ABSTRACT

Selective Recruitment of Germ Cell mRNAs by eIF4 Factors

By Andrew J. Friday

July 2015

Director: Brett D. Keiper
Department of Biochemistry and Molecular Biology
Brody School of Medicine, East Carolina University

During germ line development critical steps in the proliferation and differentiation of stem cells into oocytes and sperm are regulated by mRNA translational control. This regulation changes the subset of proteins being synthesized, which ultimately alters cell fate. In this dissertation I focused on the role of translation initiation machinery, which we have shown selects discrete subsets of mRNAs to be recruited to ribosomes, as a means to drive cell fate decisions in gametogenesis. The rate-limiting step for mRNA recruitment is the formation of the 48S translation initiation complex. Eukaryotic translation initiation factor eIF4E binds the 7-methylguanosine 5'–cap of mRNAs and recruits them to the 48S by binding to the scaffolding protein eIF4G to form a productive initiation complex. In mammalian cells, increased eIF4E-eIF4G complex leads to up-regulation of cap-dependent translation, which is required for growth and cell division. Cap-dependent mRNA translation has also been shown to be essential for sperm and oocytes development, as well as in the early embryonic cell cycle. In this body of work, I used *C. elegans* as a model for studying the contributions of eIF4 isoforms to germ cell fate decisions. Specifically my studies focused on an eIF4E isoform expressed in the germ line, IFE-1, and the cap-dependent eIF4G isoform, IFG-1 p170, and their regulation of translation initiation during sperm and egg development. Initial studies from our lab indicated that IFE-1 was required to promote maturation of spermatocytes and oocytes. Using a polysomal

bioinformatics approach, I identified 77 mRNAs preferentially translated via IFE-1, several of which have critical known roles germ cell differentiation and maturation (*gld-1, vab-1, vpr-1, rab-7, ran-1, rnp-3*). Using live worm gonad imaging techniques, I further showed IFE-1-dependent selectivity *in situ* for de-repressed mRNAs in both early and late-stage oocytes. These studies outline how IFE-1, in a coordinated effort with IFG-1 p170, exerts positive mRNA translation control toward the selective expression of germ cell proteins that influence cell fate decisions.

Selective Recruitment of Germ Cell mRNAs by eIF4 Factors

A Dissertation

Presented to

The Faculty of the Department of Biochemistry and Molecular Biology

East Carolina University

In Partial Fulfillment

Of the Requirements for the Degree

Doctor of Philosophy of Biochemistry and Molecular Biology

By

Andrew J. Friday

July 2015

Selective Recruitment of Germ Cell mRNAs by eIF4 Factors

By

Andrew J. Friday

APPROVED BY:

DIRECTOR OF DISSERTATION:_____

Brett D. Keiper, PhD

COMMITTEE MEMBER:_____

Kyle D. Mansfield, PhD

COMMITTEE MEMBER:_____

Lance D. Bridges, PhD

COMMITTEE MEMBER:_____

Christopher B. Geyer, PhD

CHAIR OF THE DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY:

_____

Joseph M. Chalovich, PhD

DEAN OF THE GRADUATE SCHOOL:_____

Paul J. Gemperline, PhD

Dedication:

To my amazing family for always supporting and encouraging me in all of my pursuits.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

4EBP	eIF4E binding protein
Apaf-1	apoptotic protease activating factor
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X protein
Bcl	B-cell lymphoma
BH	Bcl-2 homology domain
Bid	BH3 interacting-domain death agonist
BiP	binding immunoglobulin protein
caspase	cysteine aspartate directed protease
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CED	cell death defective
CGH	conserved germline helicase
CPE	cytoplasmic polyadenylation element
CPEB	CPE binding factor
CPSF	polyadenylation specificity factor
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DIC	differential interference contrast
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
eIF	eukaryotic initiation factor
ER	endoplasmic reticulum
FBF	Fem-3 mRNA binding factor
FPKM	fragments per kilobase of exon per million fragments
G1/G2	Gap 1/2
GFP	green fluorescent protein
GLD-1	Germ Line Development 1
GLP-1	abnormal germ line proliferation
gpd/GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSC	germline stem cell
H2B	histone 2B
IFE	*C. elegans* eIF4E isoform
IRES	internal ribosome entry site
MEX-3	Muscle Excess 3
MFP	mean fold change in polysome signal
MFR	mean fold change in R-value
MFT	mean fold change in total signal
Mnk-1	MAP kinase-interacting serine/threonie-protein kinase 1
MosSCI	mos1-mediated single copy insertion

mRNA	messenger ribonucleic acid
mRNP	messenger ribonucleoprotein
mTOR	mammalian target of rapamycin
NGM	nematode growth medium
OMA	oocyte maturation defective
ORF	open reading frame
p53	tumor suppressor p53
PAP	poly(A) polymerase
PABP	poly(A) binding protein
Pgk2	phosphoglycerate kinase
PGL-1	P GranuLe abnormality-1
PMSF	phenylmethylsulfonyl fluoride
POS	posterior segregation
PTBP2	polypyrimidine tract binding protein 2
PVDF	polyvinylidene fluoride membrane
qPCR	quantitative reverse transcription polymerase chain reaction
RAB-7	Rab GTPase
RAN-1	Ran GTPase
RBP	RNA binding protein
RNAi	RNA interference
RNASeq	RNA Sequencing
RNP	ribonucleoprotein
SDS	sodium dodecyl sulfate
SSC	spermatogonial stem cell
TBB-2	beta-tubulin
TBE	Tris Borate EDTA
tRNA	transfer RNA
TSAA	translational state array analysis
unc	Uncoordinated
UTR	untranslated region
VAB-1	Variable Abnormal morphology ephrin receptor

Chapter 1: Literature Review

This chapter appears in the review manuscript: *Andrew J. Friday and Brett D. Keiper. Positive mRNA translational control in germ cells by initiation factor selectivity. Biomed Research International 2015. *In Press*

Abstract

Ultimately, the production of new proteins in undifferentiated cells pushes them to new fates. Other proteins hold a stem cell in a mode of self-renewal. In germ cells in lower organisms, these decision-making proteins are produced largely from translational control of pre-existing mRNAs. To date, all of the regulation has been attributed to RNA binding proteins (RBPs) that repress mRNAs in many models of germ cell development (*Drosophila, C. elegans, Xenopus*). We focus on the selective, positive function of translation initiation factors eIF4E and eIF4G, which recruit mRNAs to ribosomes upon de-repression. Evidence now shows that the two events are not separate, but rather are coordinated through composite complexes of repressors and germ cell isoforms of eIF4 factors. Strikingly, the initiation factor isoforms are themselves mRNA selective. The mRNP complexes of translation factors and RBPs are built on specific populations of mRNAs to prime them for subsequent translation initiation. Simple rearrangement of the partners causes a dormant mRNP to become synthetically active in germ cells when and where they are required to support gametogenesis.

1. mRNA translation initiation activity matters for cell identity

Stem cell self-renewal and differentiation programs depend on gene expression. Classically the contribution of transcriptional regulation has predominated, however, there is growing evidence for regulation at the level of protein synthesis (Li and Zhao, 2014; Shyh-Chang and Daley, 2013; Wright and Ciosk, 2013). The central dogma of gene expression states that information moves from DNA to RNA to protein. Regulation of mRNA translation can be important for the differentiation of stem cells into terminal cell types, as it contributes to the identity of the resulting new cell type. Germline stem cells (GSCs) seem to make the greatest use of protein synthetic regulation to guide their differentiation into sperm and eggs (Baker and Fuller, 2007; Datla et al., 2014; Franklin-Dumont et al., 2007). Dysregulation of these protein synthetic events can lead to aberrant developmental defects including infertility, birth defects, and cancers (Berry et al., 1997; Borden, 2011; Cha et al., 2012; Culjkovic et al., 2007; Furic et al., 2010; Scheper et al., 2007; Sun et al., 2010). Proteins in the mRNA translation initiation complex (called "eIFs", eukaryotic Initiation Factors) play a key role in beginning the protein synthesis that leads to such cell fate decisions (Baker and Fuller, 2007; Contreras et al., 2008; Franklin-Dumont et al., 2007). Among these factors, eIF4E, binds the mRNA 7-methyl-GTP cap and recruits bound-mRNA to eIF4G. eIF4G scaffolds the eIF4E-bound mRNA 5'-cap, poly(A) tail, and eIF2, eIF3, eIF4A and the ribosome together. eIF4E and eIF4G together have the critical role of recruiting mRNA to the protein synthetic machinery (Figure 1.1B), and the activity of each have been implicated in multiple cancers. eIF4GI, for instance, is overexpressed in breast and lung cancers and enhances the translation of mRNAs that are involved in survival (Hif-1a, VEGF), cell signaling (cadherins), and DNA damage repair (p53, p53-BP1, PARP)

Figure 1.1

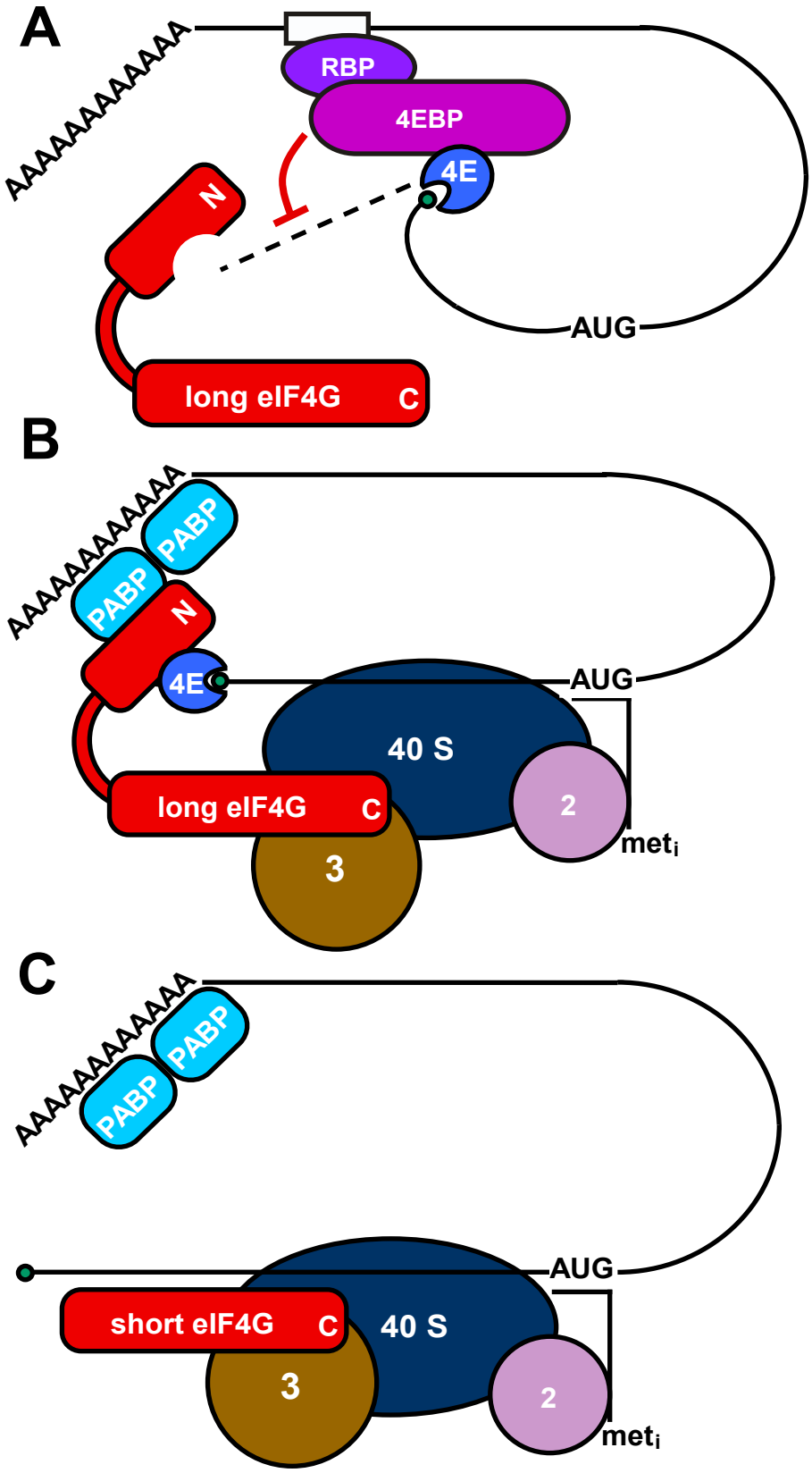

A
AAAAAAAAAA
RBP
4EBP
4E
N
AUG
long eIF4G
C
B
AAAAAAAAAA
PABP
PABP
N
4E
AUG
40 S
long eIF4G
C
2
met$_i$
3
C
AAAAAAAAAA
PABP
PABP
AUG
short eIF4G
C
40 S
3
2
met$_i$

Figure 1.1. Models of mRNA translational repression and translation initiation complexes. (A) mRNAs are translationally repressed by RBPs that bind sequence recognition motifs in the 3'UTR. Protein-protein interactions with 4EBP-eIF4E-mRNA form stable mRNP complexes that inhibit the recruitment of eIF4E-bound mRNA to eIF4G, eIF4A (an mRNA helicase), and the initiation complex. (B) Model of cap-dependent translation initiation utilizing the cap-binding protein eIF4E. Cap-bound mRNAs are recruited to the 40S ribosomal subunit by association with eIFs and PABP. (C) Model of cap-independent translation. The "short" isoform of eIF4G lacking an eIF4E-binding domain is still capable recruiting mRNA to the 40S ribosomal subunit with eIFs by binding directly to the 5' UTR.

(Badura et al., 2012; Bauer et al., 2002; Braunstein et al., 2007; Pei et al., 2011; Silvera et al., 2009; Silvera and Schneider, 2009). High levels of the cap-binding protein eIF4E promote cell growth and proliferation in cancers including ovarian, esophageal, breast, thyroid, and prostate cancers, as well as leukemias (Furic et al., 2010; Ko et al., 2009; Kouvaraki et al., 2011; Rhoads, 1991; Rinker-Schaeffer et al., 1993; Salehi and Mashayekhi, 2006). Association of eIF4E with eIF4G is under the regulation of the kinase mTOR, which phosphorylates and releases 4EBP from sequestering eIF4E (Harris et al., 2006; Hay and Sonenberg, 2004). Many mRNAs stored in germ cells have short poly(A) tails and can be activated in response to poly(A) elongation (Preiss, 2000). RNA binding proteins (RBPs), such as DAZL family members, recruit poly(A) binding protein (PABP) and initiation factors to bind to the mRNA, thus promoting translation initiation (Collier et al., 2005). Mutations in DAZL RBPs expressed in *Drosophila* testes result in limited spermatid differentiation (Eberhart et al., 1996; Ruggiu et al., 1997). Given the numerous circumstantial relationships between mRNA translational control, stem cells, gametogenesis, and cancers, it seems prudent to look for similar mRNA regulatory mechanisms that regulate cellular developmental events such as differentiation, proliferation, maturation, and apoptosis.

2. The protein synthetic needs of germline stem cells (GSCs)

How likely is it that mRNA translation initiation mechanisms are governing germ cell life, given their roles in regulating survival, growth, proliferation and DNA damage repair in oncogenesis (Badura et al., 2012; Bauer et al., 2002; Braunstein et al., 2007; Pei et al., 2011; Silvera et al., 2009; Silvera and Schneider, 2009)? We operationally define *C. elegans* stem cells as a population with the unique ability to be maintained by a self-renewal program, yet equally

capable of differentiating into a spectrum of functional cellular lineages. Somatic stem cells are present at low abundance throughout tissues and generally reside in segregated niches (Hsu and Fuchs, 2012). Their main function is to replenish localized cells following injury or disease and during normal growth. Germline stem cells (GSCs) in *C. elegans*, however, possess the unique ability to self-renew or proliferate before differentiating to enter meiosis to form autonomous gametes capable of fertilization (Kimble and Crittenden, 2006). They are the only truly immortal lineage of cells in most eukaryotes, and their role is to transmit genetic information to future progeny. These GSCs rely in part on *de novo* protein synthesis to drive their potential cell fates that include self-renewal, proliferation, differentiation, or apoptosis. Proliferation guarantees the sustained production of gamete progenitors and a renewable pool of GSCs. Differentiation programs support specialized morphing of progenitors into sperm and eggs. Each program requires novel proteins for meiosis that condense, replicate, recombine, resolve and segregate the chromosomal pool. At the same time, cytoplasmic components to supply the fertilized egg are synthesized or deposited in female germ cells (oocytes), or stripped away from male germ cells (spermatocytes). Lastly, proteins that allow each gamete to recognize, cooperate, and fuse with the other are synthesized during maturation. Apoptosis is also a germ cell fate previously underappreciated for its contribution to gamete development, and is surprisingly active in the germ line (Gumienny et al., 1999). In lower organisms, nurse cells are derived from germ cells that commit themselves to apoptosis in order to contribute cytoplasmic mRNAs, proteins, organelles, and other components to their sibling germ cells that mature into gametes (Gumienny et al., 1999; McCall, 1998).

3. Germ cells use mRNA repression/activation to control the timing for introducing new functions

Translational recruitment of mRNAs for protein synthesis in germ cells is crucial during a period of transcriptional silencing. Early oocytes accumulate and store maternal mRNAs and RBPs to be utilized for *de novo* protein synthesis during critical periods of development in oogenesis and embryogenesis. During *C. elegans* oogenesis, for example, chromosome condensation causes transcriptional silencing that remains in effect until the 2-cell embryo (Kelly and Fire, 1998; Seydoux and Fire, 1994). Genes encoding proteins required during this period are transcribed early in germ cell life, and the mRNAs stored in ribonucleoprotein particles (mRNPs) for later use. Germ cells also accumulate translational machinery, some of it unique to the germline. Stored mRNPs and translation factors provide the developing gametes and embryos with new proteins necessary for development and maturation (Dworkin and Dworkin-Rastl, 1990; Nousch and Eckmann, 2013; Tadros and Lipshitz, 2005). *Xenopus* embryos are perhaps the most extreme, as they remain transcriptionally silent through the first 12 divisions (4000 cells), at which point their cell cycle slows down and transcription resumes (Newport and Kirschner, 1984). Inability to translate stored mRNAs causes both defective gametes and embryonic lethality (Dworkin and Dworkin-Rastl, 1990; Nousch and Eckmann, 2013).

One prevalent mechanism for regulating the spatial and temporal utilization of mRNAs in development uses RBP complexes that bind to recognition motifs in mRNA 3'UTRs to repress their translation (Figure 1.1A). Dissociation of these RBPs is thought to cause de-repression of mRNPs and allow for new protein synthesis to occur where and when it is needed in germ cell progression. For example, highly conserved (from yeast to mammals and plants) PUF proteins have been implicated in maintaining mitotic proliferation and the self-renewal of GSCs in lower

organisms (Lee et al., 2007; Spassov and Jurecic, 2003). Loss of PUF protein function results in the failure of GSC asymmetric divisions and promotes their precocious differentiation as well as germ cell tumors. In the *C. elegans* germ line, an elegantly complex and progressive series of translational control events govern nearly every step in the transition of GSCs from mitosis into meiosis and through maturation. Two proteins integral to these transitions are the PUF homologs FBF-1 and FBF-2.

The choice between sperm and oocyte differentiation in *C. elegans* is also decided by mRNA translational control (Goodwin and Evans, 1997; Kraemer et al., 1999). In larval gonads, the sperm/oocyte switch is set to "sperm on" due to the repression of *tra-2* mRNA by the RBP complex GLD-1/FOG-2. In adult hermaphrodites, however, GLP-1/Notch signaling promotes the FBF/NOS-3 complex repression of *fem-3* mRNA, which switches new germ cells to "sperm off" and promotes differentiation into oocytes. The GLP-1/Notch signal itself is a product of translational control. Inverse translational repression of *gld-1* (pro-meiotic) and *glp-1* (pro-mitotic) mRNAs regulates the GSCs transition from mitosis to meiosis. The delicate balance of this mRNA regulation is such that ectopic expression of GLP-1 results in unabated germ cell mitosis and germline tumor formation with little or no germ cell differentiation (Berry et al., 1997). Furthermore, partial *gld-1* loss-of-function abolishes oogenesis, and germ cells arrest in pachytene. More extreme loss of *gld-1* (null) results in pachytene-stalled germ cells that return to the mitotic cell cycle and form germline tumors (Francis et al., 1995). In *Drosophila* GSCs, a Vasa (eIF4A-like helicase)-eIF5B complex exerts mRNA translational control that involves repression and subsequent activation to restrict the "renewal" cell fate as well. Vasa (-/-) females exhibit egg chambers with undifferentiated nurse cells and oocyte tumors, demonstrating again a pivotal role for translational regulation in GSC fate (Styhler et al., 1998; Tomancak et al., 1998).

The canonical translation factor eIF4A also has a vital role in maintaining GSC self-renewal by inhibiting the function of mRNA binding protein, BAM, in an mRNP complex. Uninhibited BAM promotes the initiation of both male and female gametogenesis in *Drosophila* (Shen et al., 2009).

Toward the end of germ cell development, oocytes and spermatocytes rely on translational control for proper growth and differentiation. In arrested stage VI *Xenopus* oocytes, progesterone signaling induces strong *cyclin-B* and *c-mos* (serine-threonine kinase) mRNA translation. New synthesis of these proteins activates Cdc-2-Cyclin B kinase to promote cell cycle resumption and meiotic maturation (Castro et al., 2001; Roy et al., 1990). In late stage arrested *C. elegans* oocytes, OMA-1 and OMA-2 (redundant RBPs) are required for progression through meiotic prophase I (Detwiler et al., 2001). At later points, the OMA-1/LIN-41 RNP is an essential regulator of oocyte growth and maturation through translational repression of target mRNAs (Spike et al., 2014; Tocchini et al., 2014). Recent data suggests that an OMA protein inhibits *wee-1.3* (Myt1 homolog) mRNA translation, preventing the inactivation of CDC-25.1 and subsequently CDK-1. This phosphatase/kinase cascade promotes cell cycle resumption in maturing oocytes (Detwiler et al., 2001; Yoon et al., 2012). At many junctures during gametogenesis, translational regulation of germ cell mRNAs plays an integral role in meiotic cell cycle checkpoints for development of viable and mature gametes.

4. mRNA recruitment for new protein synthesis

As mRNAs become de-repressed, the translation initiation machinery recognizes and recruits them to ribosomes to drive new protein synthesis required for development. Growing evidence suggests the repression events are coordinated with de-repression events to allow for precisely timed *de novo* synthesis of novel proteins as required. The limitation in a cell's utilization of de-repressed mRNAs is the recruitment of each mRNA to ribosomes via a translation initiation complex. For most mRNAs, mobilization begins with eukaryotic initiation factor 4E (eIF4E) binding the mRNA 7-methylguanosine 5'cap structure to for recruitment to the 40S ribosomal subunit (Keiper et al., 1999; Sonenberg and Gingras, 1998). eIF4E-bound mRNAs associate with the eIF4G scaffolding protein and the eIF4A helicase to form a productive eIF4 complex. Recruitment of eIF4-bound mRNAs to the 40S subunit occurs by the synergistic function of the eIF4 proteins and PABPs bound to the poly(A) tail (Tarun and Sachs, 1995) (Figure 1.1B). eIF4G coordinates eIF4E and PABP, bound to the mRNA 5'-cap and 3'-poly(A) tail, respectively, to promote the assembly of a "closed loop" circular mRNP structure. Circularization is proposed to facilitate the recycling and reinitiation of post-termination ribosomes, thus increasing the mRNA's translational efficiency (Tomek and Wollenhaupt, 2012). PABP also helps recycle 60S ribosomal subunits to the pre-initiation complex (Derry et al., 2006). Within these mechanisms, eIF4E and eIF4G play key roles in regulating mRNA translational control for cap-dependent protein synthesis.

