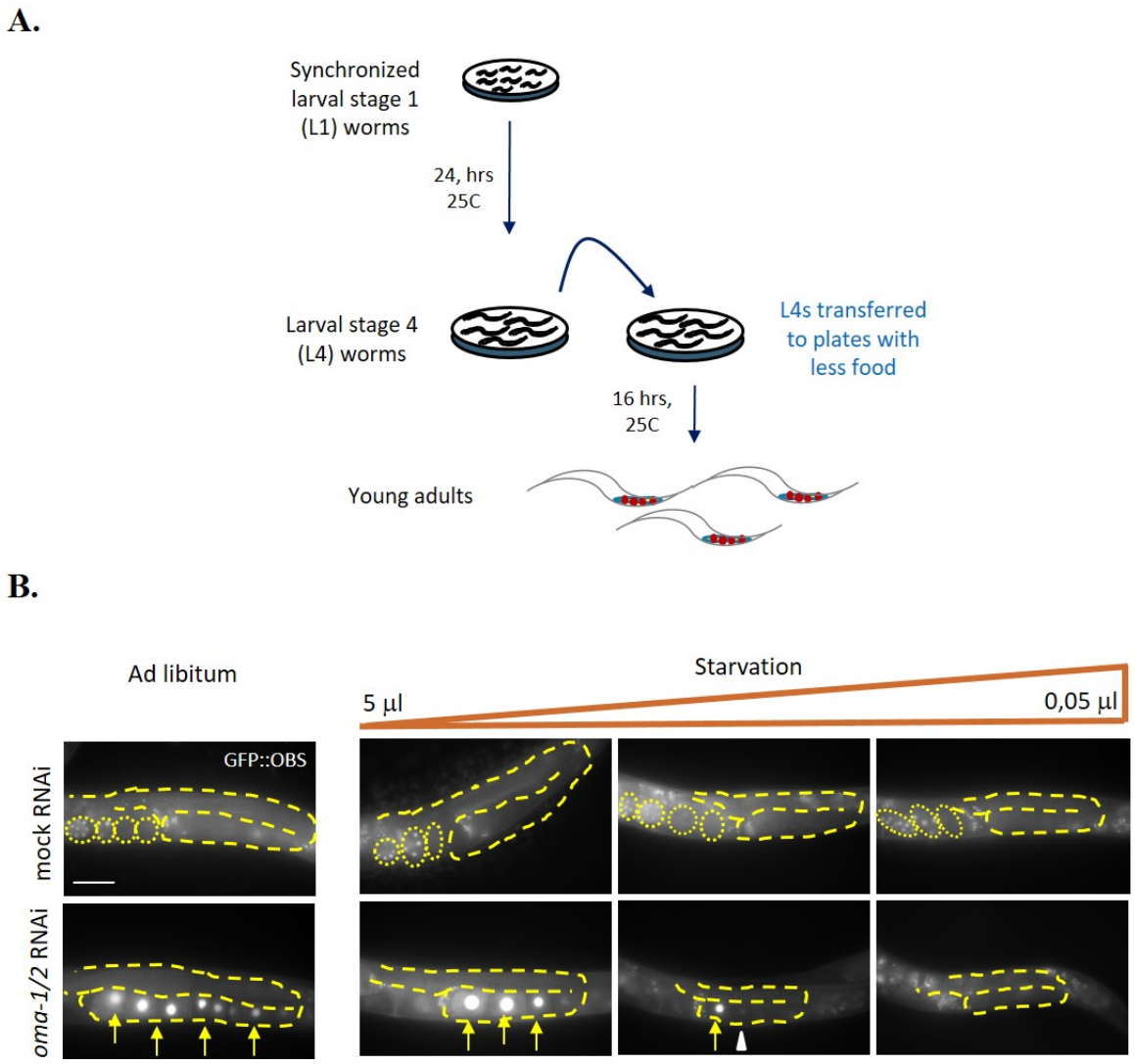


Figure 3.5 Controlled starvation interferes with reactivation of translation upon knockdown of *oma*

A) Schematics depicting the protocol for controlled starvation. Synchronized L1s were transferred to plates containing lesser amounts of food upon reaching L4 stage and were observed after reaching young adult stage. **B)** Micrographs of *C. elegans* young adults after controlled starvation, showing OBS reporter expression in nuclei (yellow arrows indicate gonad regions where the reporter is expressed. Yellow dotted line demarcates one arm of the *C. elegans* gonad and white arrow head indicate weak GFP expression. Scale bar = 50 µm. The presence of embryos in mock RNAi treated animals (indicated by yellow circles) show starvation did not arrest the development of the animals. Mock treated animals show no derepression of OBS. *oma* RNAi treated animals, show weaker derepression of OBS in oocytes after controlled starvation as compared to well-fed conditions. At least 30 worms were tested for each condition.

3.6 Specific regulation of translation by the mTORC1 complex

mTORC1 signaling works downstream of physiological cues, like nutrition and energy levels, and is known to regulate translation on a global level (Saxton & Sabatini, 2017). However, our functional screen showed that the knockdown of *aak-1*, *atx-2* and *ifet-1* derepressed only the OBS reporter but not the LRE reporter. This shows that mTORC1 pathway is not regulating all mRNAs in developing oocytes. We wanted to further investigate mTORC1 specificity in terms of mRNA regulation in the *C. elegans* germline.

Several mRNAs are spatially regulated post-transcriptionally in the *C. elegans* germline (Diag et al., 2018). In order to test the specificity of mTORC1 mediated regulation, we decided to use mRNA expressed in three different regions of the germline. We chose the mRNAs: *glp-1, gld-1,* and *pal-1* whose expression is spatially restricted to the distal, medial and proximal part of the gonad respectively **(Fig 3.6 A)**. We made use of germline GFP reporters (fused to PEST-H2B) controlled by these UTRs to analyze the role of mTORC1 pathway in their regulation. We activated mTORC1 in these reporter lines by knocking down *aak-1, atx-2* and *rict-1*.

Upon activation of mTORC1 via RNAi mediated knockdown of repressors, the expression pattern of these reporters remained nearly the same as in control RNAi treated animals **(Fig 3.6 B).** Very mild derepression can be observed in *glp-1* reporter in the oocytes upon knockdown of *atx-2* and *rict-1.* However, the derepression in these animals was not consistent. Since expression of these reporter mRNAs is not substantially affected by mTORC1 activation, the overall results suggest that there is certain level of specificity in translational regulation by mTORC1 in developing oocytes.