Both eIF4E and eIF4G are highly conserved across species (yeast to human) and each has been implicated in various cell fate decisions (see section 1). Developmental cell fates seem to be related to expression of unique eIF4E and eIF4G isoforms, or indeed unique germ cell isoforms, represented in a given cell. For example, three eIF4E proteins have been characterized in

mammals, five in *C. elegans*, three in *Xenopus*, three in plants, three in zebrafish, and eight in *Drosophila* (Browning, 1996; Hernandez et al., 2005; Keiper et al., 2000; Robalino et al., 2004; Wakiyama et al., 2001). Several are unique to, or are the predominant form in, germ cells (Joshi et al., 2004; Keiper et al., 2000; Minshall et al., 2007; Robalino et al., 2004; Wakiyama et al., 1995). eIF4E binds mRNA 5' cap and recruits bound mRNA to the translation initiation complex in association with eIF4G (Marcotrigiano et al., 1997b). eIF4E binding proteins (4EBPs) bind to the dorsal face of eIF4E and inhibit its ability to bind to eIF4G without disrupting mRNA cap-binding (Fukuyo et al., 2011; Liu et al., 2011). By this mechanism, eIF4E-bound mRNP complexes can regulate repression and de-repression of eIF4E-bound mRNAs. In *Drosophila*, of the eight eIF4E isoforms present, data suggest that eIF4E-1 uniquely regulates the translation of *oskar* mRNA, which is necessary for embryonic posterior patterning and germ cell formation (Hernandez et al., 2005; Nakamura et al., 2004). In *Xenopus* oogenesis, eIF4E1b has been identified in an mRNP complex responsible for the repression of meiotic maturation in early stage oocytes (Minshall et al., 2007). This protein associates with a novel 4EBP called eIF4E-T that transports and sequesters the cap-binding protein. In *C. elegans*, eIF4E isoforms have been shown to regulate cell fate decisions not only in the germ line (IFE-1: sperm and oocyte maturation; IFE-2: meiotic recombination; IFE-3: sperm to oocyte switch), but also in somatic tissue (IFE-2: animal longevity, IFE-4: muscle and neuron development) (Amiri et al., 2001; Contreras et al., 2008; Henderson et al., 2009; Keiper et al., 2000; Song et al., 2010; Syntichaki et al., 2007; Tavernarakis, 2007).

Likewise, eIF4G isoforms (IFG-1 p170 and p130) have also been shown to carry out distinctly differing roles in germ cell fate decisions. IFG-1 p170 is the integral scaffolding protein in the m7G-binding translation initiation complex and supports cap-dependent protein

synthesis. This "long" eIF4G form binds eIF4Es (IFEs) and promotes germ cell proliferation and oogenic differentiation (Contreras et al., 2008). Long eIF4G was also shown to be essential for translation of cell cycle mRNAs like *c-mos* in *Xenopus* oocytes (Keiper and Rhoads, 1999). Cleavage of eIF4G to a "short" form supports only the cap-independent initiation mechanism for protein synthesis. Over 70% of protein synthetic capacity remains intact in "cap-independent" *Xenopus* oocytes, but their capacity to undergo meiotic maturation (cell cycle progression) in response to progesterone is lost (Keiper and Rhoads, 1997, 1999). Similarly, the naturally occurring *C. elegans* IFG-1 p130 (short form) that lacks the eIF4E-binding domain supports the cap-independent initiation of housekeeping mRNAs and stress related mRNAs during germ cell apoptosis (Contreras et al., 2011; Morrison et al., 2014). The cap-independent mechanism was originally discovered for viral mRNAs that become translated more efficiently when eIF4G is cleaved (Prevot et al., 2003). IFG p130 cap-independent translation (e.g. of *Hsp70* and *Bcl-2* mRNAs) provides germ cells that are in distress (perhaps from mistakes/defects in meiotic DNA recombination) with an opportunity to recover and survive during a resolution period (Morrison et al., 2014). Damage that is too severe also uses cap-independent synthesis to initiate programmed cell death, or apoptosis (Contreras et al., 2008; Holcik and Sonenberg, 2005).

In *Drosophila* spermatocytes, the eIF4G homologue *Off-schedule* (eIF4G2) is essential for meiotic progression and differentiation (Franklin-Dumont et al., 2007; Sun et al., 2010). Spermatocytes depleted of eIF4G2 are small in size and accumulate CDK inhibitor protein, RUX, likely as a block of the growth checkpoint before meiotic division (Franklin-Dumont et al., 2007). In mouse spermatocytes, an *Eif4g3* mutation results in meiotic prophase arrest and the apparent loss of *Hspa2* mRNA translation. HSPA2 is necessary for activation of meiotic prophase kinase CDC2A (Sun et al., 2010). These activities suggest a model in which the ratio of

cap-dependent:cap-independent translation supports germ cell developmental events that range from growth, to recovery from cellular insult, to physiological apoptosis (Holcik and Sonenberg, 2005; Lodish, 1976).

The determinants for specific mRNA binding to eIF4 factors remain unclear. Despite their highly specific ligand binding pockets, all known cap-binding protein eIF4E isoforms have rather low binding affinities for m^7GTP and share little mRNA sequence recognition beyond the first two nucleotides (Marcotrigiano et al., 1997a, c; Miyoshi et al., 2002). Yet surprisingly, eIF4E isoforms show marked substrate specificity *in vivo* to recruit unique populations of mRNAs. The explanation for this recruitment specificity is two-fold. First, eIF4E isoforms are expressed in a tissue-specific fashion in organs that require them, and the constellation of eIF4Es present will therefore differ from tissue to tissue. For example, in *C. elegans* IFE-4 is expressed in neurons and muscle while IFE-1, IFE-3, and IFE-5 are the predominant isoforms expressed in the gonad (sperm, oocytes, and embryos) (Keiper et al., 2000). A cell-type specific isoform can obviously only translate mRNAs to which it has access. However, some eIF4Es co-exist in the same cell type but translate different mRNAs (Henderson et al., 2009). This second aspect of mRNA recruitment specificity appears to be due to the fact that each form exists in a different mRNP complex. IFE-1 is bound to an RBP known as PGL-1, which in *C. elegans* oocytes is required for IFE-1's localization with stored mRNAs in P granules (Amiri et al., 2001; Updike et al., 2014). By contrast, IFE-3 is found associated with OMA-1 mRNPs in those same oocytes (Spike et al., 2014). These two eIF4E isoforms are thus differentiated by their subcellular localization and the eIF4E binding proteins (4EBPs) with which they interact. Evidence from somatic cell translational control shows that general cap-dependent eIF4E-mediated recruitment of mRNAs is generally inhibited by 4EBPs. Sequestration allows cap-independent initiation to

prevail (Fukuyo et al., 2011; Proud, 2006). Growth factor signaling activates mTOR kinase to phosphorylate 4EBP, causing its dissociation from eIF4E and thus restoring cap-dependent protein synthesis. Among the known 4EBPs are several germ cell types that also bind mRNAs, either individually or in complexes. Maskin-CPEB binds and represses eIF4E-bound mRNAs in *Xenopus* oocytes (Stebbins-Boaz et al., 1999). Following progesterone signaling, maskin dissociates from eIF4E and the mRNA becomes actively recruited for translation initiation via eIF4G. Similarly in *Drosophila* oocytes, Cup is a 4EBP that represses *oskar* translation in an RNP complex with eIF4E (Wilhelm et al., 2003). As described above, *C. elegans* PGL-1 is a 4EBP and RBP in germ granules that binds (and presumably represses) only one eIF4E type. These represent a few instances in which RBPs coordinate with eIF4E to prevent the recruitment of mRNAs to eIF4G. Many RBPs bind recognition motifs in mRNA 3'UTRs. As the regulated mRNAs become required for translation, RBP complexes become remodeled (Figure 1.2A) such that eIF4E can associate with eIF4G and recruit the message directly to ribosomes effectively coordinating the transition from repression to activation (Cao and Richter, 2002).

Perhaps the best understood example of molecular events involved in eIF4E regulation in an mRNP complex was described for oocyte maturation in *Xenopus.* Oocyte meiosis is arrested by the translational suppression of *cyclin B* mRNA that contains a cytoplasmic polyadenylation element (CPE) in the 3'-untranslated region (Groisman et al., 2000). This suppression occurs when the eIF4E-maskin-CPEB (CPE binding factor) complex forms on *cyclin B* mRNA with a short poly(A) tail. Maskin acts as a specialized 4EBP by binding to eIF4E at the eIF4G binding site to occlude its ability to enter the translation initiation complex (Barnard et al., 2005; Pique et al., 2008; Stebbins-Boaz et al., 1999). Maskin also binds to CPEB to repress translation. To

Figure 1.2

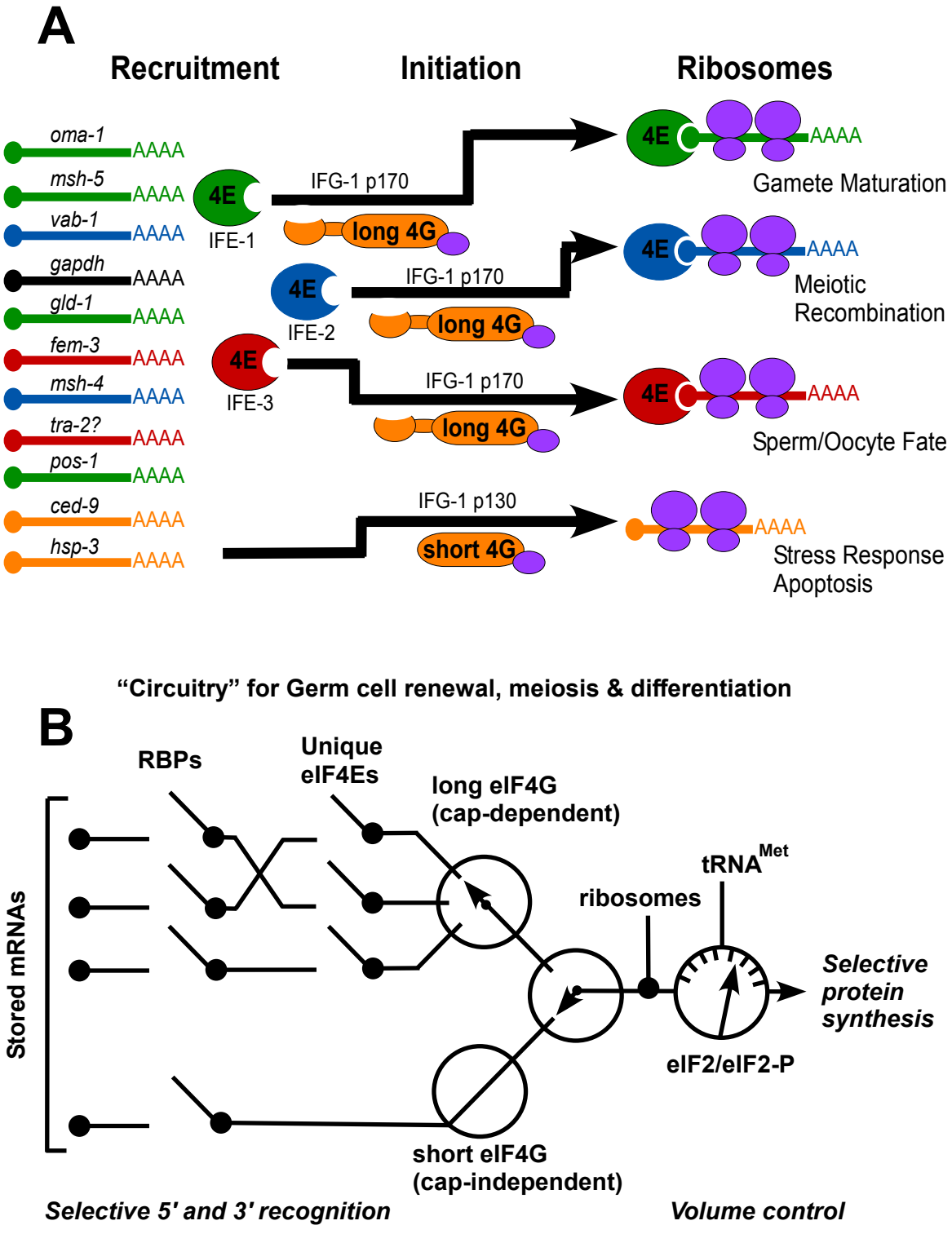

A
Recruitment
Initiation
Ribosomes
oma-1
msh-5
vab-1
gapdh
gld-1
fem-3
msh-4
tra-2?
pos-1
ced-9
hsp-3
AAAA
4E
IFE-1
IFE-2
IFE-3
IFG-1 p170
IFG-1 p130
long 4G
short 4G
Gamete Maturation
Meiotic Recombination
Sperm/Oocyte Fate
Stress Response Apoptosis
"Circuitry" for Germ cell renewal, meiosis & differentiation
B
RBPs
Unique eIF4Es
long eIF4G (cap-dependent)
tRNA^Met
ribosomes
Stored mRNAs
Selective protein synthesis
eIF2/eIF2-P
short eIF4G (cap-independent)
Selective 5' and 3' recognition
Volume control

Figure 1.2. Dynamic models for selective protein synthesis in germ cells. (A) A complex mixed population of mRNAs present in germ cells of various stages is selectively recruited for translation initiation by individual eIF4E isoforms. This positive selection occurs temporally as developing cells require new protein synthesis. Corresponding mRNA becomes de-repressed, and the eIF4E-mRNA complex is recruited to the cap-dependent translation initiation complex by the "long" isoform of eIF4G. Other mRNAs are recruited by cap-independent translation initiation via the short eIF4G isoform that lacks the eIF4E binding domain. Episodes of selective mRNA translation by individual eIF4 isoforms have been shown to drive critical germ cell fate decisions. (B) As new protein synthesis is required for germ cell renewal, growth, and differentiation, one pathway, or circuit, is activated for the translation of a certain population of stored mRNAs. mRNP complexes reach the first "translation on/off" switch at a point where bound RBPs (including eIF4 factors) undergo remodeling that results in mRNA de-repression. De-repressed mRNAs following the cap-dependent circuit must pass a switch compromised of a unique eIF4E isoform. Successfully activating this switch, eIF4E-bound mRNA is recruited to the initiation complex via the long eIF4G. Alternatively de-repressed mRNAs following the cap-independent circuit are made available for initiation recruitment via the short eIF4G in an analogous fashion. Both cap-dependent and -independent recruited mRNAs then reach a node in which eIF4A and ribosomes must be bound. (Note that in *C. elegans* and *Drosophila* germ cell mRNPs, eIF4A, or a homologous helicase, is also pre-bound.) The 40S ribosomal subunit brings with it initiator Met-tRNA bound to eIF2. This step constitutes a "rheostat" in the circuit where the volume of protein synthesis can be limited by phosphorylation of eIF2. mRNAs completing this circuit are efficiently decoded into new proteins necessary for discrete germ cell developmental events.

resume oocyte maturation, progesterone signals the activation of Aurora kinase, which phosphorylates CPEB. Active CPEB releases maskin and subsequently recruits CPSF (polyadenylation specificity factor) and poly(A) polymerase (PAP) to the 3' end of the bound mRNA. The elongated poly(A) tail attracts multiple copies of poly(A)-binding protein (PABP). Both the freed eIF4E and polymerized PABP associate with eIF4G in a productive initiation complex (Figure 1.1B), initiating the synthesis of Cyclin B and driving meiotic maturation. eIF4E's role in oocyte meiotic cell cycle progression, together with its role in regulating cell cycle and proliferation in tumor models, have made eIF4E a popular therapeutic target in cancer treatment studies, and enhanced our understanding of cancer cell translational control (Furic et al., 2010; Ko et al., 2009; Kouvaraki et al., 2011; Rhoads, 1991; Rinker-Schaeffer et al., 1993; Salehi and Mashayekhi, 2006).

Coordinated mRNP repression and de-repression is a conserved germ cell strategy in many species. The translational control of *oskar* mRNA in *Drosophila*, for example, is necessary for embryonic posterior patterning and germ cell formation. Repression and de-repression are both mediated by an eIF4E-Cup-Bru RBP complex on the *oskar* mRNA (Hernandez et al., 2005; Nakamura et al., 2004). During the early oogenesis, *oskar* is transcribed in nurse cells and repressed during its transport to the posterior pole of the oocyte to prevent precocious development (Ephrussi et al., 1991). The Bru RBP binds a sequence element in *oskar* 3' UTR as well to the eIF4E-Cup complex at the 5'cap to repress *oskar* translation. Premature translation of *oskar* mRNA prior to localization leads to embryonic patterning defects (Nakamura et al., 2004). The cell polarity established by Oskar is necessary for asymmetrical divisions and body patterning and is governed by several such proteins expressed in a gradient across the cell. The fertilized egg consequently develops organizing centers at both the anterior

and posterior poles. *bicoid* mRNA is localized to the anterior pole. Localized synthesis of Bicoid protein is necessary for head and thorax development. At the posterior pole, by contrast, *nanos* mRNA is localized. When expressed, Nanos protein binds Pumillio to form an mRNP complex that suppresses *hunchback* mRNA translation (Mosquera et al., 1993). Nanos and Pumillio have also been implicated in the suppression of *cyclin B* mRNA in early development. Yet, while these RBPs repress mRNAs together in early embryos, they may have different partners in the germ line. Nanos is required for proper germ cell migration while Pumillio is necessary for germline stem cell maintenance (Forbes and Lehmann, 1998). Pumillio represses *smaug* mRNA in the embryonic posterior pole. Smaug is itself an RBP that interacts directly with Oskar and prevents *nanos* mRNA translation (Dahanukar et al., 1999). However, the *smaug* mRNA 3' UTR is also predicted to bind other RBPs that promote its translation. One such instance of positive translational control by an RBP involves the highly conserved DAZL protein, which stabilizes mRNAs by promoting PAPB recruitment, circularization of the mRNA, and thus increased translational efficiency in developing germ cells (Collier et al., 2005). These anecdotal instances outline a diverse regulatory system in which RBPs and translation initiation factors work together for both negative and positive translational control that drive specific protein synthetic events necessary for germ cell differentiation.

5. The selective function of the eIF4 initiation complex

Differential recruitment of specific mRNAs by eIF4E and eIF4G isoforms has been studied extensively in *C. elegans* germ cells. Cap-binding eIF4E is present in five isoforms (IFE-1-5) in nematodes. Three forms (IFE-1, -3, and -5) are enriched in or exclusive to the germ line,

while two isoforms (IFE-2 and -4) are expressed primarily in somatic tissue (Keiper et al., 2000). Worm strains bearing null mutations in individual eIF4E genes have shown that each isoform has a unique subset of mRNAs that it preferentially recruits (Amiri et al., 2001; Dinkova et al., 2005; Song et al., 2010; Syntichaki et al., 2007; Tavernarakis, 2007)). Other non-regulated mRNAs (like beta-tubulin or GAPDH) appear to be indiscriminate in their choice of eIF4E isoform. A uniquely germline eIF4E (IFE-1) for instance, is a key positive translational regulator of multiple steps in sperm and oocyte progression. *ife-1* (-/-) worms are temperature-sensitive sterile due to defective cytokinesis late in spermatogenesis (Henderson et al., 2009). Hermaphrodites display substantially reduced oocyte growth, maturation, and egg fertilization. Productive fertilizations often arrest as early embryos. Follow-up studies showed that IFE-1 promotes germ cell development and preparation for embryogenesis by positively recruiting critical mRNAs for each process (e.g. *mex-1, oma-1, glp-1, gld-1, pos-1, pal-1, vab-1, rab-7, ran-1, rnp-3*) (Friday et al., 2015; Henderson et al., 2009). Selective translational recruitment by IFE-1 has been demonstrated *in situ* in live worms, as GFP reporter mRNAs become activated in a temporal and spatial manner within individual germ cells (Friday et al., 2015). Another germ cell eIF4E (IFE-3) is expressed in the same oocyte stages as IFE-1, but its subcellular localization differs. IFE-1, but not IFE-3, colocalizes to germ granules (P granules) via protein-protein binding to PGL-1 (Amiri et al., 2001; Sengupta and Boag, 2012). Loss of IFE-3 results in a distinctly different germ cell phenotype. *ife-3* (-/-) animals produce only sperm, even in hermaphrodite adults that should normally switch all germ cell differentiation to oogenesis (Subash and Keiper, unpublished). The strictly spermatogenic fate suggests that the sperm-to-oocyte switch that normally occurs in late larvae has malfunctioned. This gamete sex switch involves translational control of *tra-2* and *fem-3* mRNAs (Goodwin et al., 1993). Recent observations that IFE-3

interacts with the IFET-1/CGH-1/LARP-1 complex on *fem-3* mRNA suggests another instance of a transitional mRNP complex involving a specific eIF4E (Boag et al., 2008; Sengupta et al., 2013; Zanin et al., 2010). Loss of still another eIF4E isoform (IFE-2), which is expressed at very low levels in germ cells, leads to temperature-sensitive meiotic catastrophes. Specifically, *ife-2* (-/-) germ cells have a severe defect in chromosome crossover resolution and repair during meiosis (Song et al., 2010). The repair activities are due to IFE-2-mediated recruitment of key mRNAs (*msh-4/him-14*, *msh-5*) required for proper meiotic chromosome segregation. In just these few examples of distinct roles for three nematode eIF4Es, it is apparent that the translational apparatus itself carries out critically important mRNA selections that alter germ cell fates.

Translational regulation of mRNAs in *C. elegans* germ cells is not limited to the cap-binding activity of eIF4Es. Orthologs of eIF4G (IFG-1), which joins mRNA to the ribosome, also exert preferential mRNA recruitment and influence germ cell fate. Two isogenic forms of eIF4G are expressed in worms, a long p170 IFG-1 that has binding sites for IFEs, and an N-terminally truncated short p130 IFG-1 that does not (Figure 1.1B,C). Depletion of both p130 and p170 IFG-1, and thus both cap-dependent and cap-independent translation, suppresses the initial expansion of GSCs that occurs in early L2 larval worms as well as the somatic growth and molting of the young worms (Contreras et al., 2008). Immature worms live nearly a complete lifespan but are unable to grow and appear fully arrested in development. Most interesting, however, are the consequences of altering the balance between the cap-dependent (p170) and cap-independent (p130) IFG-1 activities. These consequences are manifested primarily in the germ line. RNAi or genetic depletion of IFG-1 p170 (long eIF4G, Figure 1.1) alone amplifies the natural proportion of germ cell apoptotic events in differentiating oocytes (Contreras et al., 2011). The deaths are not spurious collateral damage from constrained protein synthesis. Rather,

they require apoptotic signaling through the apoptosome via Apaf-1 (*ced-4*) and caspase (*ced-3*). Germ cell death appears to be driven by IFG-1 p130-sustained, cap-independent translation of mRNAs that signal stress, recovery, and eventually apoptosis. Enhanced translation of the chaperone BiP (*hsp-3*) and the apoptotic regulator Bcl-2 (*ced-9*) mRNAs occur in a background of less efficient translation of many "normal" mRNAs (Morrison et al., 2014). Overall, the integrated positive contributions of four selective germ cell eIF4Es and two eIF4Gs, in concert with the better-known RBP repressors, leads to a circuitry of translational control that has great latitude for mRNA types and temporal events in germ cell life (Figure 1.2A). There is also considerable evidence from *Xenopus* oocyte studies for the involvement of eIF2. eIF2 brings the initiator Met-tRNA to the mRNA complex, and is subject to phosphorylation by the GCN2 kinase to regulate the volume (overall output) of protein synthesis in late oogenesis and at meiotic maturation (Akkaraju et al., 1991; Allende et al., 1988; Alves et al., 2009; Carvallo et al., 1988; Patrick et al., 1989; Winkler et al., 1985). This multifaceted mRNA handling system in germ cells maintains the sophistication of the translation initiation functions of eIF4 and eIF2 factors, in conjugation with repressor RBPs, to carefully govern the identity, timing, and abundance of proteins synthesized (Figure 1.2B). Evidence from unique cases of mRNA translational control observed in lower organisms has led to a paradigm in which the translation initiation machinery itself acts as integral part of the regulatory pathway at multiple critical transitions in germ cell progression.

Positive translational control of mRNAs is quickly becoming recognized as equally as important as repression by RBPs for translational control in developmental contexts. Because this mode of post-transcriptional gene regulation predominates in germ cells of many species and continues through early embryonic development, the cooperative nature of eIFs and RBPs will

merit further exploration (Mendez and Richter, 2001). Key regulatory proteins for both positive and negative mechanisms of translational control are highly conserved in many sexually-reproducing animal species that have been studied (Gebauer and Hentze, 2004; Gingras et al., 1999; Kyrpides and Woese, 1998; Mendez and Richter, 2001). Given the conserved molecular themes in translational control, the interplay between eIFs and RBPs can be explored in a broad range of animals to yield general principles for regulation of germ cell mRNAs.

6. Outline of the C. elegans germ line

My thesis takes advantage of the nematode worm, *C. elegans*, to study protein synthesis regulation in germ cells. *C. elegans* exist in two sexes: hermaphrodites which produce both sperm and oocytes; and males which only produce sperm (Kimble and Crittenden, 2005). The reproductive organs (gonads) make up the majority of the *C. elegans* adult body mass. Hermaphrodites have a bi-lobed gonad that produces (at different times) both sperm and oocytes from the same stem cell population. The distal end of each lobe of the gonad contains a population of germ line stem cells (GSCs) (Kimble and Crittenden, 2005). During the final larval stage, the hermaphrodite gonad produces sperm from these stem cells. By young adulthood the sperm are stored in the spermatheca for future fertilization events, and the gonad switches to producing oocytes from the same stem cell pool. These continue to proliferate into early adulthood in the mitotic region of the gonad (Kimble and Crittenden, 2005). As they progress linearly through the gonad, they transition into meiosis and grow in size as nuclei that are partially surrounded by cell membrane but share a continuous cytoplasm (syncytium). Oocyte nuclei arrest in meiotic prophase during this growth phase. Resumption of meiotic cell

cycle occurs upon maturation, when major sperm proteins (MSP), released from the sperm in the spermatheca (or inseminated sperm from male worms), signals for MAPK activation (Cheng et al., 2008; Miller et al., 2003). Maturing oocytes are pushed through the spermatheca where they are fertilized by the sperm residing there.

Chapter 2: Spatial and Temporal Translational Control of Germ Cell mRNAs via an eIF4E Isoform, IFE-1

This chapter appears in the primary manuscript: *Andrew J. Friday, Melissa A. Henderson, J. Kaitlin Morrison, and Brett D. Keiper. Spatial and Temporal Translational Control of Germ Cell mRNAs by an eIF4E Isoform, IFE-1. Journal of Cell Science 2015. In Revision

Abstract

Regulated mRNA translation is vital for germ cells to produce new proteins in spatial and temporal patterns that drive gamete development. Translational control involves the de-repression of stored mRNAs and their recruitment by initiation factors (eIFs) to ribosomes. *C. elegans* expresses five eIF4Es (IFE-1-5); several were shown to selectively recruit unique pools of mRNA. Individual IFE knockouts yield unique phenotypes due to inefficient translation of certain mRNAs. Our studies identified mRNAs translationally recruited by germline-specific eIF4E isoform, IFE-1, and some of the developmental events in which they are involved. Differential polysome microarray analyses identified 77 mRNAs recruited by IFE-1 for translation initiation. Among the IFE-1-regulated mRNAs are several required for late germ cell differentiation and maturation. Polysome association of *gld-1, vab-1, vpr-1, rab-7, and rnp-3* mRNAs relies, at least in part, on IFE-1. Live animal imaging showed IFE-1-dependent spatial and temporal translation of several germline mRNAs. Altered MAPK activation in various oocyte stages suggested dual roles for IFE-1 both in promoting and in suppressing oocyte maturation at different stages. This single eIF4E isoform exerts positive, selective translational control to drive germ cell development throughout gametogenesis for both sperm and egg.

Introduction

Cell fate, proliferation, and differentiation are dependent on gene expression, often at the level of protein synthesis (Kimble and Crittenden, 2006). Dysregulation of these processes is associated with human pathologies including infertility, birth defects, and cancers (Graff et al., 2008; Song and Lu, 2011). Clinical studies show that eukaryotic translation initiation factor eIF4E is overexpressed in multiple cancers including ovarian, esophageal, breast, thyroid, and prostate (Furic et al.; Ko et al., 2009; Kouvaraki et al.; Salehi and Mashayekhi, 2006). Its overexpression upregulates the translation of cell cycle/growth mRNAs contributing to the cancer phenotype. eIF4E is essential for cap-dependent protein synthesis to recruit mRNAs to the ribosome for translation. Specifically, eIF4E binds both the 7-methylguanosine 5'-cap of mRNAs and the scaffolding translation initiation factor eIF4G (Marcotrigiano et al., 1997a). eIF4G assembles initiation factors with the 40S ribosomal subunit on mRNA to form the 48S complex, the rate limiting step for protein synthesis (Gingras et al., 1999; Lamphear et al., 1995; Liu et al.). The selection of cap-dependent mRNAs for translation by eIF4E can alter cell fate by promoting growth and proliferation (De Benedetti and Graff, 2004; De Benedetti and Rhoads, 1990). eIF4E-mediated recruitment of mRNAs can be inhibited by eIF4E binding proteins (4EBPs), which bind to the dorsal side of eIF4E to prevent association with eIF4G. Cap-dependent recruitment is thereby inhibited, allowing cap-independent initiation to prevail (Contreras et al., 2008; Fukuyo et al., 2011; Marcotrigiano et al., 1997a; Proud, 2006). Growth factor signaling activates mTOR kinase to phosphorylate 4EBP, causing a dissociation from eIF4E and promoting cap-dependent protein synthesis (Hay and Sonenberg, 2004; Long et al., 2004).

Five isoforms of eIF4E (IFEs 1-5) are expressed in the nematode *C. elegans* (Jankowska-Anyszka et al., 1998; Keiper et al., 2000). For clarity, according to prescribed *C. elegans* nomenclature, the gene name is written in small, italic letters, "*ife-1*", while all capital letters, "IFE-1" refers to protein. Mutants are referred to by the gene name, followed by the allele designation in parentheses, "*ife-1*(bn127)"; in further references the allele designation is dropped. "*ife-1*(bn127)" is specifically referring to an *ife-1* deletion allele. We have published that individual isoforms regulate translation of a unique subset of mRNAs in a tissue-specific manner (Amiri et al., 2001; Dinkova et al., 2005; Henderson et al., 2009; Kawasaki et al., 2011; Song et al., 2010). Since all IFEs bind 5' mRNA caps, these findings suggest a selective cap-dependent regulation that goes beyond mTOR regulation. For example, though IFE-2 and IFE-4 are expressed in somatic tissue, they regulate different mRNAs whose products drive unique developmental events. IFE-2 regulates aging, while IFE-4 regulates nerve and muscle tissue function (Dinkova et al., 2005; Syntichaki et al., 2007). IFEs 1, 3, and 5 (and to a lesser extent IFE-2) are expressed in the germ line and are involved in gamete development (Henderson et al., 2009; Keiper et al., 2000; Song et al., 2010). Early germ line and embryonic development require production of essential proteins for cellular differentiation, maturation, and viability. During early gametogenesis, stored mRNAs are utilized when transcription is largely silenced due to chromosomal condensation (Kelly and Fire, 1998; Seydoux et al., 1996). Studies in oocytes from multiple species suggest that available ribosomes translate these mRNAs as they become de-repressed, providing proteins necessary for development and maturation (Dworkin and Dworkin-Rastl, 1990; Goodwin and Evans, 1997; Macdonald and Smibert, 1996; Mendez and Richter, 2001; Wormington, 1993). In *Xenopus laevis* oocytes, for example, meiotic maturation is arrested by translational suppression of cyclin-B mRNA that contains a cytoplasmic

polyadenylation element (CPE) in the 3'-untranslated region (3'UTR). The translational machinery is involved in the translational repression event. Repression of cyclin-B mRNA occurs when the eIF4E-maskin-CPEB (CPE binding factor) complex forms. Maskin acts as a specialized 4EBP that inhibits recruitment of bound mRNA to a translation initiation complex via eIF4G (Barnard et al., 2005). To resume oocyte maturation, progesterone activates a signaling cascade that results in CPEB phosphorylation, Maskin dissociation from eIF4E, and cap-mediated recruitment of the de-repressed mRNA. Translation of these products reinitiates the cell cycle and oocyte maturation (Barnard et al., 2005; Cao and Richter, 2002). mRNA translational control provides a substantive link to oncogenesis and reproductive health.

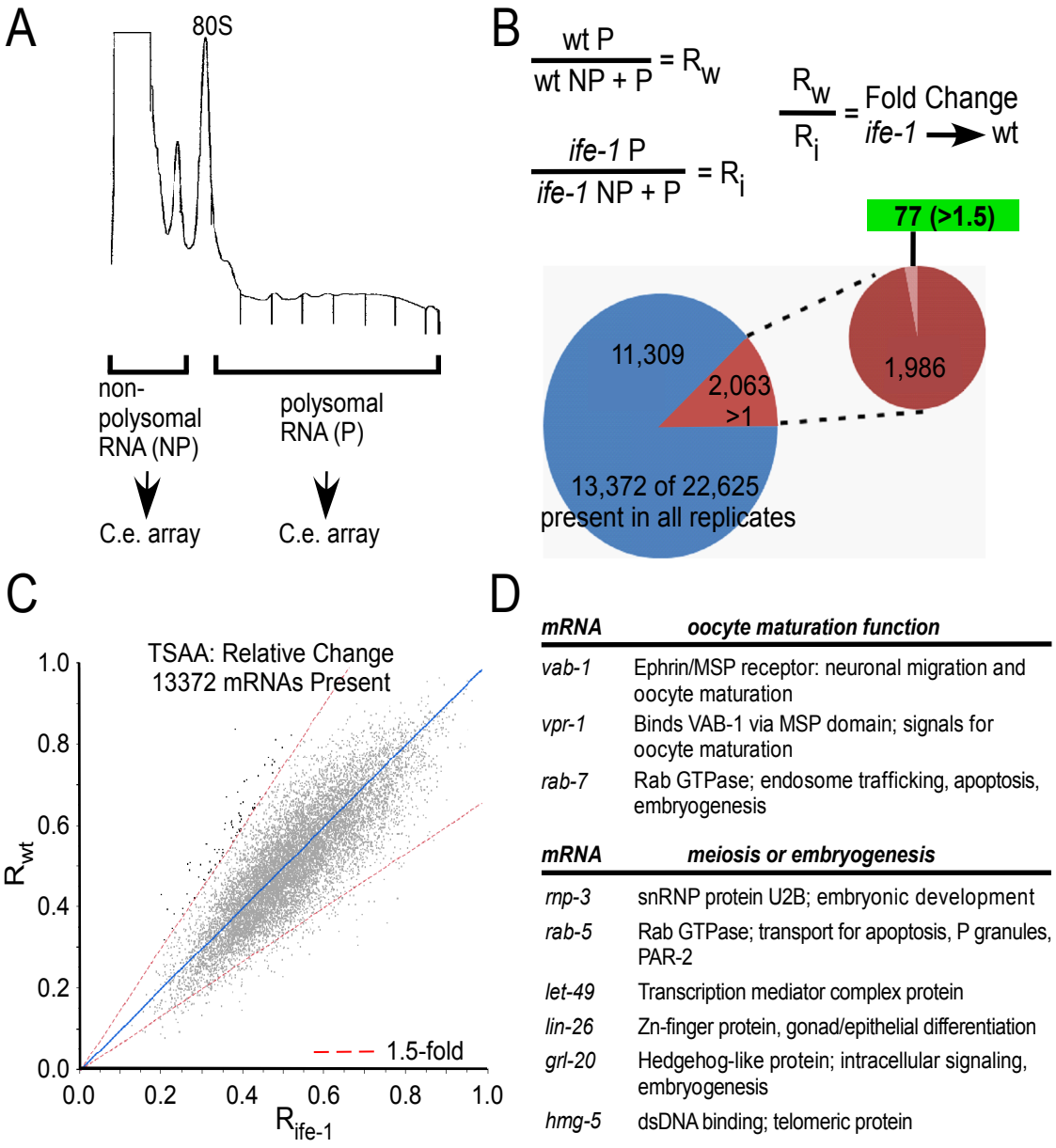
IFE-1 is a nematode eIF4E isoform bound to a 4EBP-like protein, PGL-1 (Amiri et al., 2001). Together, these proteins reside in P granules, which are germ line-specific ribonucleoprotein (mRNP) complexes containing stored mRNAs (Kawasaki et al., 1998). Based on localization in these mRNPs, we hypothesize that IFE-1 may be uniquely involved in the subsequent translation of stored mRNAs during germ cell development. Supporting this, *ife-1* worms have significant developmental defects during gametogenesis and embryogenesis. These include a temperature-sensitive defect in cytokinesis in secondary spermatocytes, which results in a complete lack of mature sperm (Henderson et al., 2009). Even when sperm is present, *ife-1* worms still exhibit an 80% reduction in fecundity. The latter results from a reduced rate of oocyte production, compromised egg viability, and early embryonic lethality. We have used a genomic approach called translation state array analysis (TSAA) to identify 77 mRNAs that rely on IFE-1 for positive translational control. Several of these mRNAs encode proteins with roles in gamete development. Furthermore, IFE-1 carries out spatiotemporal recruitment of mRNAs involved in sex determination, MAPK activation, oocyte maturation, and embryogenesis.

Results

IFE-1 regulates the translational efficiency of a distinct population of mRNAs.

Our previous studies showed that IFE-1 is required for the efficient translation of several developmentally controlled mRNAs in the *C. elegans* germline (*pos-1, oma-1, mex-1, pal-1* and *glp-1*) (Henderson et al., 2009). We set out to identify the entire population of messages that are IFE-1-dependent using the TSAA. (Chappell et al., 2013; Dinkova et al., 2005; Song et al., 2010). Using Affymetrix microarrays, we compared the probe signal output of every mRNA present with and without ribosomes bound. We calculated the relative changes (R value) in polysome loading of each mRNA between wild type and *ife-1* strains. By tracking both the polysomal (P) and non-polysomal (NP) partitioning of mRNAs, rather than just the amount resolved in polysomes, we directly assessed the translational efficiency of each mRNA, independent of changes in mRNA abundance (Figure 2.1A). The mRNA subset (mean fold-total, MFT) with the largest change in total (NP + P) signal substantially overlaps with the subset of mRNAs (mean fold-polysomal, MFP) with the largest change in polysome (P) signal. Calculating a mean fold change in R value (MFR) allowed us to prioritize a group that reflects changes in translational efficiency. It shares just 8 target mRNAs with the polysome MFP group (Figure 2.2). This demonstrates that assessing translational control by simply measuring how much of an mRNA resides in polysomes without considering abundance changes would have been misleading.

Figure 2.1



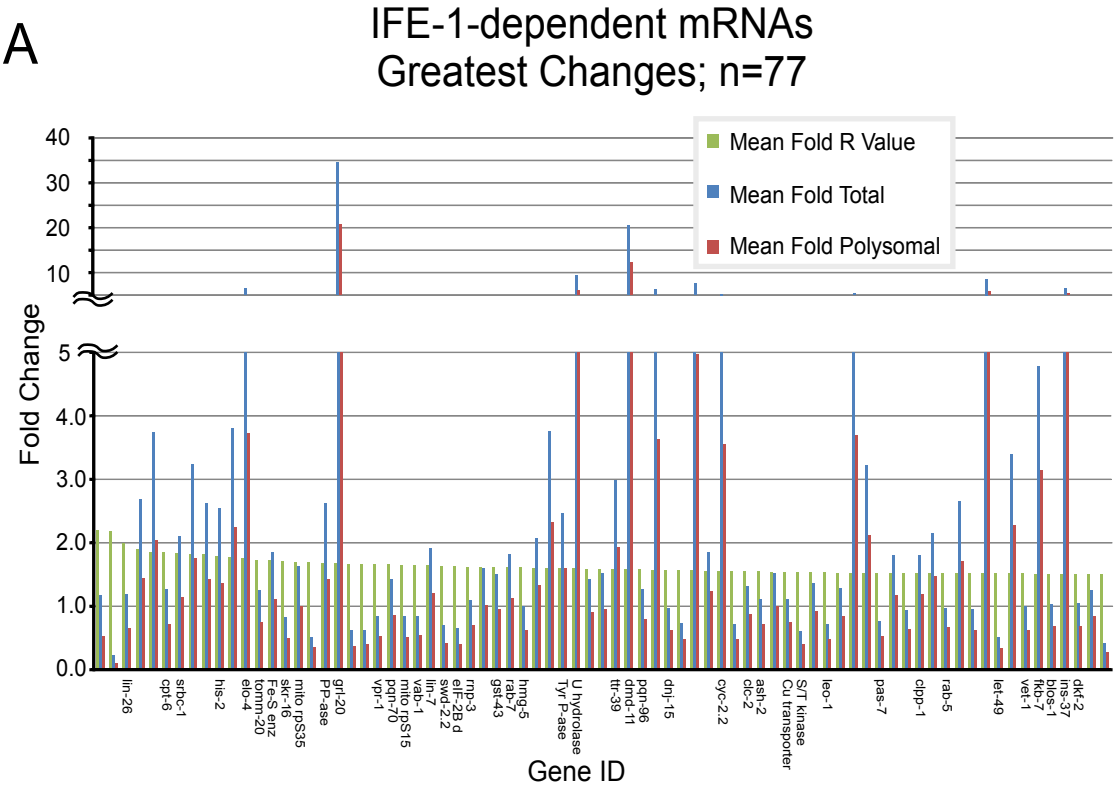
mRNA	*oocyte maturation function*
vab-1	Ephrin/MSP receptor: neuronal migration and oocyte maturation
vpr-1	Binds VAB-1 via MSP domain; signals for oocyte maturation
rab-7	Rab GTPase; endosome trafficking, apoptosis, embryogenesis

mRNA	*meiosis or embryogenesis*
rnp-3	snRNP protein U2B; embryonic development
rab-5	Rab GTPase; transport for apoptosis, P granules, PAR-2
let-49	Transcription mediator complex protein
lin-26	Zn-finger protein, gonad/epithelial differentiation
grl-20	Hedgehog-like protein; intracellular signaling, embryogenesis
hmg-5	dsDNA binding; telomeric protein

Figure 2.1. **Translational State Array Analysis (TSAA) identified mRNAs translationally regulated by IFE-1.** (A) Polysome profile of whole worm lysates resolved on a 10-45% sucrose gradient. Absorbance was monitored at 254nm during fractionation. RNA derived from non-polysomal (NP) fractions (1-4) and polysomal (P) fractions (6-11) were pooled separately for the TSAA. (B) Mean fold change of relative polysomal probe signal (MFR) was calculated using customized Excel data sheets with formulas created by the authors. MFR values were sorted and culled from the initial 22,629 probe output signals to a list of 77 mRNAs with an MFR>1.5 and an MFR–S.D.>1.0. (C) Bivariate response of mean Rw by mean Ri for the 13,372 mRNAs statistically "present" in all three biological replicates, derived in JMP Pro10 software. mRNAs with a fold change greater than 1.5 are highlighted in bold. (D) Table representing mRNAs of known germ line or embryonic regulatory function, derived by cross-referencing mRNAs with WormBase.

Figure 2.2

A


IFE-1-dependent mRNAs
Greatest Changes; n=77
Mean Fold R Value
Mean Fold Total
Mean Fold Polysomal
Fold Change
40
30
20
10
5
4.0
3.0
2.0
1.0
0.0
lin-26
cpt-6
srbc-1
his-2
elo-4
Fe-S enz
tomm-20
skr-16
mito rpS35
PP-ase
grl-20
vpr-1
pqn-70
mito rpS15
vab-1
lin-7
swd-2.2
elF-2B d
rnp-3
gst-43
rab-7
hmg-5
Tyr P-ase
U hydrolase
ttr-39
dmd-11
pqn-96
dnj-15
cyc-2.2
clc-2
ash-2
Cu transporter
S/T kinase
leo-1
pas-7
clpp-1
rab-5
let-49
vet-1
lkb-1
blos-1
ins-37
dkf-2
Gene ID

B

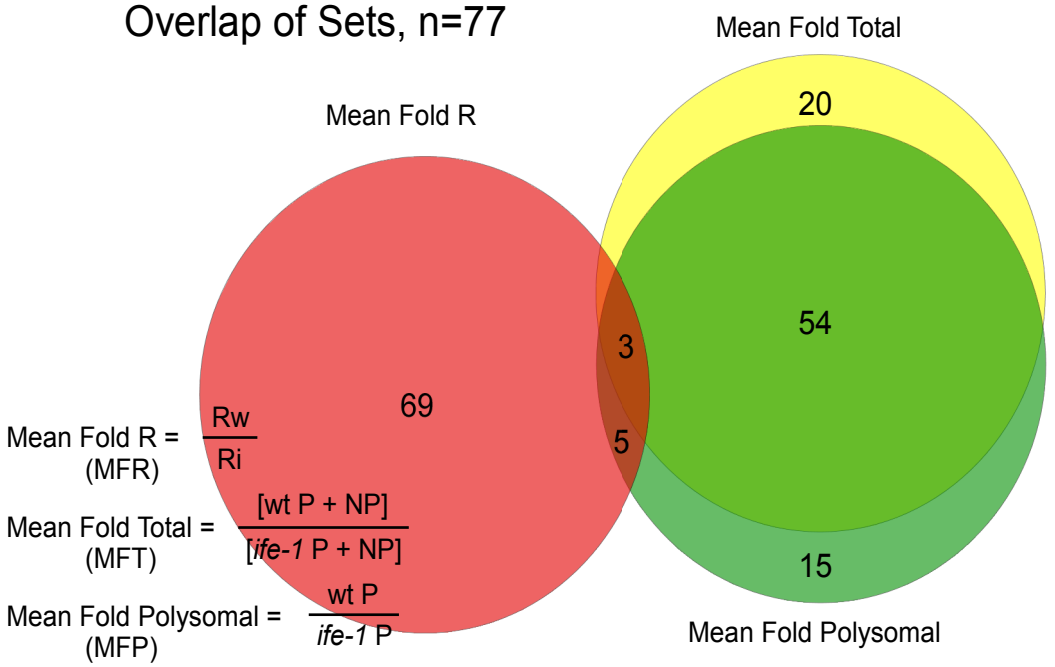

IFE-1-dependent mRNAs
Overlap of Sets, n=77
Mean Fold Total
Mean Fold R
Mean Fold Polysomal
20
54
3
69
5
15
Mean Fold R = Rw / Ri (MFR)
Mean Fold Total = [wt P + NP] / [ife-1 P + NP] (MFT)
Mean Fold Polysomal = wt P / ife-1 P (MFP)

Figure 2.2. IFE-1 Dependent mRNA. Of the 22,625 total probe output signals from Affymetrix Microarrays, 13,372 mRNAs were considered statistically "present" in all three polysome gradient replicates. From this list, mRNAs were prioritized by their mean fold R values with the restriction that the mean fold R value minus the standard deviation must be greater than 1.0. 77 mRNAs had a mean fold change greater than 1.5 (A). Plotted in bar graphs, the 77 genes with ranked mean fold R (MFR) changes were plotted with their changes in mean total (MFT) and polysomal fold change (MFP). Gene IDs provided from Affymetrix were cross referenced with WormBase for identification of known genes (X axis). (B) When the 13,372 mRNAs were prioritized by mean R, total, and polysomal fold change independently, the identities of 77 mRNAs with greatest of each fold change were compared in a Venn diagram derived in JMP Pro10. The substantial overlap shows a strong correlation between mRNAs with a large change in both total mRNA and polysomal mRNA amount.