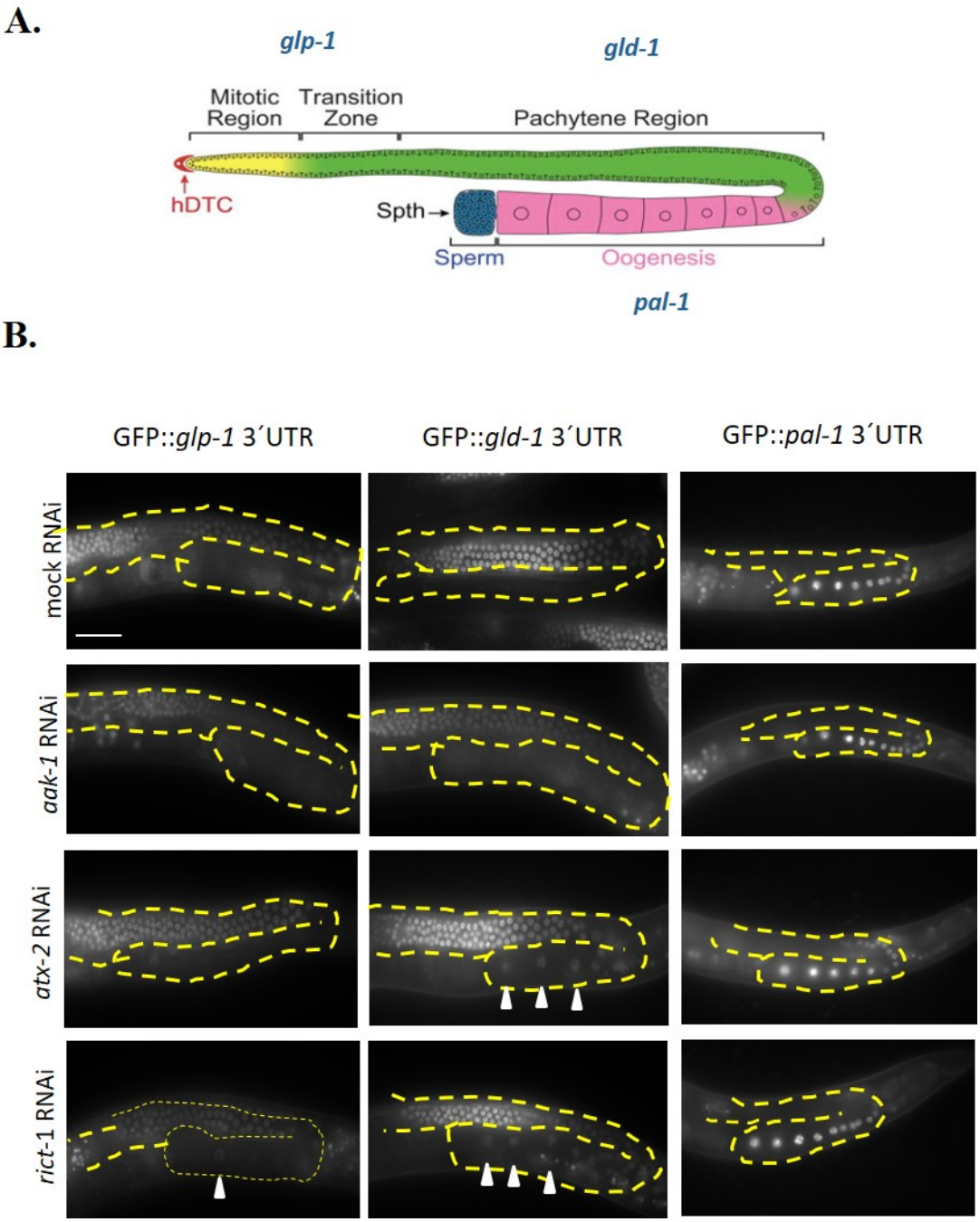


Figure 3.6 Specificity of mRNA regulation via mTORC1 complex

A) Schematic depicting the *C. elegans* germline. *glp-1* expression is restricted to the distal part of the gonad, *gld-1* expression is restricted in the medial part, and *pal-1* expression is restricted to the proximal part of the gonad. [Figure adapted from: (Morgan et al., 2010)]. **B)** Micrographs of *C. elegans* young adults treated with *aak-1*, *atx-2*, *rict-1* and mock RNAi, showing OBS reporter expression in nuclei (yellow arrows indicate gonad regions where the reporter is expressed). Yellow dotted line demarcates one arm of the *C. elegans* gonad and white arrow heads indicate weak GFP expression. Scale bar = 50 µm. At least 30 worms were tested for each condition.

4. Discussion

4.1 Reporters for functional screen to understand the mechanism underlying mRNA regulation by LIN-41 and OMA proteins

For studying regulation via OMA proteins, we used a reporter with a 3′ UTR with OBSs. This 3′ UTR is from *cdc-25.3*, a gene expressed in the germline. Most known direct targets of OMA like *cdc-25.3, zif-1, rnp-1,* etc. are regulated by both OMA and LIN-41 (Detwiler et al., 2001). Using a native OMA target in the germline as a candidate, we found two conserved mRNA regulating pathways that play a role in OMA and LIN-41 mediate mRNA regulation.

For studying regulation via direct binding of LIN-41 to mRNAs, we used a reporter with a 3' UTR with LREs. As currently there is no known validated direct target of LIN-41 in the germline we used the 3' UTR of *mab-10,* which is a somatic target of LIN-41. This 'non-native' target of LIN-41 is regulated as expected in the germline but the expression level as such was very low. Furthermore, using this reporter, we were not able to find many protein partners that function in its regulation. Also, the identified regulators did not belong to any common complex or pathway. It is possible that because of its 'somatic properties' it is not expressed properly in the germline. The overall results from the screen suggests that the LRE reporter may not be a good reporter to use in our screen. In future, when a LIN-41 target in the germline is validated, we can repeat the screen to identify more players participating in regulation of LIN-41 direct targets.

4.2 Different mechanisms deployed by LIN-41

Results from our functional screen suggests LIN-41 regulates its direct (via binding through LREs) and indirect (via binding through OMA or associated proteins) targets by independent mechanisms as no single protein was involved in the regulation of both LRE and OBS reporters. This is intriguing that the same RBP can elicit different mechanisms depending on the nature of binding to mRNA, either direct or indirect. However, at this point we do not have further insight into the mechanisms involved in the regulation of direct LIN-41 targets.

4.3 Specific translational regulation by mTORC1

The association of LIN-41 and OMA with mTORC1 pathway components and our analysis suggest that these RBPs likely repress translation by recruiting a 4EBP (IFET-1). Recruitment of 4EBP to an mRNA will prevent the binding of eIF4G to the cap complex and subsequent recruitment of ribosomes. In this way 4EBP acts as a translational repressor that prevents the interaction between the mRNA and the translational machinery (Richter & Lasko, 2011). However, upon removal of 4EBP, eIF4G facilitates the circularization of mRNA through PABP and the translational machinery is recruited to the mRNA (Qin et al., 2016). One way to remove 4EBP from the cap complex is by phosphorylating it as phosphorylation renders it inactive to bind to eIF4E. PI3-kinase/Akt pathway and mTOR kinase pathways are known to phosphorylate 4EBP (Qin et al., 2016).

IP experiments showed that LIN-41 and OMA proteins also associate with AAK-1 and ATX-2. Both AAK-1 and ATX-2 have been shown to function as inhibitors of mTORC1 (Bar et al., 2018). We hypothesize that inhibition of mTORC1 via these proteins may be required for a continued repression of translation. Inactivation of mTORC1 by rapamycin treatment and controlled starvation prevented reactivation of OBS reporter translation in *oma* RNAi treated animals.
These results suggest that active mTORC1 is required for reactivation of translation and mTORC1 might be functioning via phosphorylating 4EBP.

mTORC1 is known to regulate translation on a global level (Muckenthaler & Preiss, 2006). Our functional screen showed that the OBS reporter is regulated via mTORC1 pathway but not the LRE reporter suggesting some levels of specificity. To test the specificity further, we also analyzed three more mRNA reporters (*glp-1, gld-1* and *pal-1*) that are expressed in a spatially restricted manner in the germline. We found that activation of mTORC1 via different genetic means did not affect the expression of those reporters significantly. Therefore, instead of the general perception that mTOR signaling regulates global cellular translation, our results suggest that specific mRNAs can also be regulated via the mTORC1 pathway.

We can imagine two scenarios by which the mRNA is being kept in a translationally repressed state. In the first scenario, the RBPs, LIN-41 and OMA, may sequester the mRNA in a local environment in the cytoplasm which is inaccessible to mTORC1. In this local environment the translation is repressed by the 4EBP, IFET-1 **(Figure 4.1 A).** In the second scenario, mTORC1

inhibitors, AAK-1 and ATX-2, are recruited to the mRNA and they continuously inactivate mTORC1 in the vicinity of the mRNA. Thus, continuing the translational repression **(Figure 4.1 B)**.