We assessed the partitioning of mRNAs into polysomal and non-polysomal pools. A prioritized list of 77 IFE-1-dependent mRNAs was derived from the 13,372 probe sets present in all three biological replicates. Each of these mRNAs showed greater than a 1.5-fold increase in relative polysome loading (MFR) when IFE-1 was present (Figure 2.1B,C), which was largely maintained between individual replicates (Figure 2.3). Several of the 77 IFE-1-dependent mRNAs (*vab-1, vpr-1,* and *rab-7*) that are involved in the regulation of MAP kinase in late stage oocyte maturation (Figure 2.1D). Others (*lin-26, rab-5, rnp-3, let-49, hmg-5 and grl-20*) are essential for meiotic progression or embryonic development following fertilization. The identification of these mRNAs was consistent with previous genetic and biochemical characterizations of IFE-1's role in protein synthesis, specifically in oocytes and early embryos. Sperm-specific mRNAs, such as those encoding the 28 known major sperm proteins (MSPs) were not among those identified by the TSAA. Spermatogenesis was compromised in the *ife-1* mutant even at the permissive temperature used in the TSAA (Henderson et al., 2009). MSPs were shown by proteomic analysis to be deficient in *ife-1* worms (Kawasaki et al., 2011). By segregating the MFR and MFT for all identified *msp* mRNAs, our microarray data show that *msp* mRNAs are underrepresented in the total population but are not changed in their proportional polysome loading (Figure 2.4, C,D). Decreased MSP protein in IFE-1-deficient worms is therefore due to the lack of mRNA rather than translational control. Comparison of changes in total mRNA abundance (MFT) showed a trend for a slight loss in total mRNA content in the absence of IFE-1 (Figure 2.5, 2.6).

Figure 2.3

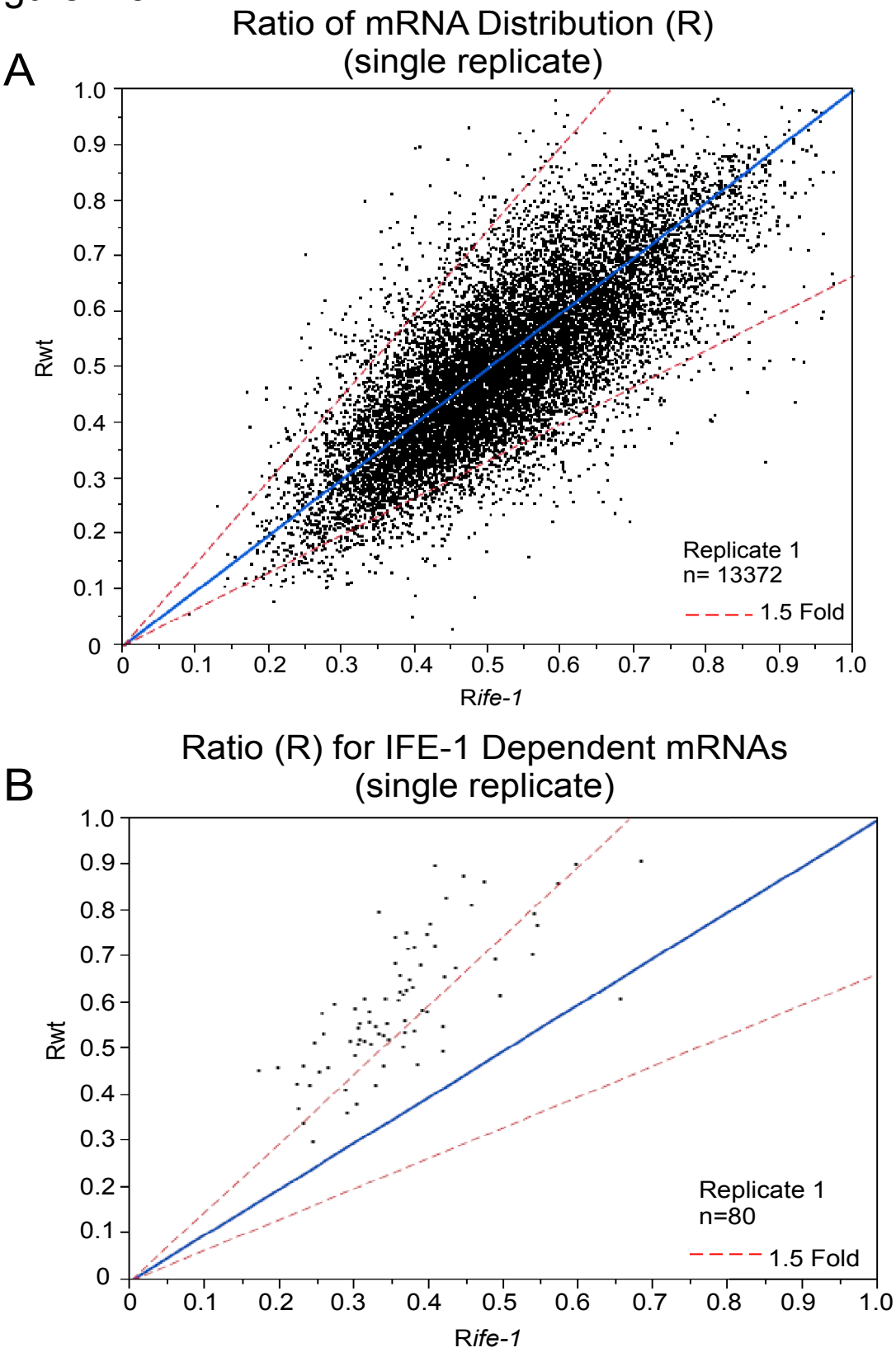

A
Ratio of mRNA Distribution (R)
(single replicate)
Rwt
Rife-1
Replicate 1
n= 13372
1.5 Fold
B
Ratio (R) for IFE-1 Dependent mRNAs
(single replicate)
Rwt
Rife-1
Replicate 1
n=80
1.5 Fold

Figure 2.3. Distribution of mRNAs by Fold R Value. (A) A bivariate response of Rw by Ri for all "present" mRNAs (13,372) in a single replicate. This shows the range in R fold values within a single replicate, compared to that of the mean fold change presented in Figure 2.1C. (B) The bivariate response of Rw by Ri for only the 77 mRNAs with a mean R fold change greater than 1.5. This is a representation of the variance observed within a single replicate.

Figure 2.4

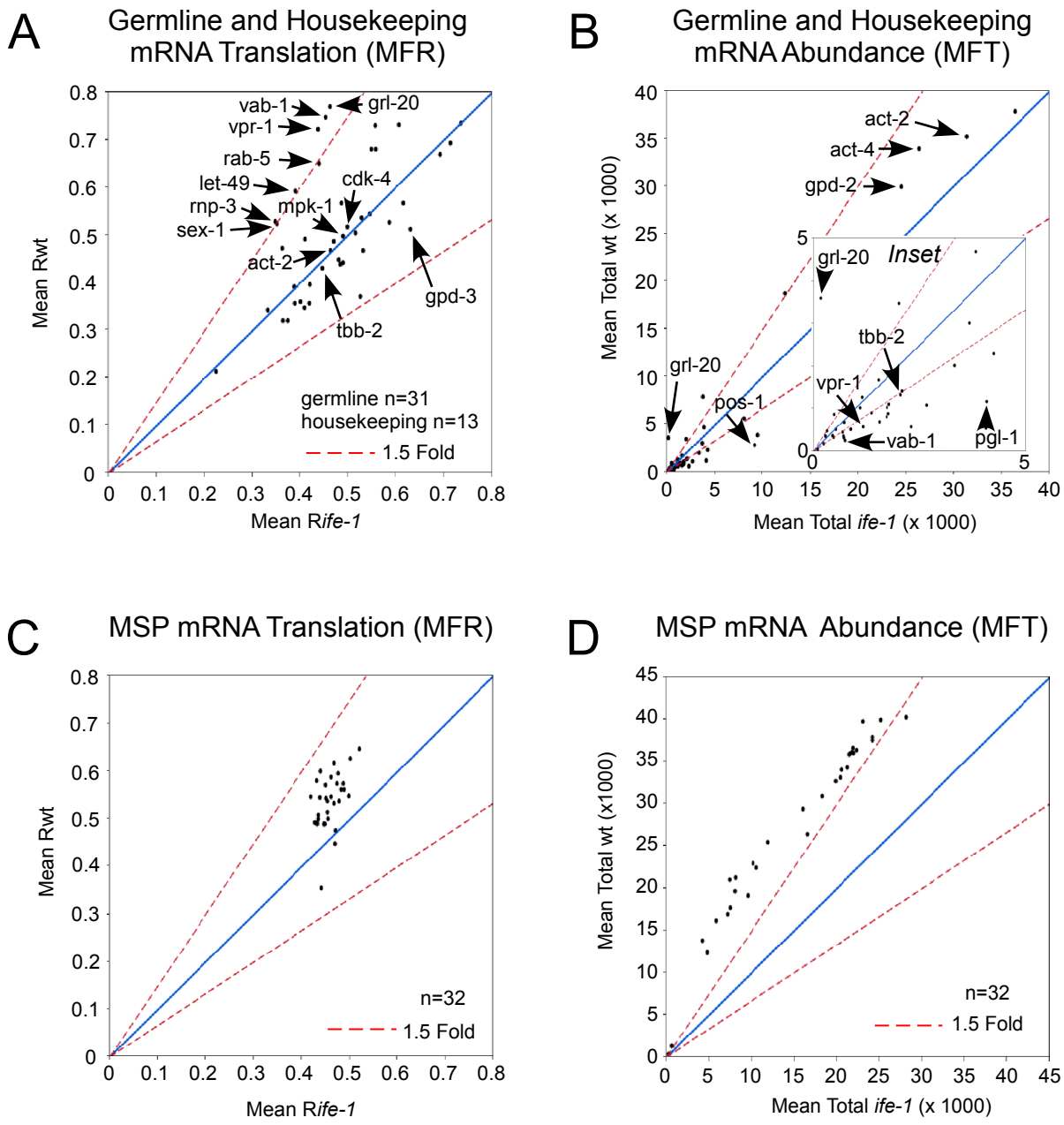

A
Germline and Housekeeping mRNA Translation (MFR)
Mean Rwt
Mean Rife-1
vab-1
grl-20
vpr-1
rab-5
let-49
cdk-4
rnp-3
mpk-1
sex-1
act-2
gpd-3
tbb-2
germline n=31
housekeeping n=13
1.5 Fold
B
Germline and Housekeeping mRNA Abundance (MFT)
Mean Total wt (x 1000)
Mean Total ife-1 (x 1000)
act-2
act-4
gpd-2
grl-20
Inset
tbb-2
vpr-1
grl-20
pos-1
vab-1
pgl-1
C
MSP mRNA Translation (MFR)
Mean Rwt
Mean Rife-1
n=32
1.5 Fold
D
MSP mRNA Abundance (MFT)
Mean Total wt (x1000)
Mean Total ife-1 (x 1000)
n=32
1.5 Fold

Figure 2.4. Distributions of Germline and Housekeeping Genes. A graphical distribution of 31 germline and 13 housekeeping genes for both mean Rw by mean Ri (A) and mean total wt by mean total *ife-1* (B). Of note, housekeeping genes used as controls in polysome profiles and 3'UTR reporter assays, *gpd-3* and *tbb-2* respectively, have little change in R fold values when IFE-1 is knocked out. *gpd-3* mean R fold change = 0.90 ± 0.12; *tbb-2* mean R fold change = 0.97 ± 0.17. Major sperm protein (MSP) mRNAs were also analyzed for mean R fold change (C) and mean total fold change (D). For all 32 MSPs identified, there was an increase in total mRNA when IFE-1 is present, but insignificant change in translational efficiency.

Figure 2.5

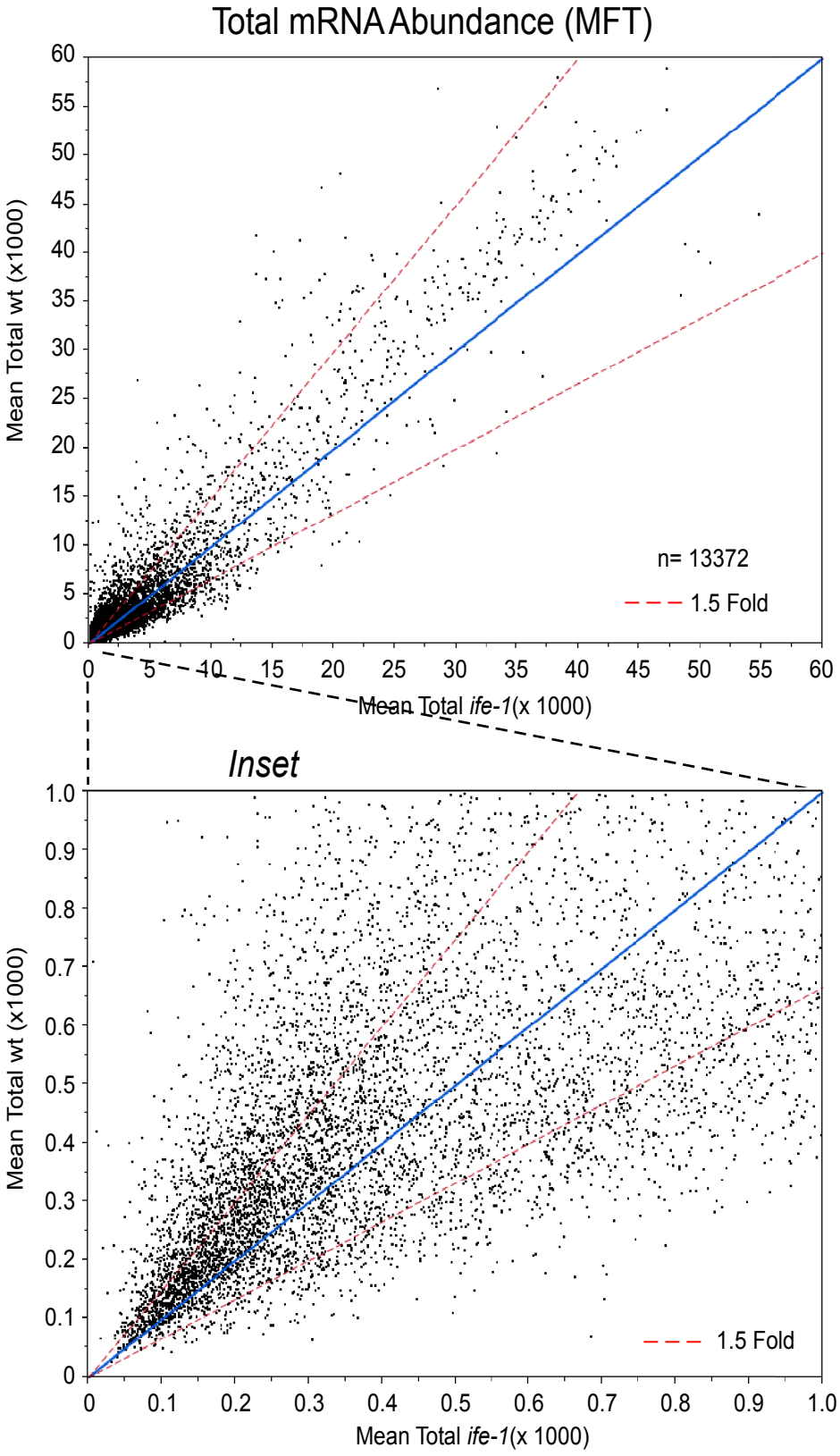

Total mRNA Abundance (MFT)
Mean Total wt (x1000)
Mean Total ife-1(x 1000)
n= 13372
1.5 Fold
Inset
Mean Total wt (x1000)
Mean Total ife-1(x 1000)
1.5 Fold

Figure 2.5. Distribution of mRNAs by Mean Fold Total Values (MFT). A bivariate response of mean total wt by mean total *ife-1* for the 13,372 "present" mRNAs, created in JMP Pro10. Data points are the sum of P + NP from the Affymetrix Array. This data shows a trend for a slight loss in total mRNA content when IFE-1 is absent. The inset shows a magnification of the lower-left quadrant (values 1000 x 1000), where most mRNAs reside.

Figure 2.6

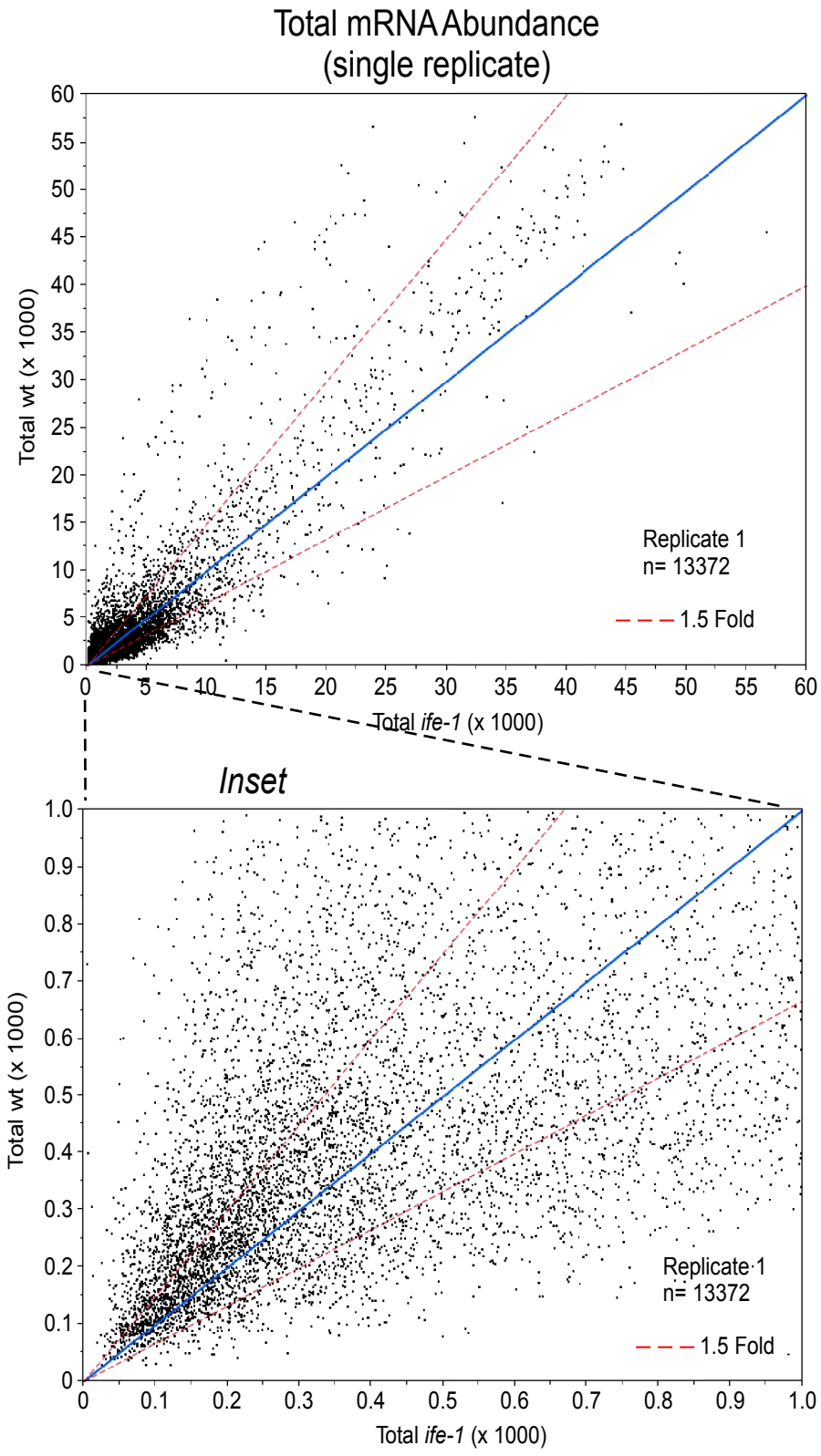

Total mRNA Abundance
(single replicate)
Total wt (x 1000)
Total *ife-1* (x 1000)
Replicate 1
n= 13372
1.5 Fold
Inset
Total wt (x 1000)
Total *ife-1* (x 1000)
Replicate 1
n= 13372
1.5 Fold

Figure 2.6. Distribution of mRNAs by Fold Total Values (Single Replicate). A bivariate response of total wt by total *ife-1* for the 13,372 "present" mRNAs, created in JMP Pro10. Data points are the sum of P + NP from the Affymetrix Array. This data shows a trend for a slight loss in total mRNA content when IFE-1 is absent. The inset shows a magnification of the lower-left quadrant (values 1000 x 1000), where most mRNAs reside. This is a representation of the variance observed within a single replicate.

IFE-1 recruits mRNAs that regulate germ cell differentiation and maturation events.