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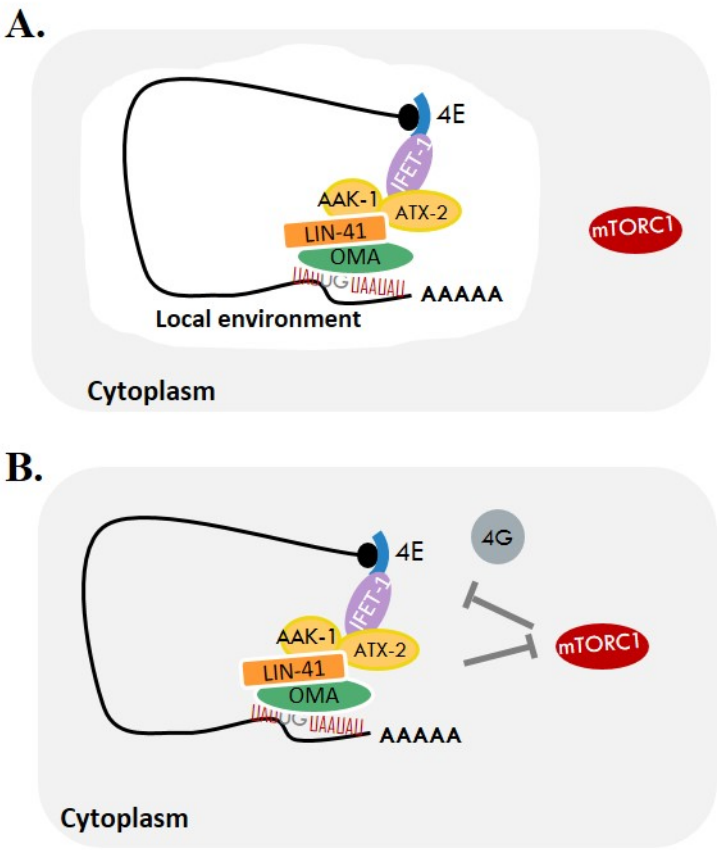

Figure 4.1 Schematics presenting possible mechanisms of translational repression

A) OMA and LIN-41 recruit IFET-1 (4EBP) to repress the translation of target mRNA. The target mRNA is sequestered in a local environment in the cytoplasm, which is inaccessible to mTORC1, thus preventing translation reactivation. B) OMA and LIN-41 recruit IFET-1 (4EBP) to repress the translation of target mRNA. mTORC1 inhibitors, AAK-1 and ATX-2, present in the RNP inhibit mTORC1 and prevent it from inactivating IFET-1, thus continuing translational repression. The balance between the two inhibitory events (inactivation of mTORC1 vs inactivation of IFET-1) will decide the fate of the mRNA.

5. Conclusion and future prospects

OMA and LIN-41 are RBPs functioning in developing oocyte. They regulate oocyte maturation and OET via post-transcriptional regulation of target mRNAs. LIN-41 co-regulates several OMA targets by associating with them through either OMA or other proteins. LIN-41 also regulates mRNAs via direct binding. In order to understand how LIN-41 and OMA proteins regulate mRNA targets we identified their partner proteins and analyzed their roles in mRNA regulation. We did not get any insight into protein complexes that regulate direct targets of LIN-41. In future, our functional screen could be repeated with a valifated germline direct target of LIN-41. We found that an mRNA containing OBSs in its 3'UTR which is regulated by both OMA and LIN-41 proteins is regulated via CCR4-NOT deadenylase pathway and mTORC1-4EBP pathway.

The CCR4-NOT deadenylase complex plays a minor role in regulation of the OBS reporter. RT-qPCR experiments conducted to observe the status of poly(A)- tail of the reporter mRNA did not yield significant results likely due to inconsistency in sampling. For better qPCR results, worm gonads could be dissected to avoid the presence of embryos in the samples. In addition, a PAT (poly(A) test) could be carried out to measure the length of the poly(A)-tail of the reporter mRNA in different conditions (Sallés & Strickland, 1999). These experiments will give us a better idea about the contribution of deadenylation in regulation of OBS reporter.

The OBS reporter is mainly repressed by the 4EBP, IFET-1. Our results suggest the mRNA requires active mTORC1 signaling for reactivation of translation. We currently have two models wherein either the mRNA is physically sequestered from mTORC1 or mTORC1 inhibitors present in the RNP continually inhibit mTORC1. To differentiate between these two scenarios, we need to have a way to visualize mTORC1 and mTORC1 activity in the germline. If we see that the OBS reporter is sequestered in intracellular zones where mTORC1 is absent the first scenario would be correct. If we see that mTORC1 activity is inhibited close to OBS reporter then the second scenario is correct.

6. References

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Appendix 1 - Buffer and media

10X M9 buffer (10 L)

KH_2PO_4	300 g
$Na_2HPO_4 \cdot 2H_2O$	752 g
NaCl	500 g
$MgSO_4 \cdot H_2O$	4.937 g

Dissolve substances in 7.5 L ddH_2O and then make up to 10 L with ddH_2O.

To make 1X M9 buffer: dilute 10X M9 buffer in ddH_2O and autoclave before use.

Bleaching solution (1 L)

Sodium hypochlorite, 5% Chlorine	100 ml
5M KOH	50 ml
ddH_2O	850 ml

Store at 4 °C.

NG 2% plates (5 L)

ddH_20	4.9 L
Difco-Agar, Granulated (BD 214530)	100.0 g
Bacto-Peptone (BD 211677 / BD 211820)	12.5 g
NaCl	15.0 g

Autoclave and cool down to 50 °C, then add:

Cholesterol (5 mg/ml EtOH) (sterile) (Sigma C3045)	5.0 ml
1.0 M $CaCl_2$ (sterile)	5.0 ml
1.0 M $MgSO_4$ (sterile)	5.0 ml
1.0 M Potassium phosphate buffer, pH 6.0 (sterile)*	125.0 ml

And pour the plates.

*1 M Potassium phosphate buffer is made by mixing 132 ml of 1 M K_2HPO_4 and 868 ml of 1 M KH_2PO_4 / 1M KH_2PO_4, pH 6.0 (adjusted by KOH)

RNAi plates - NG 2% plates with 1 mM IPTG and 50 μg/ml Carbenicillin (5 L)

ddH_20	4.9 L
Difco-Agar, Granulated (BD 214530)	100.0 g
Bacto-Peptone (BD 211677 / BD 211820)	12.5 g
NaCl	15.0 g

Autoclave and cool down to 50 °C, then add:

Cholesterol (5 mg/ml EtOH) (sterile) (Sigma C3045)	5.0 ml
1.0 M $CaCl_2$ (sterile)	5.0 ml
1.0 M $MgSO_4$ (sterile)	5.0 ml
1 M Potassium phosphate buffer, pH 6.0 (sterile)	125.0 ml
1 M IPTG (sterile)	5.0 ml
Carbenicillin (100 mg/ml) (sterile)	2.5 ml

And pour the plates.

LB plates with Ampicillin and Tetracycline (1 L)

Yeast extract	5 g
NaCl	10 g
Tryptone peptone	10 g
ddH_2O	1 L

Adjust pH to 7.2 with 5 M NaOH

Add 15 g Agar

Autoclave and cool down to 50 °C, then add:

100 mg/ml Ampicillin	1 ml
12.5 mg/ml Tetracycline	1 ml

And pour the plates

Appendix 2 – List of protein partners tested in the functional screen

Table 7.1 Table with complete list of protein partners tested in the functional screen using LRE and OBE reporter. 'No' indicates no GFP derepression; 'yes' indicates GFP derepression; + and ++ indicate stronger GFP derepression.

	LRE reporter	OBS reporter
PAN-2	No	No
PAN–3	No	No
PAN–2/3	No	No
GLD–2	No	No
RNP–8	No	No
CPB–3	No	Yes
MEX–5	No	No
MEX–6	No	No
ALG–1	No	Yes
ALG-2	No	No
PUF–3	No	No
PUF–5	No	No
PGL-1	No	Yes (+)
PQN–21	No	No
NFYB–1	No	Yes (+)
C06E1.9	Yes	No
GEI–4	No	Yes
SEC–16	No	No
GLA–3	No	No
MTR–4	No	Yes
HLH–1	No	No
C34C12.2	No	No
SPAT–2	No	No
PHAT–4	Yes (+)	No
SDHA–2	No	No
NRDE–2	No	Yes
C16A3.4	No	No

C17E7.4	No	No
F16B12.6	Yes	No
F56C.9	No	No
XND–1	No	Yes
UBR–1	No	Yes
SCD–1	No	Yes
SRG–22	No	Yes
AAK–1	No	Yes (++)
PQN–39	No	Yes
T16H12.4	No	Yes
PQN–59	No	No
DKF–2	No	Yes
LGL–1	No	Yes
ATX–2	No	Yes (++)
UCR –2.3	No	No
RICT – 1	No	Yes (++)