We analyzed the ribosomal loading of mRNAs by resolution on sucrose gradients in order to determine the detailed translational efficiency of specific IFE-1-dependent mRNAs. Based on known *ife-1* mutant phenotypes, we focused on known mRNAs that regulate germ cell fates during meiosis and oocyte maturation and showed decreased translational efficiency in the absence of IFE-1 in the TSAA. Lysates of wild type and *ife-1* animals were resolved via sucrose gradient centrifugation. Polysome profiles that resolve mRNAs by incremental ribosome loading were recorded (Figure 2.7A-B). mRNAs at the top of the fractionated gradient, above the 80S peak, do not have ribosomes bound and are not translating. mRNAs that sediment past the 80S peak have multiple ribosomes bound. These sediment progressively further with greater translational efficiency. It should be noted that ribosome loading increases exponentially rather than linearly along the gradient. mRNAs that shift from "light polysome" fractions to "heavy polysome" fractions in the gradient have undergone a greater change in protein synthesis efficiency than a similar shift near the top or middle of the gradient. Only a modest decrease in the total polysome content is observed for *ife-1*. This is to be expected because global protein synthesis (even cap-dependent) is still being supported via other eIF4E isoforms present.

vab-1, *vpr-1*, *rab-7* and *ran-1*- Isolated RNA from each sucrose fraction was subjected to real time PCR to determine changes in the translational efficiency of mRNAs identified as IFE-1-dependent in our TSAA. Primers were used to detect *vab-1* (MSP receptor), *vpr-1* (MSP domain protein), and *rab-7* (Rab GTPase), which are genes implicated in the regulation of late stage oocyte maturation. VAB-1 is a major sperm protein receptor that suppresses MAP kinase activity required for late stage oocyte maturation. In the *ife-1* worms there was a reduction in heavy polysomal *vab-1* mRNA (fractions 8-10) with a corresponding increase in non-translating

Figure 2.7

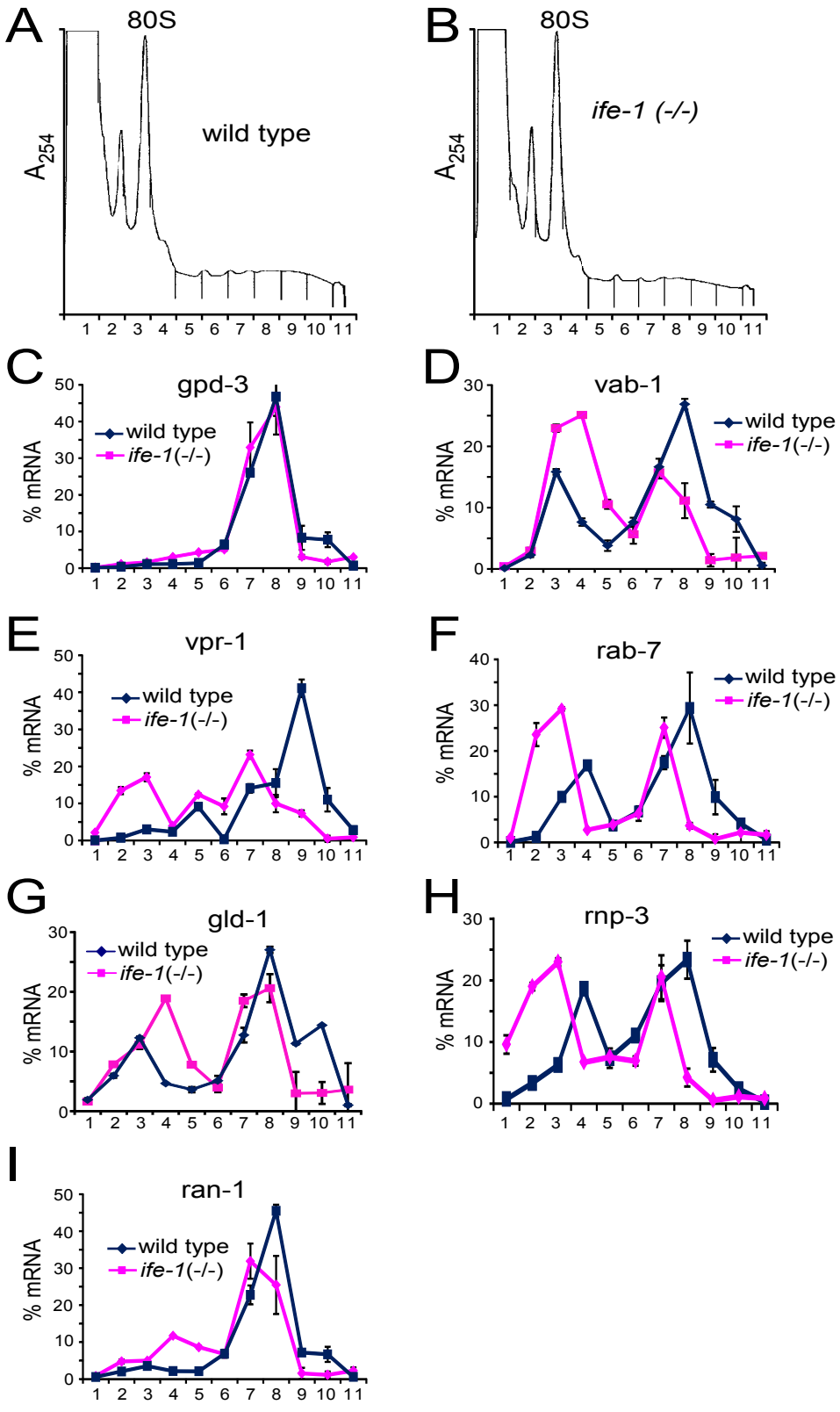

A
80S
wild type
A_{254}
1 2 3 4 5 6 7 8 9 10 11
B
80S
ife-1 (-/-)
A_{254}
1 2 3 4 5 6 7 8 9 10 11
C
gpd-3
wild type
ife-1(-/-)
% mRNA
D
vab-1
wild type
ife-1(-/-)
% mRNA
E
vpr-1
wild type
ife-1(-/-)
% mRNA
F
rab-7
wild type
ife-1(-/-)
% mRNA
G
gld-1
wild type
ife-1(-/-)
% mRNA
H
rnp-3
wild type
ife-1(-/-)
% mRNA
I
ran-1
wild type
ife-1(-/-)
% mRNA

Figure 2.7. Polysome fractionation analysis of germ line mRNAs. Polysome profiles for wild type (A) and *ife-1* null (B) whole worm lysates resolved on a 10-45% sucrose gradient. Profiles are derived by continuously monitoring absorbance at 254nm during fractionation. Profiles represent 1 of 3 biological replicates. Resolution is such that individual polysomes were apparent to at least the 6-mer size, and the monosome (80S) peak absorbance is the largest. Each subsequent peak depicts the addition of another bound ribosome. The quantitative distribution of individual mRNA sedimentation throughout the gradients. mRNA encoding *gpd-3* (C), *vab-1* (D), *vpr-1* (E), *rab-7* (F), *gld-1* (G), *rnp-3* (H), and *ran-1* (I), was quantified by qRT-PCR to indicate changes in translational efficiency. mRNA signal was normalized to total RNA content in each fraction.

and light polysomal *vab-1* mRNA (fractions 3-5; Figure 2.7D). This decrease in translational efficiency indicates that *vab-1* utilizes primarily IFE-1 for initiation. mRNAs encoding VPR-1, an MSP-type protein that binds VAB-1 receptor, and RAB-7, a Rab GTPase proposed to regulate VAB-1 trafficking, also show a marked decrease in heavy polysome loading, with a corresponding increase in the distribution of mRNA in non-translating or poorly translating fractions (Figure 2.7E,F). The decrease in translational efficiency of *vpr-1* and *rab-7* indicates that, in addition to *vab-1*, two mRNAs involved in VAB-1 signaling are also translationally recruited by IFE-1. General mRNA translation in the absence of IFE-1 is not perturbed, as evidenced by the lack of a change in the distribution of the housekeeping *gpd-3* (GAPDH) mRNA (Figure 2.7C). This suggests that other eIF4E isoforms are sufficient to support translation initiation of general housekeeping mRNAs. These data show that a set of proteins (VAB-1, VPR-1, RAB-7) that regulate oocyte MAP kinase utilize IFE-1 for their synthesis.

RAN-1, similar to RAB-7, is a GTPase that regulates VAB-1 trafficking in maturing oocytes. The dependence of *ran-1* mRNA on IFE-1 measured by TSAA showed only a 1.2-fold MFR increase in the presence of the isoform. However, when assessing the translational efficiency by resolved polysome gradient RNA, a more complex distribution of *ran-1* mRNA was evident. In the absence of IFE-1, *ran-1* showed a modest decrease in heavy polysome loading (fractions 8-10) with an increase in the light polysome region (fractions 4-7; Figure 2.7I). However, since most of its redistribution was among polysomes of varying sizes, rather than out of polysomes altogether, such changes in *ran-1* translation would not have been reflected in the TSAA. This points out the limitation of assessing simple "on/off" state translation assays using pooled RNA fractions, and suggests that the TSAA probably underestimates the translational impact. For *ran-1* mRNA (and others), this complex redistribution could indicate that translation

initiation via other IFE isoforms is sufficient to maintain modest synthesis in all tissues. Alternatively, IFE-1 may strongly recruit *ran-1* mRNA in the germline, while other IFEs initiate *ran-1* translation in the somatic tissue. These observations suggest that multiple mRNAs encoding meiotic maturation proteins selectively rely on IFE-1 for positive translational control.

gld-1- Much earlier in oogenesis, germ cells transition from mitotic expansion to meiotic differentiation. GLD-1 is an RBP that suppresses mitosis and promotes meiosis in immature oocytes by repressing the translation of key regulatory mRNAs, like *glp-1* (Hansen et al., 2004b; Jones et al., 1996). The translational efficiency of *gld-1* mRNA in *ife-1* worms was diminished, as indicated by a shift in its distribution from heavy polysomes (fractions 9-10; Figure 2.7G) to light polysomes (fractions 4-5). The decrease in heavy polysomal *gld-1* mRNA at the bottom of the gradient with a subsequent increase in the upper fractions indicated a dramatic decrease in the extent of initiation. These results corroborate our previous findings for *pos-1* and *oma-1*, showing that IFE-1 recruits mRNAs that regulate both early and late fate decisions throughout *C. elegans* germ cell development (Henderson et al., 2009).

rnp-3- RNP-3 is a small nuclear ribonucleoprotein (snRNP) that was identified as IFE-1-dependent in the TSAA. Loss of RNP-3 activity has been shown to result in modest embryonic lethality (Saldi et al., 2007). Like *vab-1*, *vpr-1* and *rab-7* mRNAs, *rnp-3* translation was much less efficient in the absence of IFE-1. The bulk of its mRNA shifted from heavy polysomes (fractions 8-9; Figure 2.7H) and monosomes (fraction 4) into the non-translating region of the gradient (fractions 1-3). The increase in non-translating *rnp-3* indicates that initiation events did not occur in the absence of IFE-1. Overall changes in *rnp-3* ribosome loading confirmed the 1.6-fold decrease observed in the TSAA, but also provide further information on the way in which its translation is hindered in the absence of IFE-1.

IFE-1 mediates spatial and temporal translational control throughout the gonad

Translation of *gld-1* mRNA in distal oocytes. Many germline mRNAs in *C. elegans* are regulated by repression via proteins binding to their 3' UTRs. These mRNAs need to be activated to bind ribosomes following de-repression. We hypothesize that as de-repression occurs, IFE-1 plays an active role in recruiting these mRNAs for translation initiation in a spatiotemporal manner. To determine IFE-1's ability to recruit de-repressed mRNAs in the proper context (time and place), we used a 3' UTR reporter assay. We visually monitored the translation of GFP-fused transgene mRNAs to observe changes in protein expression when IFE-1 was depleted by RNA interference (RNAi). As the reporter 3' UTR becomes de-repressed, GFP should be expressed in appropriate germ cells. If regulation of these mRNAs is mediated solely by de-repression of 3' UTR binding, their translation will be unaffected by the loss of any one eIF4E isoform. However, if only IFE-1 is able to recruit these mRNAs as they become de-repressed, GFP expression from these mRNAs will not occur. This assay provides the distinct advantage of being able to monitor translational control of an mRNA within single cells as they progress through the germline in live worms.

GLD-1 is expressed in immature oocytes in the distal end of the gonad where it promotes meiosis, and its expression is translationally controlled by the 3' UTR (Francis et al., 1995; Hansen et al., 2004b). Having established that *gld-1* mRNA was substantially dependent on IFE-1 by polysome profile analysis (Figure 2.7G), we then determined that translation of GLD-1 was regionally dependent on IFE-1. We used an *in vivo* reporter assay with an amino-terminal fusion to GFP, *gld-1* ORF, and the *gld-1* 3' UTR. Fluorescence microscopy showed that *gfp::gld-1* mRNA became de-repressed in immature oocytes in syncytium (Figure 2.8A). Upon depletion of IFE-1 by RNAi, the fluorescence in the whole distal region of the gonad was visibly diminished.

Figure 2.8

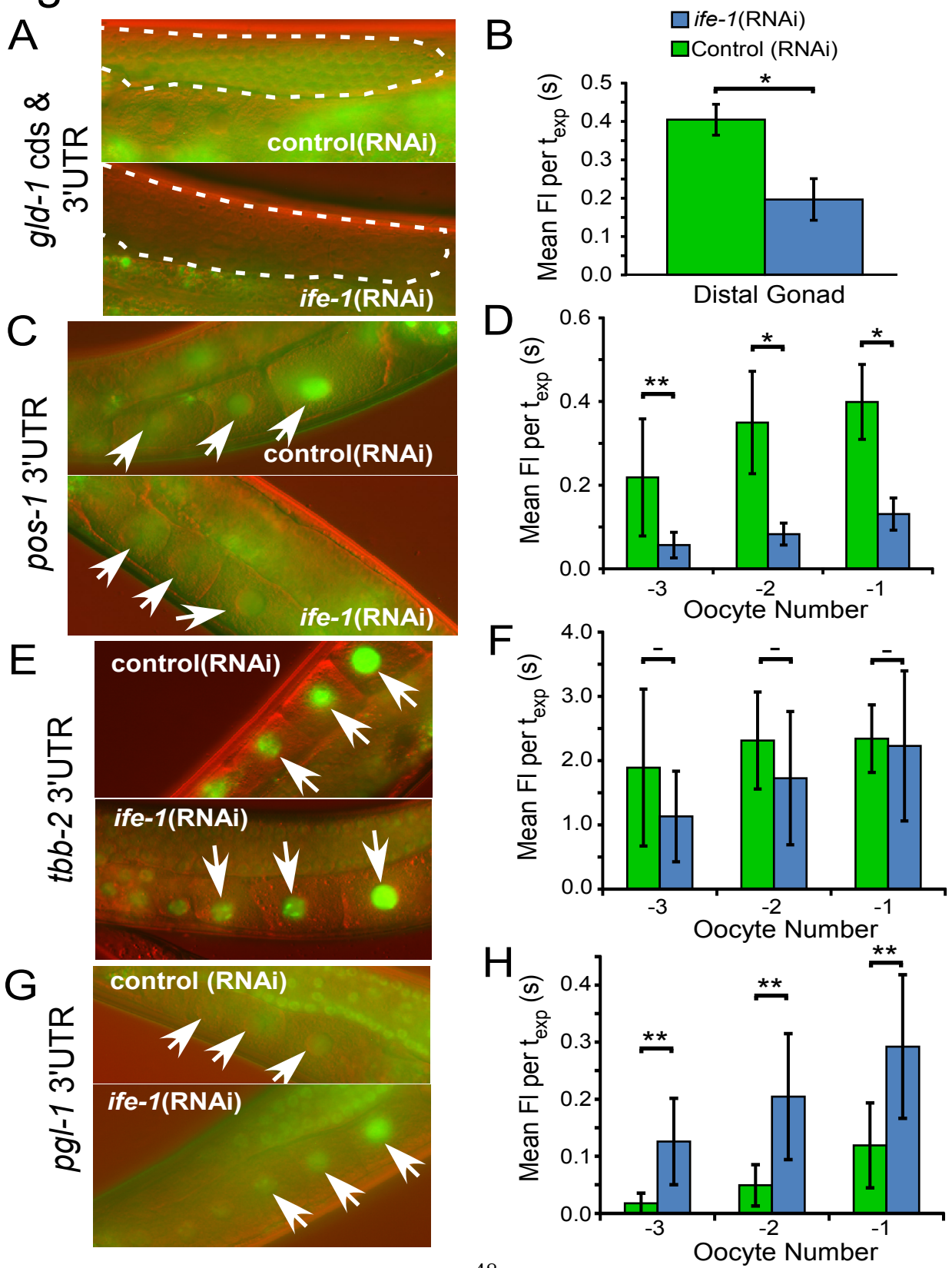

A
gld-1 cds & 3'UTR
control(RNAi)
ife-1(RNAi)
B
ife-1(RNAi)
Control (RNAi)
Mean FI per t_exp (s)
Distal Gonad
C
pos-1 3'UTR
control(RNAi)
ife-1(RNAi)
D
Mean FI per t_exp (s)
Oocyte Number
E
tbb-2 3'UTR
control(RNAi)
ife-1(RNAi)
F
Mean FI per t_exp (s)
Oocyte Number
G
pgl-1 3'UTR
control (RNAi)
ife-1(RNAi)
H
Mean FI per t_exp (s)
Oocyte Number

Figure 2.8. IFE-1 regulates mRNAs in a spatiotemporal manner in the germ line.

Microscopy of *in vivo* reporter constructs translationally regulated by 3'UTRs for *gld-1* (A), *pos-1* (C), *tbb-2* (E), and *pgl-1* (G). In the absence of IFE-1, de-repressed reporters under IFE-1 translational regulation were not efficiently expressed (B,D). Quantification of fluorescence was utilized from minimally five exposures, and mean fluorescence normalized to exposure time (Fl/t) was linear. This fluorescence measure showed that the *tbb-2* 3'UTR (F) reporter construct had no significant change in GFP expression (n=10). The *pos-1* 3'UTR (D) (n=11) and *gld-1* 3'UTR (B) (n=12) reporter constructs had a significant decrease in GFP expression while *pgl-1* (H) exhibited a significant increase in GFP expression in the absence of IFE-1 (n=11). "**" indicates $p<0.002$, "*" indicates $p<0.0001$, and "-" indicates no significant difference.

We determined there was a reproducible two-fold increase in GLD-1::GFP expression when IFE-1 was present by quantifying the mean GFP fluorescence intensity per exposure time (Fl/t) (Figure 2.8B). Our data show that even in immature oocytes, IFE-1 significantly contributes to positive translational control of *gld-1* mRNA. Importantly, these findings demonstrate IFE-1's role in the earliest known germ cell translational control event.

Spatial and temporal translation of *pos-1*, *tbb-2* and *pgl-1* mRNAs. For subsequent assays, we tested reporter constructs that fuse GFP to histone H2B and bear the 3' UTRs of various mRNAs, so that the fluorescence becomes localized in the oocyte nuclei. We previously demonstrated that *pos-1* mRNA requires IFE-1 to achieve even moderately efficient translation (Henderson et al., 2009). *pos-1* (posterior segregation) mRNA is natively repressed throughout most of oogenesis. It is de-repressed only in late stage oocytes so newly synthesized POS-1 protein can be utilized to establish early embryonic polarity. The *pos-1* 3' UTR reporter strain showed accumulation of the H2B::GFP product only in nuclei of the three largest oocytes of live worms. Following IFE-1 knockdown, H2B:GFP expression was nearly absent from oocytes of all stages (Figure 2.8C). Quantification of GFP (Fl/t) showed a three-fold increase in *pos-1* 3'UTR reporter translation by IFE-1 in each of the largest three oocytes (Figure 2.8D). This indicates that when the *pos-1* mRNA becomes natively de-repressed in the -3, -2, and -1 oocytes, only IFE-1 can recruit the mRNA for translation.

We tested a *tbb-2* 3'UTR reporter to demonstrate that IFE-1-mediated regulation applies primarily to translationally controlled mRNAs. *tbb-2* encodes β-tubulin and is expressed at high levels in the *C. elegans* germ line (Lu et al., 2004). When IFE-1 was depleted there was no visible decrease in H2B:GFP expression from the *tbb-2* 3'UTR reporter construct (Figure 2.8E). Comparison of GFP (Fl/t) in the late stage oocytes (-3, -2, and -1 oocytes) showed no significant

difference in H2B:GFP expression (Figure 2.8F). This was expected because *tbb-2* is not a translationally-repressed mRNA in germ cells (Merritt et al., 2008). It is clear that *tbb-2* mRNA is not IFE-1-dependent, and other IFEs can recruit it to ribosomes with equal efficiency. Thus, the observed temporally-regulated mRNA translation is specific to IFE-1 and not the result of general translation initiation activity.

PGL-1 is an mRNA binding protein that localizes IFE-1 protein to P granules. An H2B:GFP reporter with *pgl-1* 3'UTR was fully active for translation even in early meiotic germ cells (distal) from control RNAi-treated worms, and remained modestly abundant in late stage oocytes. Interestingly, when IFE-1 was depleted, GFP from *pgl-1* 3' UTR mRNA continued to be expressed in early germ cells and appeared to increase in all of the later stage oocytes (Figure 2.8G). Quantification showed a significant increase (more than two-fold) in GFP in the -3, -2, and -1 oocytes after IFE-1 knockdown (Figure 2.8H). Increased *pgl-1* mRNA translation in the absence of IFE-1 suggests that other IFE isoforms more efficiently recruit that mRNA for translation in the absence of IFE-1. By depleting IFE-1 *in vivo* from these transgenic GFP reporter strains, we identified a unique role for IFE-1 in the translational control of certain mRNAs within individual germ cells at critical junctures during development. Furthermore, IFE-1 regulates these mRNAs in conjunction with their 3' UTR sequences and therefore may coordinate with the proteins mediating their repression.

IFE-1 regulates MAPK activation in maturing oocytes.

Without IFE-1, oocytes grow slowly and mature inefficiently (Henderson et al., 2009). In *C. elegans*, growing oocytes naturally arrest in meiotic prophase (McCarter et al., 1999). MSPs induce the lysosomal degradation of a maturation-suppressing receptor protein, VAB-1,

primarily in the most proximal oocyte. As a result, oocyte maturation is induced with the activation of mitogen-dependent protein kinase (MAPK), a marker of cell cycle progression. Wild type gonads display activated MAPK only in the maturing (-1) oocyte when immunostained for diphospho-MAPK (Figure 2.9A). In worms depleted of IFE-1 by RNAi, gonads were devoid of activated MAPK (Figure 2.9C). In *vab-1* animals, there was no suppression of MAPK from the -1 through the -5 fully grown oocytes (Figure 2.9B), as previously published (Miller et al., 2003). Removing the suppressive role of VAB-1 permits a precocious oocyte maturation phenotype. When IFE-1 was knocked down in worms devoid of VAB-1, we observed even broader MAPK activation, earlier than the -5 oocyte, back to pachytene stages (Figure 2.9D). This indicated that IFE-1 activity can suppress MAPK activity independent of VAB-1 in very early oocytes. In the absence of both IFE-1 and VAB-1, the intensity of MAPK activation in all the later stage oocytes (-1 through -5) was diminished relative to the -1 oocyte in gonads with IFE-1 (Figure 2.9A), but greater than those lacking only IFE-1 (Figure 2.9C). This indicated that IFE-1 activity may suppress MAPK activation in all immature oocytes both VAB-1-dependent and independent, while still promoting MAPK activation in the late stage (-1) oocyte. Alternating suppression and activation of MAPK likely involves recruitment of several mRNAs in the immature vs the maturing oocytes. Since IFE-1 is expressed throughout all germ cell stages (Amiri et al., 2001), it may be exerting multiple phases of positive mRNA translational control along the path of oogenesis (Figure 2.9E).

Figure 2.9

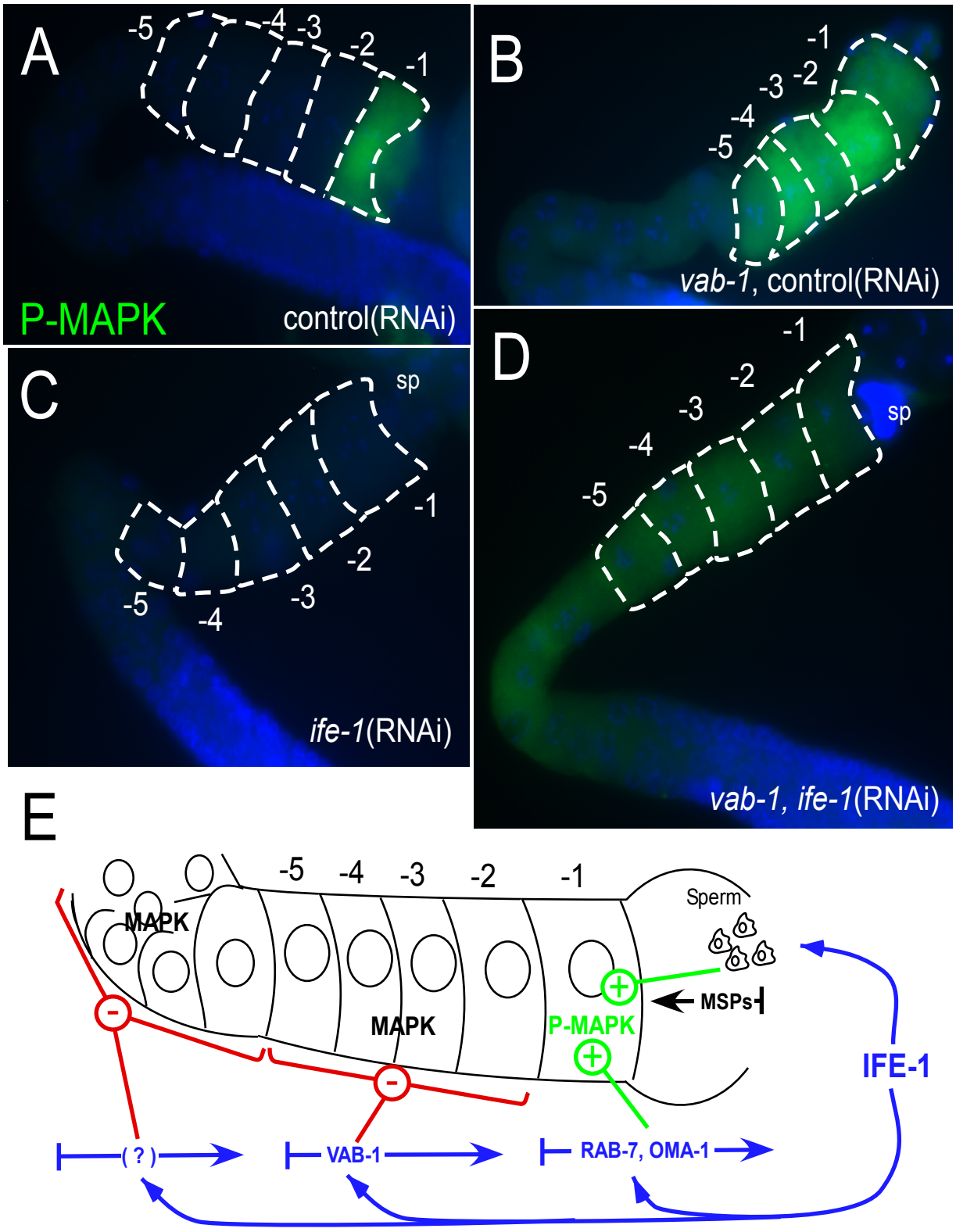

A
-5
-4
-3
-2
-1
P-MAPK
control(RNAi)
B
-1
-2
-3
-4
-5
vab-1, control(RNAi)
C
sp
-1
-2
-3
-4
-5
ife-1(RNAi)
D
-1
-2
-3
-4
-5
sp
vab-1, ife-1(RNAi)
E
-5
-4
-3
-2
-1
Sperm
MAPK
MAPK
P-MAPK
MSPs
IFE-1
(?)
VAB-1
RAB-7, OMA-1

Figure 2.9. Analysis of MAPK activation in early and late stage oocytes. Wild type (A) and *vab-1* null (B) dissected worm gonads were fixed and immunostained for activated (di-phosphorylated) MAPK after treatment with control RNAi. Similarly, *ife-1* RNAi knockdown in wild type (C) and *vab-1* null (D) exhibited a loss of MAPK activation in the -1 oocyte (C) and precocious low levels of MAPK activation in *vab-1* null *ife-1*(RNAi) oocytes (D). (E) Proposed model for IFE-1 regulation of late oocyte proteins and MAPK regulation in maturing oocytes.

The contribution of sperm signals versus cell-autonomous maturation competence becomes important as oocytes become fully grown. Given the maturation defect in the absence of IFE-1, we sought to determine which is compromised. We have previously shown that *ife-1*(bn127) worms do not make viable sperm at 25°C (Henderson et al., 2009). However, the loss of sperm is not observed in the *ife-1*(RNAi) phenotype (25°C; Figure 2.9D). We visualized MAPK under conditions in which wild type (viable) sperm could substitute for mutant sperm to rule out that the absence of MAPK activation in the -1 oocyte (Figure 2.9C) was due to insufficient or crippled sperm. *vab-1* worms crossed with wild type males (Figure 2.10A) exhibited the same extent of MAPK activation out to the -5 oocyte as uncrossed *vab-1* worms (Figure 2.10B). The same immunostaining pattern suggests that the sperm present in the *vab-1* mutant alone are sufficient to promote MAPK activation. We then depleted IFE-1 from *vab-1* worms crossed with wild type sperm. Again MAPK was activated beyond the -5 position (Figure 2.10B), as exhibited by the *vab-1 ife-1*(RNAi) gonads (Figure 2.9D). The lack of both VAB-1 and IFE-1 permits this broad, suboptimal MAPK activity throughout the whole proximal gonad.

We also examined MAPK activation in gonads of *ife-1* worms raised at 15°C (permissive for viable sperm) and 25°C (no sperm; Figure 2.10C,D) to address whether sperm-oocyte signaling may itself be altered when IFE-1 is absent. In both the presence and absence of isogenic sperm, *ife-1*(bn127) fully suppressed MAPK activation. This may be owing to lower number of sperm (Henderson et al., 2009) or the under-representation of *msp* mRNAs in worms lacking IFE-1 (Figure 2.4 D). TSAA data showed, however, *msp* mRNA translation did not depend on IFE-1 (Figure 2.4 C). When wild type sperm were presented to *ife-1* worms at 15°C (Figure 2.10I) and 25°C (Figure 2.10J), we observed a substantial resumption of MAPK activation in post-pachytene oocytes. This suggests that IFE-1-mediated suppression of MAPK

Figure 2.10

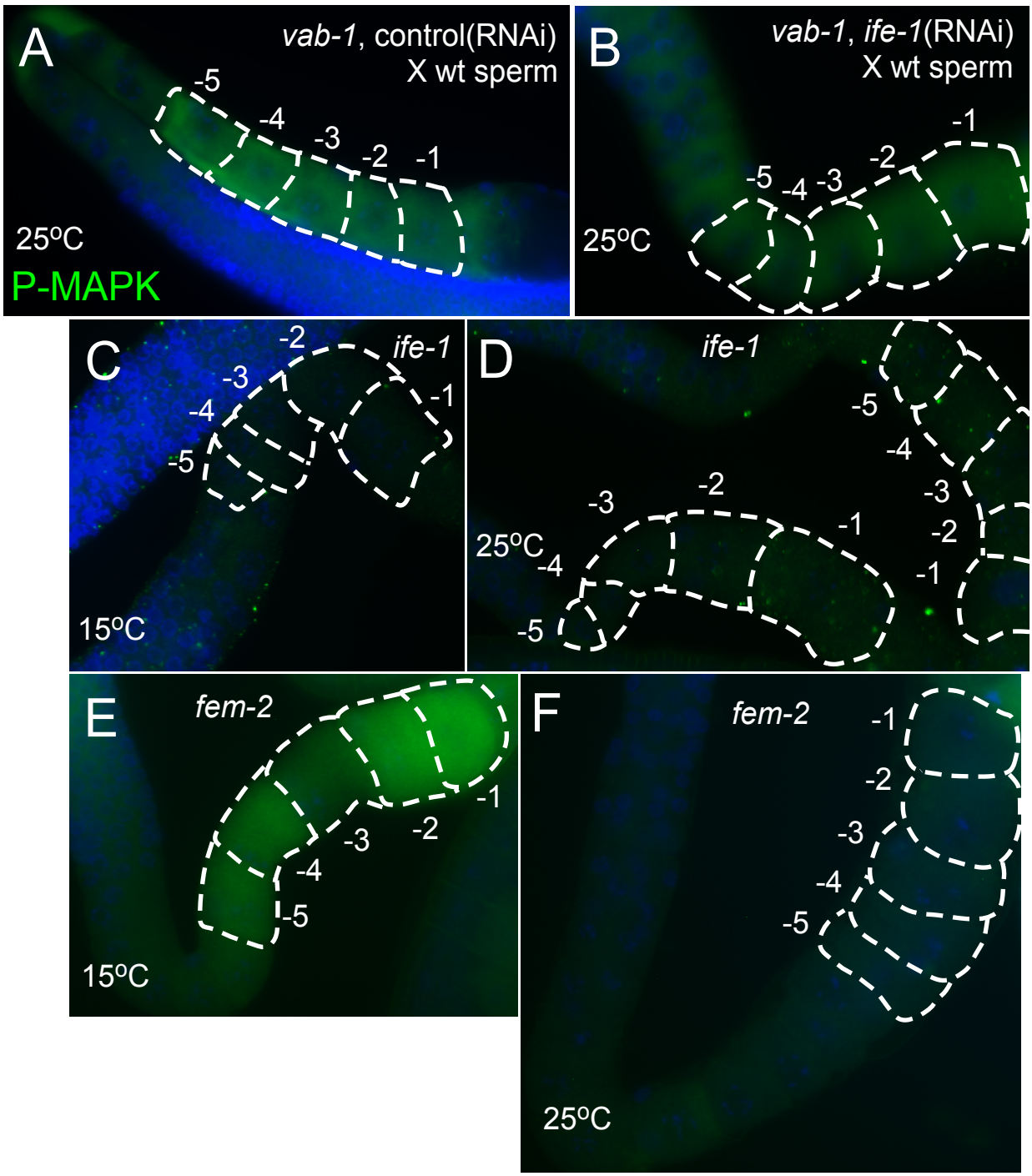

A
vab-1, control(RNAi)
X wt sperm
-5
-4
-3
-2
-1
25ºC
P-MAPK
B
vab-1, ife-1(RNAi)
X wt sperm
-1
-2
-5
-4
-3
25ºC
C
ife-1
-2
-3
-4
-5
-1
15ºC
D
ife-1
-5
-4
-3
-2
-1
25ºC
-4
-3
-2
-1
-5
E
fem-2
-1
-2
-3
-4
-5
15ºC
F
fem-2
-1
-2
-3
-4
-5
25ºC

Figure 2.10 - Continued

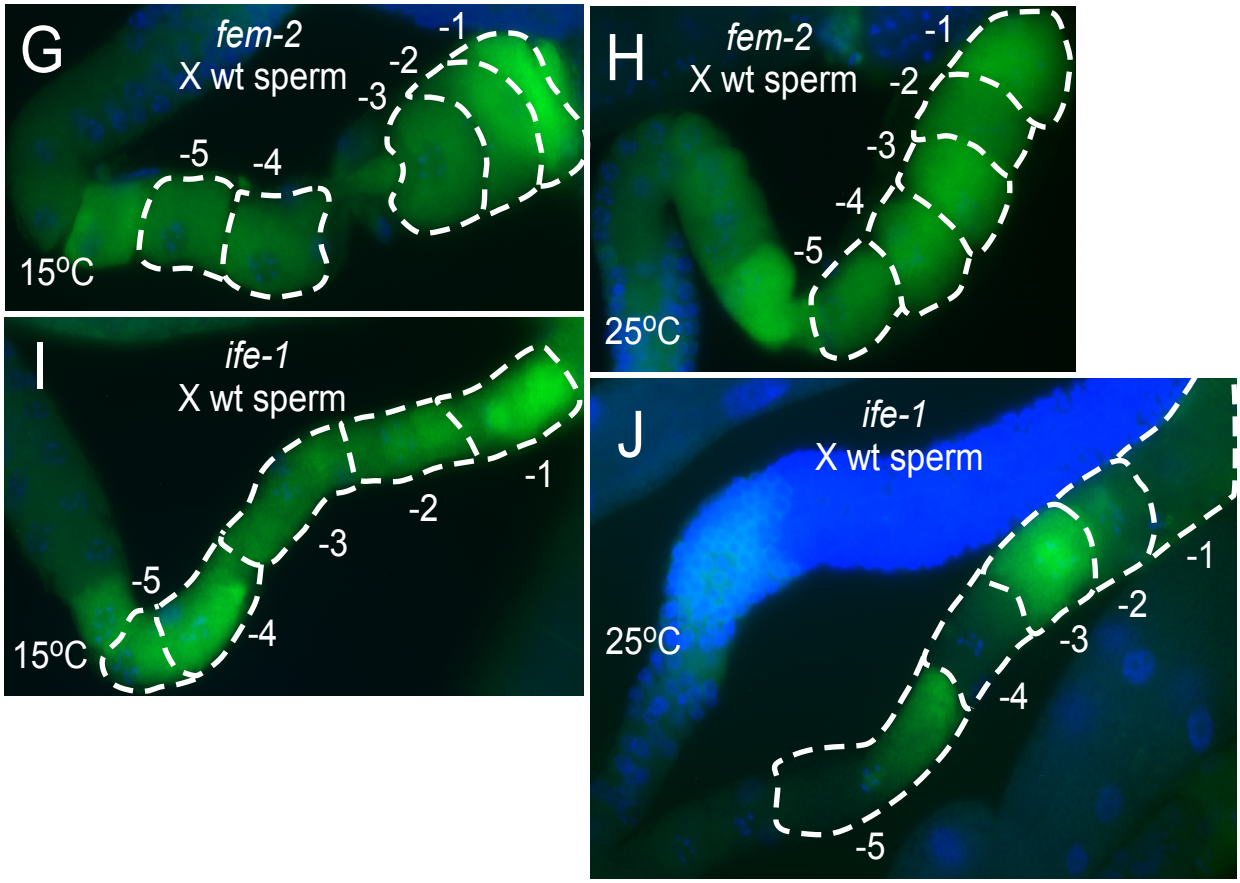


Figure 2.10. Analysis of MAPK activation in the presence of wild type sperm. *vab-1* null worms treated with control (A) and *ife-1* (B) RNAi were crossed with wild type males. Dissected gonads were fixed and immunostained for activated MAPK. Each germ line exhibited moderate MAPK activation profiles similar to that of uncrossed *vab-1* null mutants (Figure 2.9B,D). *ife-1* worms were grown at the sperm-permissive temperature (C) 15°C and at the sperm restrictive temperature (D) 25°C (no sperm). These gonads displayed a complete loss of MAPK activation as seen in wild type *ife-1* knockdown worms (Figure 2.9C). *fem-2* worms grown at sperm-permissive temperature exhibited MAPK activation in the -1 through -5 oocytes (E), which was lost in feminized gonads without sperm (F). *fem-2* mutants crossed with wild type sperm had activated MAPK in -1 through -5 oocytes (G) that was maintained when feminized (H). *ife-1* worms grown at 15°C (I) and 25°C (J) and crossed with wild type sperm restored modest MAPK activation that was lost in the *ife-1* null and knockdown worms.

activation may be overcome by wild type MSP signaling. To address how a deficiency in the sperm MSP signal might affect kinase signaling in proximal oocytes, we looked for MAPK activation in a *fem-2*(b245) strain that also fails to make viable sperm at 25°C. Given the translational role of IFE-1 during meiotic maturation, we expected to see a MAPK pattern that reflected primarily the loss of -1 oocyte kinase activation. Remarkably, *fem-2* loss of function completely recapitulates the MAPK pattern seen in oocytes lacking IFE-1. MAPK activity was absent in *fem-2* oocytes of all stages at the restrictive temperature (Figure 2.10F), but became broadly activated even beyond the -5 oocyte in the presence of sperm (Figure 2.10G,H). This mimicked MAPK activation for worms lacking IFE-1 (Figure 2.10C,D) and the derestriction of kinase activation found in *ife-1* oocytes in response to normal sperm (Figure 2.10I,J). This suggests that *fem-2* acts downstream or in parallel to *ife-1*. The simplest interpretation would be that *fem-2* mRNA is likewise IFE-1-dependent. However, TSAA data show an MFR of 0.99 +/- 0.25, making it unlikely that IFE-1 translationally controls *fem-2* mRNA directly. Perhaps other mRNAs involved in FEM-2 signaling are translationally regulated by IFE-1. These data suggest that in the absence of IFE-1, MSP signaling can override suppression of MAPK activation. Our current and previous findings support a model in which IFE-1-dependent mRNAs play a role in promoting MAPK activation in the mature (-1) oocyte and suppressing MAPK activation in immature oocytes (Figure 2.9E).

Discussion

During *C. elegans* germline development, proliferation and differentiation of the stem cells into competent eggs and sperm are regulated by mRNA translational control. The selective protein synthesis of these germ cell determinants is governed temporally and spatially by both sequence-specific mRNA repression and translational activation. Much has been described about mRNA repression by RNA-binding proteins (e.g. GLD-1, FBFs, OMA-1, CPEB, Nanos, Pumilio) and how they are critical for germ cell development (reviewed in (Mendez and Richter, 2001; Nousch and Eckmann, 2013; Parisi and Lin, 2000). This repression, however, represents only the first half of the regulation. Our studies focus on positive translational control of mRNAs and the subsequent developmental activities. Distinct isoforms of translation initiation factor eIF4E carry out selective translational recruitment of mRNAs (Amiri et al., 2001; Dinkova et al., 2005; Henderson et al., 2009; Keiper et al., 2000; Miyoshi et al., 2002; Song et al., 2010). Here, we identified the entire compliment of mRNAs recruited by the eIF4E isoform IFE-1 in germ cells. Our data implicate IFE-1-dependent positive translational control in several temporal phases of sperm and oocyte progression, including entry into meiosis and maturation (Figure 2.11).

IFE-1 recruits a unique set of mRNAs, distinct from the four other eIF4E isoforms (IFEs 2-5) expressed in *C. elegans*. The deletion of *ife*-1 is neither sufficient to substantially disrupt overall protein synthesis, nor the mRNA cap-dependent recruitment step (Figure 2.7A-C). Three different eIF4E isoforms, IFE-1, -3 and -5, are enriched in the germline (Keiper et al., 2000). Most individual *ife* mutant strains are viable and display unique developmental phenotypes. Defects in *ife-1* worms include temperature-sensitive secondary spermatocyte arrest, reduced oocyte production and viability, and embryonic arrest (Henderson et al., 2009). Such phenotypes

Figure 2.11

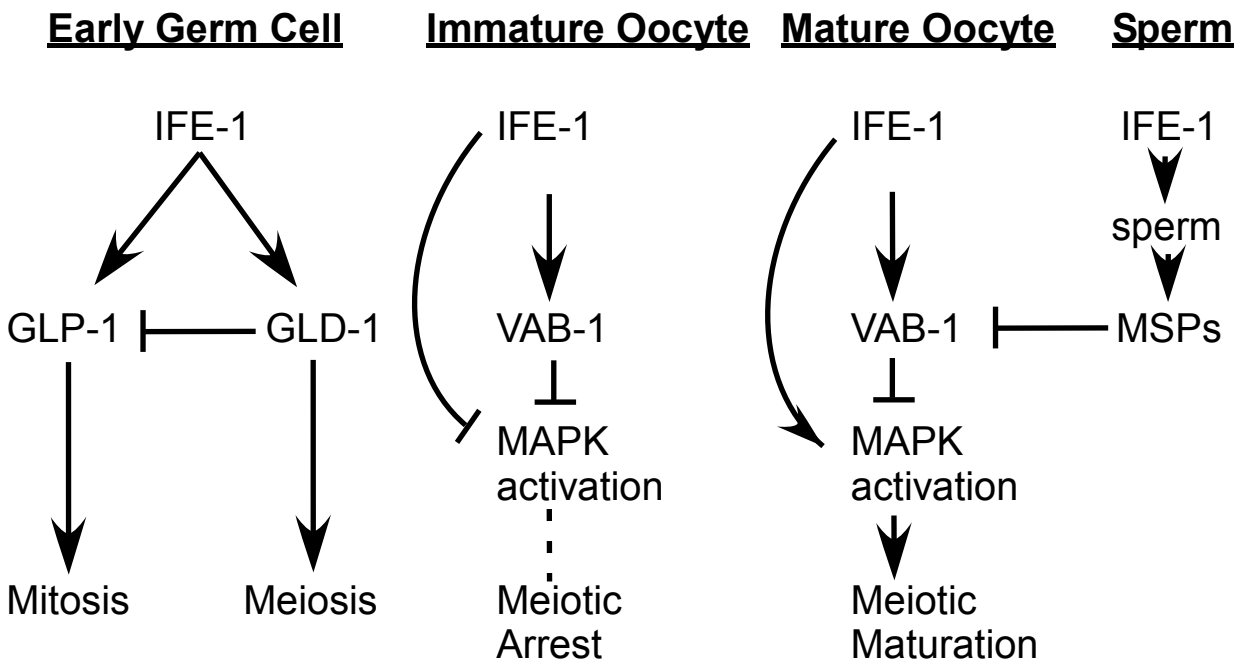

Early Germ Cell
Immature Oocyte
Mature Oocyte
Sperm
IFE-1
IFE-1
IFE-1
IFE-1
sperm
GLP-1
GLD-1
VAB-1
VAB-1
MSPs
MAPK
activation
MAPK
activation
Mitosis
Meiosis
Meiotic
Arrest
Meiotic
Maturation

Figure 2.11. Working model for IFE-1's role in early germ cell meiosis, MAPK suppression and activation, and maturation. Our data supports a model in which IFE-1 natively suppresses MAPK activation in immature oocytes by promoting *vab-1* mRNA translation. In the maturing oocyte, most directly influenced by MSP signaling, IFE-1 also appears to play an opposing role in the maturing (-1) oocyte by promoting MAPK activation through substrates other than *vab-1* mRNA. Finally, IFE-1's role also includes promoting viable sperm MSP production and subsequent signaling of MAPK activation in the maturing oocyte by destroying VAB-1 signaling.

suggest that IFE-1 is responsible for translating a defined subpopulation of mRNAs. Our TSAA identified 77 mRNAs that specifically require IFE-1 for efficient translation. The identified mRNAs are involved in a range of cellular activities such as GTPases (*ran-1, rab-5, rab-7*) required for mitotic spindle formation or vesicle trafficking, Ephrin receptor tyrosine kinase activity (*vab-1, vpr-1*) that regulates oocyte maturation, and transcription/splicing factors (*rnp-3, let-49, lin-26*) necessary for embryonic gene expression (Figure 2.1D).

IFE-1 recruits individual mRNAs that are translationally repressed in mitotic and early meiotic germ cells, as demonstrated by polysome analyses here and in our previous study (Henderson et al., 2009). The *glp-1* and *gld-1* mRNAs, which are inversely translationally repressed, both require IFE-1 for efficient translation. GLD-1 has been identified as an integral regulator in the mitosis/meiosis switch in immature oocytes. GLD-1 binds and represses mitotic mRNAs including *glp-1* (Hansen et al., 2004a; Marin and Evans, 2003). In the absence of GLD-1, mitotic germ cells fill the gonad, forming a germ cell tumor (Jeong et al., 2011). Polysome fractionation demonstrated that *gld-1* mRNA requires IFE-1 for efficient translation *in vivo*. Results from GFP reporter experiments showed that IFE-1 mediates translational control via the 3'UTR and does so appropriately in early meiosis (Figure 2.8A). Diminished levels of both in the absence of IFE-1 may be offsetting, since this eIF4E isoform promotes synthesis of pro-mitotic GLP-1 and pro-meiotic GLD-1. Accordingly, mitotic germ cell tumors do not form in *ife-1* worms. GLD-1 acts in conjunction with other compensatory factors (GLD-2, GLD-3) in the distal gonad, such that the phenotypic outcomes will depend on levels of these proteins as well (Eckmann et al., 2004). However, it is clear that IFE-1 contributes to the native homeostasis of immature germ cell protein synthesis necessary for meiotic progression.

Oocytes naturally arrest in prophase before maturation and fertilization (McCarter et al., 1999). In *C. elegans,* the meiotic arrest is due to signaling from VAB-1, which suppresses mitogen-dependent protein kinase (MAPK) (Cheng et al., 2008; Miller et al., 2003). VAB-1 is the Ephrin receptor tyrosine kinase expressed both in germ cells and their somatic neighbors. MSPs released from sperm signal for VAB-1 to be relocalized and degraded (Cheng et al., 2008). VAB-1 degradation in oocytes restores MAPK activation, which induces cyclin-B and causes resumption of the cell cycle, culminating in oocyte maturation (Miller et al., 2003). We show that IFE-1 promotes synthesis of VAB-1 in late-stage, non-maturing oocytes. Furthermore, IFE-1 translationally controls not only *vab*-1 mRNA, but also mRNAs encoding several VAB-1 regulators. These include VPR-1, the VAP ortholog containing an MSP domain that binds VAB-1 *in vitro*; RAN-1, a Ran GTPase that promotes trafficking of VAB-1 to the endocytic recycling vesicles where VAB-1 is predicted to suppress MAPK activity; and RAB-7, a Rab GTPase predicted to promote trafficking of VAB-1 to lysosomes for degradation (Cheng et al., 2008). Thus, one eIF4E isoform acts through cap-dependent positive mRNA translational control to induce both positive and negative signaling for oocyte maturation. Additionally, the various patterns of MAPK activation show that IFE-1 has a role in suppressing MAPK activation in young post-pachytene oocytes (prior to the -5 position) that is independent of VAB-1, suppresses MAPK in proximal (-5 to -1) oocytes through VAB-1, and plays a converse role in promoting sperm-induced MAPK activation in the mature (-1) oocyte.

An interesting clue to IFE-1's larger role in early/late MAPK regulation came from mating experiments with wild type sperm. Both *ife-1* and *fem-2* prevent sperm production and fail to activate MAPK in the mature (-1) oocyte. However, *ife-1* "females" exposed to wild type sperm again elicited a broad, but dampened MAPK activation in all late stage oocytes. The

identical aberrant MAPK activation was seen in *fem-2* "females" crossed with wild type males (Figure 2.10H). These data suggest that *ife-1*-dependent mRNAs may act in a pathway to MAPK activation that includes *fem-2*. FEM-2 is a putative protein phosphatase that is known to function in the sperm/oocyte switch (Hodgkin, 1986), but it is unclear what role it plays in post-pachytene oocyte maturation. As a protein phosphatase, it is conceivable that FEM-2 has a role in suppressing MAPK through dephosphorylation events. We also previously showed that, unlike *ife-1*, which slows oocyte growth and fertility even at permissive temperatures, *fem-2* has no demonstrable effect on oocyte production or fertility (Henderson et al., 2009). Evidence suggests that *ife-1* and *fem-2* act in the same pathway to maturation, but the relationship between them is unclear.

We propose that IFE-1 mediates the recruitment of most translationally-regulated mRNAs in late stage oocytes (e.g. *vab-1*, *rab-7*, *oma-1*, *mex-1*, and *pos-1*) (Henderson et al., 2009). Other mRNAs identified in our TSAA screen, and verified by polysome resolution of endogenous mRNAs, are also interesting in light of *ife-1* phenotypes. *rnp-3* mRNA, for example, encodes an snRNP-associated spliceosomal protein RNP-3/U2B required for embryonic viability. Although redundantly active with RNP-2/U1A, knockout of *rnp-3* alone is sufficient to induce low levels of embryonic lethality (Saldi et al., 2007). The inability of *ife-1* worms to efficiently translate *rnp-3* mRNA may help explain the embryonic arrest and overall reduced fecundity (Amiri et al., 2001). These data indicate that IFE-1 has a substantially broader translational affect than the characterized RBPs. IFE-1 induces the selective synthesis of a series of proteins that both promote and suppress cell differentiation, depending upon where and when IFE-1 encounters their mRNAs.

We recognize that the preferential recruitment of an mRNA by one IFE isoform over another is not likely due to sequence binding specificity at the 5' mRNA cap. eIF4Es bind just the 5'-cap and first two nucleotides of an mRNA (Marcotrigiano et al., 1997a). Even though IFE isoforms show preferences for either monomethylated or trimethylguanosine 5'-caps (Miyoshi et al., 2002), this level of discrimination is insufficient to explain the unique mRNA selectivity we have now confirmed for IFE-1, IFE-2 and IFE-4 (Dinkova et al., 2005; Henderson et al., 2009; Song et al., 2010) and this study). Instead, preferential mRNA recruitment may come from interactions between the dorsal or lateral faces of eIF4Es with various 4EBPs (Igreja et al.). Of the germline eIF4E isoforms, IFE-1 uniquely associates with one such 4EBP in P granules, known as PGL-1 (Amiri et al., 2001). Protein-protein complexes involving other eIF4Es and 4EBPs have been shown to recognize motifs in mRNA 3'UTRs and repress the translation of the bound message (Pique et al., 2008; Stebbins-Boaz et al., 1999). As bound mRNAs become required, 3'UTR complexes become remodeled such that eIF4E can associate with eIF4G and recruit the message to ribosomes (Cao and Richter, 2002). In *C. elegans,* the *gld-1* mRNA is bound and translationally repressed by a Pumilio homolog (FBF) in the mitotic region of the germ line (Marin et al., 2003). When germ cells migrate away from the niche, *gld-1* mRNA becomes de-repressed and subsequently recruited for translation by IFE-1. As germ cells approach the meiotic region, newly synthesized GLD-1 protein represses the translation of *glp-1* mRNA via 3'UTR interactions. The repression of *glp-1* is an important event in the transition from mitosis to meiosis (Hansen et al., 2004a). We have shown that IFE-1 plays a direct role in both processes by recruiting each mRNA temporally in turn. This paradigm for the mitosis to meiosis switch is just one example of how IFE-1-mediated translational control may be integral to cell fate decisions in the germ line (Figure 2.11).

Biochemical polysome fractionation allowed us to directly assay the translational efficiency of individual mRNAs, but it provided no information on where or when each mRNA was being recruited by IFE-1. Instead, the use of a histone H2B::GFP 3'UTR reporter assay permitted us to observe translational control events *in situ* and *in vivo.* We monitored spatiotemporal translational activation of each reporter mRNA within individual living worm gonads with single cell resolution. More importantly, since the worm gonad contains germ cells at all stages of development, even a single worm observation showed the entire developmental course of IFE-1-mediated translational control. Our data suggest that IFE-1 acts early to establish the germ cell shift from mitosis (stem cells) to meiosis (differentiated oocytes) by regulation of *gld-1* and *glp-1* mRNAs. Additionally *pos-1* mRNA became de-repressed in late stage oocytes, and this too was dependent on IFE-1. Similar late stage oocyte synthesis of MEX-1 protein in the -3, -2, and -1 oocytes was previously observed to be IFE-1 dependent (Henderson et al., 2009). Both observations suggest that other eIF4E isoforms present are not sufficient to efficiently recruit either *pos-1* or *mex-1* mRNAs to ribosomes. Not all late oocyte mRNAs require IFE-1, evidenced by identical expression of the *tbb-2* 3'UTR control. Interestingly, *pgl-1* 3'UTR mRNA actually increased in expression in the absence of IFE-1. Thus, not only is IFE-1 not required for the efficient *pgl-1* mRNA translation, but it may in fact, compete (e.g. for initiation complexes or free ribosomes) with the other IFEs that mediate PGL-1 synthesis. Thus far, we can define three classes of germline mRNAs relative to eIF4E isoform IFE-1: 1) those that uniquely require this isoform following de-repression, 2) those that do not require the IFE-1 isoform at all, 3) and those that use other IFEs to greater efficiency/advantage than IFE-1. Collectively these data suggest that each IFE selectively activates individual messages in a spatial and temporal manner.

Our studies have shown that a single eIF4E isoform, IFE-1, preferentially initiates the translation of a unique subset of mRNAs relative to other germ line eIF4Es. IFE-1 temporally regulates the recruitment of mRNAs for translation at the mitosis/meiosis transition in the distal gonad (*gld-1, glp-1*), at MAPK induced meiotic maturation in the proximal gonad (*vab-1, vpr-1, rab-7, ran-1, oma-1*), and in preparation for embryogenesis (*pos-1, mex-1*). As such, IFE-1 guides a unique class of protein synthetic events that punctuate germ cell development throughout gametogenesis for both sperm and egg.

Chapter 3: Loss of Bcl-2 (*ced-9*) induces germ cell apoptosis and eIF4G (IFG-1 p170) cleavage

***The data in this chapter represent my experimental contributions included in the two published manuscripts: Contreras, V.C. et. al., (2011). Cap-independent translation promotes *C. elegans* germ cell apoptosis through Apaf-1/CED-4 in a caspase-dependent mechanism.. PlosOne Vol 6. Issue 9. 0024444. Morrison, J.K. et al., (2014). Induction of cap-independent BiP (*hsp-3*) and Bcl-2 (*ced-9*) translation in response to eIF4G (IFG-1) depletion in *C. elegans*.. Translation (Landes Bioscience) Vol 2. Issue 1. Text of Chapter 3 is my own.**

Introduction

Translational control mechanisms, both cap-dependent and cap-independent, are substantial determinants of germ cell fates. As described previously (Chapter 1: Literature Review), two isoforms of eukaryotic translation initiation factor 4G (eIF4G) have unique roles in supporting cellular protein synthesis and development. The "long" eIF4G form (IFG-1 p170) is a scaffolding protein that catalyzes the cap-dependent joining of mRNA to ribosomes. Long eIF4G coordinates the cap-binding protein eIF4E (IFEs 1-5 in *C. elegans*) and RNA helicase, eIF4A, with the mRNA and 40S ribosomal subunit (Keiper et al., 1999). In germ cells, cap-dependent translation promotes proliferation, growth, and oocyte differentiation (Henderson et al., 2009; Song et al., 2010). During apoptosis a signaling cascade results in caspase-mediated eIF4G cleavage to a cap-independent "short" form (Nicholson et al., 1995). Short eIF4G lacks the eIF4E binding domain, yet it is still able to bind eIF3, eIF4A, and mRNA to support cap-independent translation initiation (Pestova et al., 1996).

Cap-independent translation is not limited to cells undergoing apoptosis or virus infection. Healthy cells have a constitutive cap-independent isoform, p97, which is capable of recruiting mRNAs to the ribosome by means of internal ribosome entry sites (IRESes) or other cap-independent mechanisms (Nevins et al., 2003). The recruitment of mRNAs for cap-independent translation via IRES recognition was originally discovered in poliovirus and has

been observed in most picornaviruses (Pelletier and Sonenberg, 1988). During poliovirus infection, eIF4G cleavage results in an increase in cap-independent translation and thus a preferential translation of IRES-containing viral mRNAs. A similar example of cap-independent translation contributing to cell fate determination is the programmed cell death process of apoptosis. During apoptosis, a cascade of proteolytic events leads to the activation of caspase 3, which specifically cleaves long eIF4G in the hinge region to disrupt cap-dependent protein synthesis (see Figure 1.1). However, this same cleavage event actually enhances the activity of cap-independent translation initiation because of the greater prevalence of "short" eIF4G forms. This change in translation initiation mechanism has been shown to enhance the translation of IRES-mediated mRNAs. Among them are pro-apoptotic mRNAs, such as those encoding apoptotic peptidase-activating factor 1 (Apaf-1) and "death associated protein 5" (DAP5), thus promoting an apoptotic fate (Nevins et al., 2003). Interestingly, IRES-mediated cap-independent recruitment also promotes the translation of some anti-apoptotic mRNAs, such as Bcl-2, which provides cells with an opportunity to recover from cellular insult before being committed to an apoptotic fate. Recent findings show that the pro-apoptotic and anti-apoptotic mRNAs are activated sequentially, presumably reflecting that effort to survive (Graber and Holcik, 2007; Sherrill et al., 2004).

Apoptosis is a naturally occurring mechanism for removing superfluous, infected, or damaged cells in somatic tissues. During fetal development, for example, the extraneous cells that populate tissue between fingers during limb growth are targeted for removal via apoptosis in order to prevent webbing (Mori et al., 1995). The apoptotic pathway is highly conserved throughout animal species. In mammals, Bcl-2 (and several related BH3-domain proteins) inhibit apoptosis during development and cellular stress by maintaining the membrane integrity

of mitochondria and endoplasmic reticulum (ER). Upon sufficient insult to the cell, so called "BH3-only" domain pro-apoptotic proteins (e.g. Bax, Bad, Bak) are activated, often by caspase-mediated proteolysis. These bind and disrupt anti-apoptotic proteins (such as Bcl-2 and Bcl-XL) residing in the ER and mitochondrial membranes to destroy the membrane integrity (Datta et al., 2000; Sattler et al., 1997).Once inserted, Bax and Bak are free to oligomerize and form pores in the ER and mitochondrial membranes. ER pores release calcium, which then permeates the inner mitochondrial membrane and can result in mitochondrial rupture (Szalai et al., 1999). Mitochondrial pores, created by Bax and Bak, release cytochrome c into the cytoplasm. Cytochrome c stimulates the formation of a large structure called the apoptosome by binding Procaspase-9 and Apaf-1. A formed apoptosome activates associated procaspase-9 by self-cleavage. Active caspase-9 subsequently cleaves and activates other procaspases, including caspase-3 and caspase-7, resulting in the spread of the cell death signal in a caspase cascade. (Li et al., 1997). This cascade culminates with active executioner caspases that cleave many substrates including cytoskeletal proteins, a DNA fragmentation factor that mediates genomic DNA degradation, and transcription and translation factors, to ultimately promote apoptosis. The proteolytic event most meaningful for protein synthesis is the caspase-3-mediated cleavage of eIF4G into its short cap-independent form, which lacks the N-terminal eIF4E and PABP binding domains (Bushell et al., 1999). This disrupts cellular growth-promoting cap-dependent mRNA translation and eventually further potentiates the cap-independent synthesis of pro-apoptotic proteins.

The *C. elegans* Bcl-2 homolog, CED-9, is an anti-apoptotic factor that acts upstream of the Apaf-1 homolog, CED-4, and the pro-caspase CED-3 in the well characterized cell death (*ced*) pathway that leads to physiological germ cell apoptosis in worms. CED-9 suppresses

apoptosis in all cell types. Unlike somatic tissue, however, roughly 50% of all germ cells in the *C. elegans* hermaphrodite gonad are naturally committed to physiological apoptosis in order to contribute mRNAs and cytoplasmic components to their sibling germ cells (Gumienny et al., 1999). This occurs primarily in the reflexed loop, a so-called "apoptotic region" of the gonad, after oocytes enter the pachytene stage of meiosis. In oocytes selected for apoptosis, the binding of CED-9 to CED-4, the Apaf-1 homolog, is lost. CED-4 self-assembles to form the apoptosome and associate with pro-caspase CED-3. Once bound to the apoptosome, CED-3 auto-activates by self-cleavage. Activated CED-3 is released and begins to cleave essential cellular machinery, including translation machinery, committing the oocyte to an apoptotic fate. Our lab demonstrated that, similar to caspase-3 in mammals, one of the substrates CED-3 cleaves is the eIF4G long isoform (IFG-1 p170) (Contreras et al., 2011). The result is an increased abundance of a short IFG-1 p130-like isoform. As described above, pre-existing IFG-1 p130 resides in a cap-independent initiation complex (Contreras et al., 2008). The shift toward only cap-independent mRNA translation complexes is thought to facilitate the synthesis of pro-apoptotic proteins in germ cells that are destined to die.

Results

CED-9 suppresses apoptosis in C. elegans germ cells in vivo

The purpose of experiments presented in this chapter was to develop a conditional mutant worm strain in which we could both induce physiological germ cell apoptosis and observe the germ cell deaths *in situ*. Such a strain would be very useful for the overall study of mRNA translation promoted by cap-independent translation in apoptosis germ cells (Morrison et al., 2014). The

strain was further useful as a live "positive control" for comparison to germline apoptotic events induced by direct disruption of IFG-1 p170 (Contreras et al., 2011; Contreras et al., 2008) In order to study the extent of CED-9-mediated suppression of apoptosis during oogenesis, we explored multiple avenues to genetically knock down *ced-9* in wild type and *ced-3* (caspase deficient) worms. The parent worm strains used (MD701, KX84) expressed a CED-1::GFP fusion protein in the gonad sheath from the *lim-7* promotor. CED-1 is a transmembrane protein in the gonad sheath that promotes phagocytic cells and germ cell corpse engulfment (Shen et al.). This fusion protein provides a sensitive method for visualizing germ cell corpses in individual whole worms *in vivo*. For engulfment, the somatic sheath cells invades the germ cell layer and cluster around dying germ cells during engulfment (Schumacher et al., 2005). We sought to confirm that loss of anti-apoptotic CED-9 was sufficient to induce physiological apoptosis (Gumienny et al., 1999), and events could be visually monitored in real time in our CED-1::GFP fusion strain. To this end, we constructed a *ced-9* RNAi construct by subcloning *ced-9* cDNA (nt 195-630) into the pL4440 plasmid to drive dsRNA expression in *E. coli*. Worms were fed the dsRNA-expressing bacteria as a food source ("fed RNAi" plates) as previously described (Contreras et al., 2008). Wild type worms depleted of CED-9 exhibited a nearly three-fold increase in CED-1::GFP-decorated germ cell corpses, suggesting the induction of the pro-apoptotic caspase cascade [*ced-9*(RNAi), Figure 3.1]. In an isogenic strain lacking CED-3, however, depletion of CED-9 did not induce germ cell apoptosis. These preliminary results supported the conclusion that CED-9 is necessary to suppress the pro-apoptotic signaling cascade during oogenesis through the caspase, CED-3.

Figure 3.1

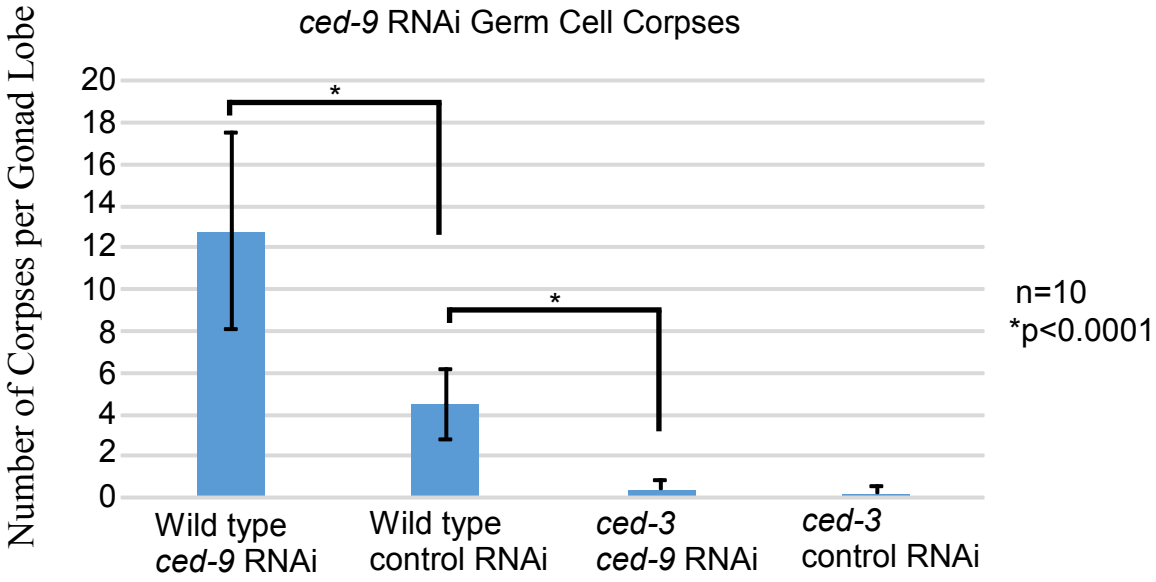

ced-9 RNAi Germ Cell Corpses
Number of Corpses per Gonad Lobe
20
18
16
14
12
10
8
6
4
2
0
*
*
Wild type *ced-9* RNAi
Wild type control RNAi
ced-3 *ced-9* RNAi
ced-3 control RNAi
n=10
*p<0.0001

Figure 3.1. CED-9 Knockdown-Induced Germ Cell Apoptosis is CED-3 Dependent.

Graphical representation of germ cell corpses counted after 24 hours of RNAi feeding. Wild type (MD701) and *ced-3* (KX84) worm strains express CED-1::GFP transgene that allows for visual detection of germ cell corpses during engulfment. Strains were control vector or *ced-9* RNAi plasmid induced HT115. CED-9 knockdown in wild type worms induced a significant increase in apoptosis. *ced-3* worms have very few germ cell corpses with no significant increase following CED-9 knockdown.

Developing a stable, conditional ced-9(ts) ced-1::gfp strain and comparative fluorescence microscopy

Our preliminary data indicated that *ced-9*(RNAi) was sufficient to induce broad physiological germ cell apoptosis *in vivo*. This pilot study assayed the development of germ cell corpses at a single time point following *ced-9* knockdown. In order to study the induction of germ cell apoptosis in a large population of worms over a longer time course, it was necessary to develop a stable, conditional *ced-9 ced-1::gfp* mutant. This temperature sensitive mutant was also used in future experiments to determine changes in mRNA translational efficiency following the induction of germ cell apoptosis (performed by Dr. Morrison). My contribution was to carry out genetic crosses and evaluate the enhanced germ cell apoptosis in the new worm lines. *ced-1::gfp* (MD701) males were generated by crossing *ced-1::gfp* hermaphrodites with wild type males. The F1 males were evaluated by visual microscopy for positive GFP expression in the gonad. *ced-1::gfp* males were subsequently crossed with *ced-9*(n1653ts) (MT3970) hermaphrodites. F2 generation hermaphrodite progeny that were also GFP positive were pooled and allowed to self-cross. F3 generation hermaphrodites (64 isolates) were cloned and self-crossed. Mothers were allowed to lay eggs, and then screened for gonad sheath GFP expression and germ cell corpses. Of the 64 clones, 16 of the F4 progeny expressed CED-1::GFP and exhibited a larger number of germ cell corpses. From these, the four most stable F4 founder lines were isolated and 12 worms from each were grown at 25°C (the non-permissive temperature) and subsequently scored for germ cell corpses. The two founder lines with the strongest and most stable CED-1::GFP expression (4.1 and 36.1) were established as new *ced-9(ts) ced-1::gfp* clonal lab strains (KX110 and KX111). The two new strains, along with wild type (MD701) and *ced-3 ced-1::gfp* worms (previously developed by Vince Contreras), were subjected to a 56 hour time

course to compare the timing and extent of germ cell apoptosis induction. Germ cell corpses were counted by visual inspection upon fluorescence microscopy of whole, unfixed worms (Figure 3.2). *ced-9(ts) ced-1::gfp* mutants had a marked increase in germ cell corpses compared to wild type worms. New corpses became prominent within 24 hours after conditional loss of *ced-9* (Bcl-2) function (Figure 3.3). 48 hours at the non-permissive temperature, both *ced-9*(ts) worm strains developed four-fold more germ cell corpses than wild type. Additionally, caspase-deficient (*ced-3*) worms had few appreciable germ cell corpses throughout the entire time course. These data suggested that worms lacking CED-9 have a sustained increase in physiological germ cell apoptosis that established after 24 hours at 25°C and was sustained for up to 56 hours. Those lacking the ability to induce proteolysis were unable to produce any germ cell corpses. Furthermore, by developing a stable, conditional *ced-9* strain with a fluorescent apoptotic reporter, we could now systematically induce physiological germ cell apoptosis, and visually monitor germ cell corpse engulfment, for all subsequent translational control studies, including polysome resolution (see publication (Morrison et al., 2014). Although both established strains were equivalent, subsequent experiments used strain KX110 exclusively.

IFG-1 p170 becomes cleaved in the ced-9(ts) mutant

It was next of interest to determine if induction of physiological apoptosis by the *ced-9(ts)* mutation resulted in the N-terminal cleavage of IFG-1 p170. Cleavage of non-mammalian eIF4Gs by a caspase had not previously been demonstrated. As stated previously, functional CED-9 (Bcl-2) inhibits physiological germ cell apoptosis upstream of the caspase cascade that results in the activation of CED-3. Among its targets, CED-3 might be predicted to cleave the long IFG-1 p170 isoform in oocytes during the course of apoptotic progression. The resulting short IFG-1 p130 would support cap-independent translation initiation and potentially further

Figure 3.2

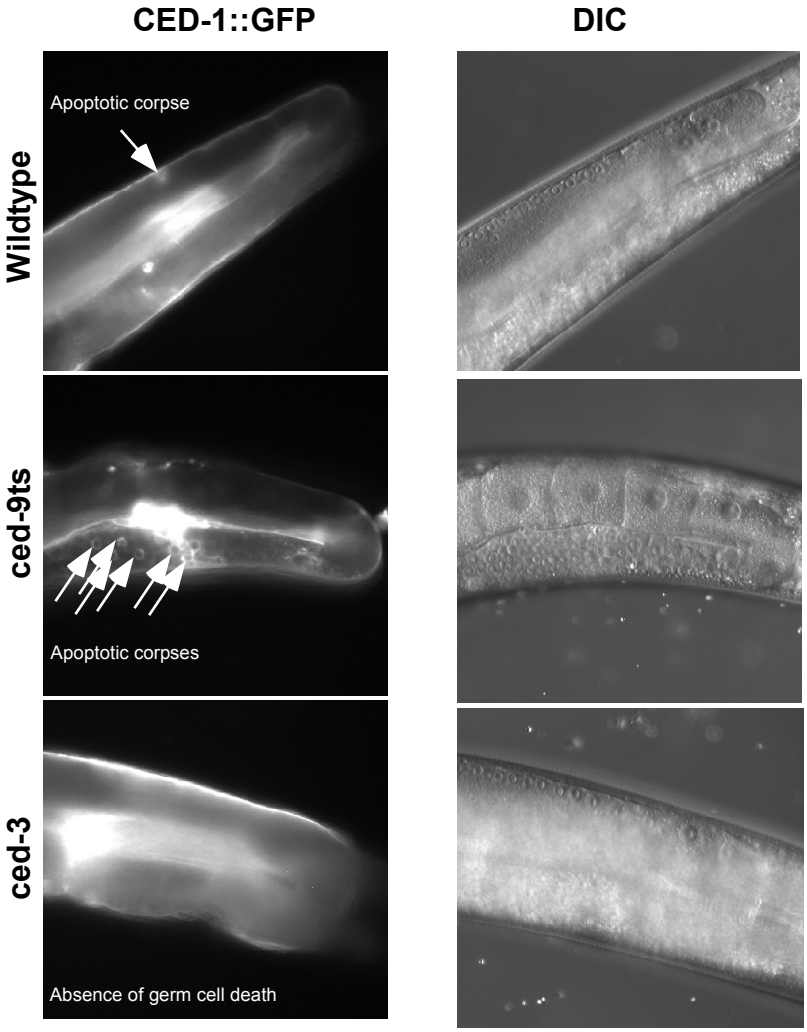

CED-1::GFP
DIC
Wildtype
Apoptotic corpse
ced-9ts
Apoptotic corpses
ced-3
Absence of germ cell death

Figure 3.2. Physiological Apoptosis was Increased in *ced-9(ts)* Mutant Following 25°C Shift. (A-C) Fluorescence images depicting germ cell corpses and (D-F) their accompanying DIC overlay. Each image shows an adult gonad lobe expressing CED-1::GFP. Germ cells visually being engulfed are indicated with a white arrow. Following 24 hour at 25°C (A,D) wild type worms undergo low basal levels of apoptosis, while *ced-3* (C,F) typically have no germ cell corpses. *ced-9(ts)* worms, by contrast, have a robust increase in physiological apoptosis.

Figure 3.3

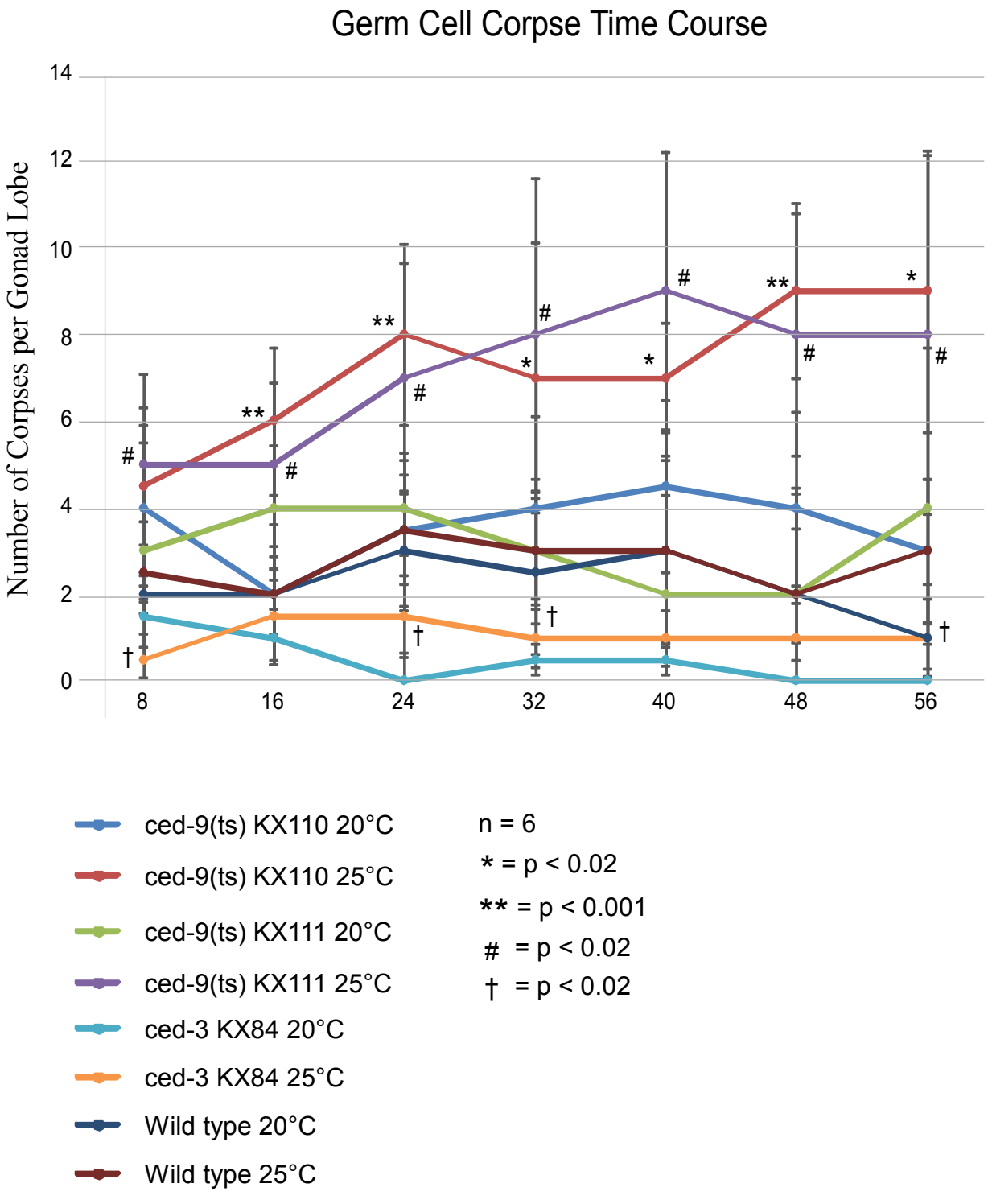

Germ Cell Corpse Time Course
Number of Corpses per Gonad Lobe
14
12
10
8
6
4
2
0
8
16
24
32
40
48
56
ced-9(ts) KX110 20°C
ced-9(ts) KX110 25°C
ced-9(ts) KX111 20°C
ced-9(ts) KX111 25°C
ced-3 KX84 20°C
ced-3 KX84 25°C
Wild type 20°C
Wild type 25°C
n = 6
* = p < 0.02
** = p < 0.001
# = p < 0.02
† = p < 0.02

Figure 3.3. *ced-9(ts)::ced-1:gfp* Mutants Have a Temperature-sensitive Increase in Apoptosis. Time course monitoring the presence of germ cell corpses in the gonad. Worm strains *ced-9(ts)* (KX110, KX111), *ced-3* (KX84), and wild type (MD701) were grown at 20°C and 25°C. All strains expressed CED-1::GFP as a visual marker for germ cell corpses. Corpses were counted at time points every 8 hours over the course of 56 hours. Statistical t-test was performed to compare corpse counts at 25°C from *ced-9(ts)* KX110 to wild type ("*" = $p < 0.02$ "**" = $p < 0.001$), *ced-9(ts)* KX111 to wild type ("#" = $p < 0.02$), and *ced-3* KX84 to wild type ("†" = $p < 0.02$).

promote the cell's commitment to an apoptotic fate. Preliminary analysis of the *ced-9(ts) ced-1::gfp* mutants (KX110 and KX111) established a sustained increase in germ cell corpses after 24 hours at the non-permissive temperature (25°C). We then set out to demonstrate that during this phase of increased apoptosis, CED-3 was actively cleaving the IFG-1 p170 N-terminal domain. Whole worm lysates were prepared from wild type and *ced-9(ts)* strains grown at 25°C for 48 hours. By western blot analysis using an antibody recognizing the core domain common to p170 and p130, we showed that both *ced-9(ts)* mutant strains showed significant cleavage of IFG-1, evident by two novel N-terminal cleavage products (Figure 3.4). [Note that IFG-1 p170 may also be resolved into multiple bands that derive from minor splice variants (Contreras et al., 2008)]. This analysis indicated the *ced-9(ts)* strains I generated would be useful as a tool to study a physiological change in IFG-1 p170/p130 representation in genetically identical, homogeneous large populations of worms. Using this and other derived *ced-1::gfp* strains, Dr. Kaitlin Morrison further pursued the study of how this physiological change in IFG-1 p170/p130 ratio altered translation initiation and germ cell survival in a paper we published in 2014 (Morrison et al., 2014).

One focus of the subsequent germ cell apoptosis research in the lab was to assess changes in the translational efficiency of individual mRNAs following the disruption of IFG-1 p170. To do this, Dr. Morrison and I resolved polysomes from whole worm lysates via sucrose gradient sedimentation (Figure 3.5). Standard gradients were used to assess the relative translational efficiencies of BiP, Bcl-2, p53 and Apaf-1 mRNAs, among others (see (Morrison et al., 2014). Addition of EDTA to a separate set of polysome gradients demonstrated that mRNAs which sediment in the lower fractions of the gradient were, in fact, associated with ribosomes (require Mg^{++}), and were not due to mRNP complexes (Figure 3.5). This was performed on wild type,

Figure 3.4

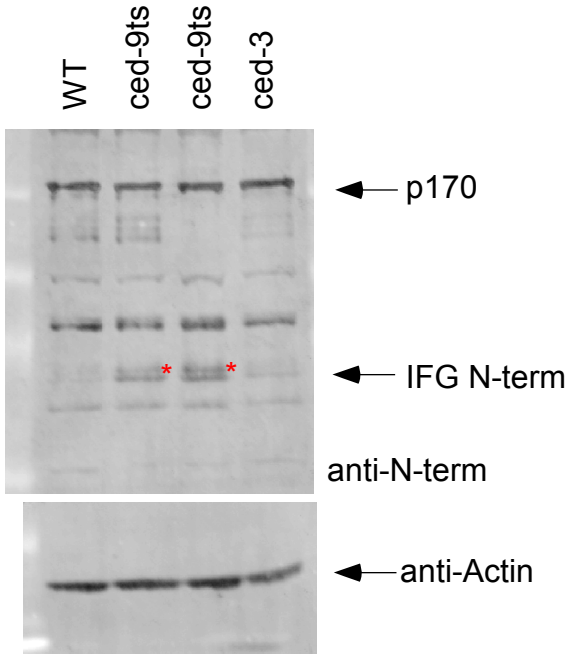


Figure 3.4. IFG-1 was Cleaved During *C. elegans* Apoptosis. Western blot of IFG-1 p170 cleavage *in vivo* in *ced-9(ts)* adult worms. Worms treated 48 hours at 25°C were homogenized and the extract analyzed by 8% SDS-PAGE. Two independent lines of *ced-9(ts)* worms were analyzed. Immunoblotting was performed using IFG-1 N-terminal and actin antibodies. N-terminal cleavage products are indicated (*). (blotting performed by Dr. Keiper from experimental samples prepared by A. Friday)

Figure 3.5

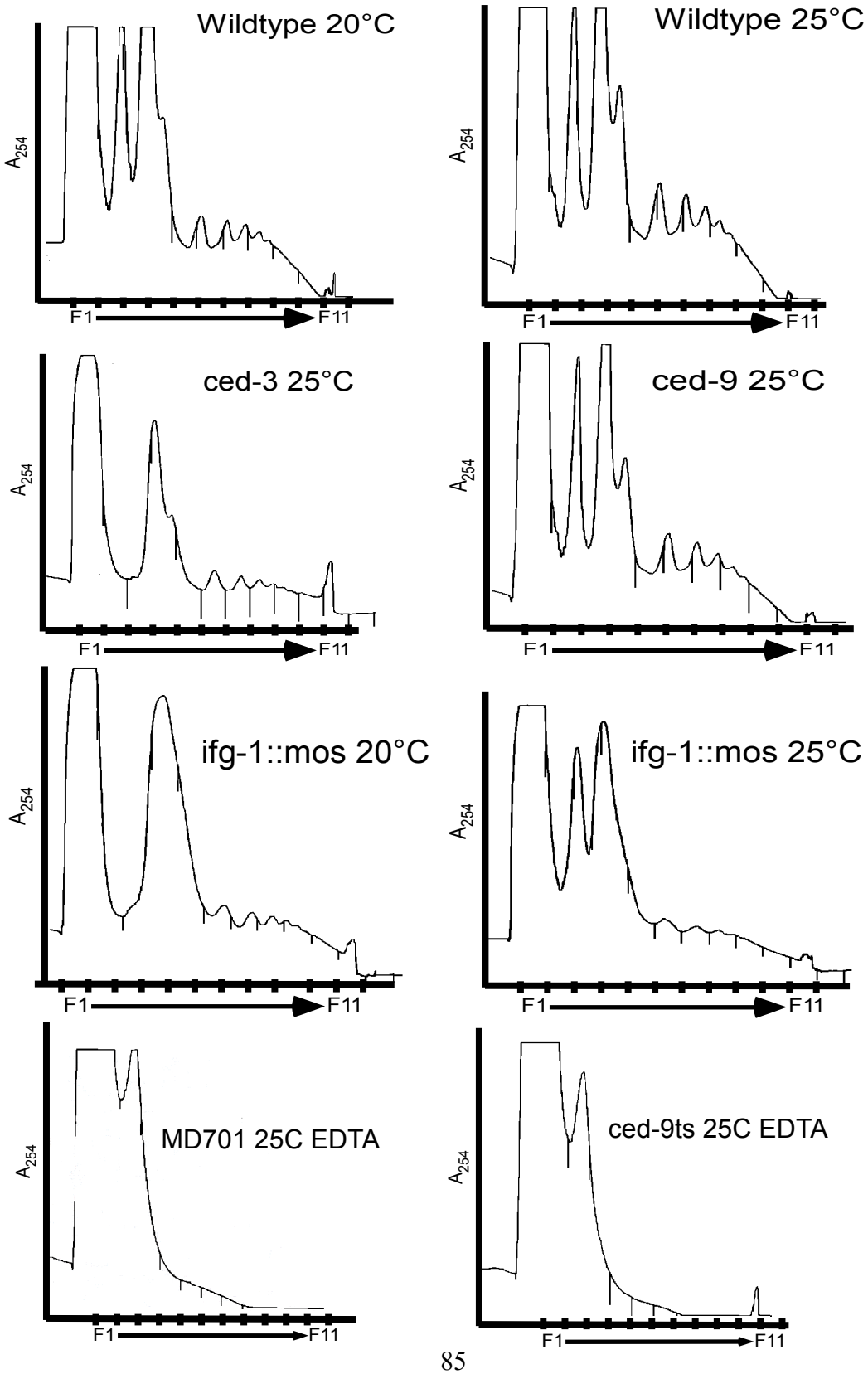

Wildtype 20°C
Wildtype 25°C
ced-3 25°C
ced-9 25°C
ifg-1::mos 20°C
ifg-1::mos 25°C
MD701 25C EDTA
ced-9ts 25C EDTA
A254
F1
F11

Figure 3.5. Sucrose Gradient Fractionation of Polysomes and EDTA Release Gradients. Absorbance profiles for fractionation of 10-45% sucrose gradients of (A-C) wild type, (D) *ced-3*, (E, F) *ced-9(ts)*, and (G,H) *ifg-1::mos* whole worm lysates. In order to discount mRNP complexes being responsible for mRNAs identified in the heavy polysomes, EDTA release gradients were performed in wild type and *ced-9(ts)* strains (C,F). These gradients were further used by Dr. Morrison to determine the translational efficiency of select mRNAs during her thesis project.

ced-9(ts) ced-1::gfp, and *ifg-1:mos ced-1::gfp* (strain KX54 created by Dr. Henderson) worm lines. Dr. Morrison was then able to carefully identify changes in mRNA translational efficiencies in worms with disrupted IFG-1 p170 cap-dependent translation initiation.

Discussion

We hypothesized that translational regulation of mRNAs in *C. elegans* germ cells is driven by both cap-dependent and cap-independent protein synthesis. These two modes of translation initiation promote different outcomes in germ cell life. The long IFG-1 p170 isoform supports cap-dependent protein synthesis, and this mode of mRNA translation promotes germ cell proliferation and oogenic differentiation (Contreras et al., 2008). The short IFG-1 p130 isoform supports cap-independent mRNA translation and promotes germ cell stress responses and apoptosis through a caspase-dependent mechanism (Contreras et al., 2011; Morrison et al., 2014). We utilized the versatility of the *C. elegans* genetics, fluorescence microscopy, and biochemical fractionation to show that loss of Bcl-2 function leads to germ cell apoptotic events and substantial cleavage of IFG-1 p170. My contribution to this study was the creation of a *ced-9(ts) ced-1::gfp* apoptosis-sensitized reporter strain, as well as the preliminary results using depletion of *ced-9* by RNAi to show that germ cell deaths were highly sensitive to Bcl-2. Furthermore, this same project led us to genetically disrupt IFG-1 p170 via Mos transposon insertion (*ifg-1::mos ced-1::gfp*), and this likewise gave rise to a strain with a significant increase in germ cell corpses (Morrison et al., 2014). The development of these strains provided us with powerful biological tools to study germ cell apoptosis by perturbing the apoptotic signaling pathway directly (Bcl-2/CED-9) or by perturbing the mechanism of mRNA translation initiation

directly through IFG-1 p170. These data together showed that changing the balance between IFG-1 p130 and p170 can itself enhance germ cell apoptotic fates. The versatility of the *C. elegans* system allowed us to visually monitor the induction of individual germ cells undergoing apoptosis in live worms, and correlate that with a decrease in the IFG-1 p170 to p130 ratio. We have further shown that IFG-1 acts as an upstream regulator of apoptosis in germ cells, rather than merely as a final consequence (Contreras et al., 2011). Natural variation or mutations that disrupt the IFG-1 p170/p130 ratio (cap-dependent/cap-independent, respectively) are therefore sufficient to alter the proportion of germ cells that survive to complete gametogenesis. Further experimentation identifying specific changes in mRNA translational efficiencies during germ cell apoptosis was the project of Dr. Morrison. In our published paper, her experiments showed enhanced translational efficiency for *hsp-3* and *ced-9* mRNAs when IFG-1 p170 cap-dependent initiation was disrupted (Morrison et al., 2014). A logical continuation of the *in vivo* cap-independence project was to identify all mRNAs that are preferentially translated either cap-dependent or cap-independently via RNASeq translational state array analysis. My contribution and preliminary results from the polysomal bioinformatics approach that allowed this characterization is included in Chapter 5.

Chapter 4: Generating transgenic worms expressing Flag-tagged:IFE-1 and Flag-tagged:IFE-5 for biochemical and genetic studies.

Introduction

The purpose of the work described in this chapter is to develop a means for biochemical isolation of germline specific eIF4E isoforms, IFE-1 and IFE-5, and subsequent identification of bound regulatory proteins. The *flag::ife-1* and *flag::ife-5* constructs were created with a *pie-1* promoter. This promoter expresses exclusively in the germ line (Merritt et al., 2008), allowing us limit the expression of *flag::ife-1* to the oocytes and sperm. Thus, complexes isolated by anti-Flag batch binding would be germline complexes only, and not contain any somatic cell proteins. This is of particular importance with regard to future experiments with eIF4E isoforms, whose native expression is not limited to the germ line (e.g. IFE-2, IFE-4). The most pressing reason to develop transgenic tagged IFEs is that anti-IFE-1, -2, -3, -4, and -5 anti-peptide antibodies, while available and highly isotype-specific, have low binding affinity. In previous co-IP experiments, these antibodies co-precipitated many non-specific proteins under conditions when an IFE was immunoprecipitated (B.D. Keiper, unpublished). Developing Flag-tagged isoform-specific transgenic strains will allow the isolation of IFE-complexes under previously defined conditions. Furthermore, a *flag::ife-1* transgenic strain can be crossed into an *ife-1* null background to potentially rescue the oocyte and spermatocyte defects, as well as enrich the proportion of Flag:IFE-1 in germline complexes. Finally, transgenic Flag::IFE-1 will allow us to make site-directed mutants in the future that will be used to identify structural determinants of protein binding interactions (e.g., IFE-1-IFG-1, IFE-1-PGL-1) *in vivo*. These will also allow

determination of structural requirements within the mRNA cap-binding cleft that are essential for cap-dependent recruitment.

To develop a strategy for employing tagged IFEs in *C. elegans* mRNA translational control, we considered what was already known about eIF4E structure and biological function. Eukaryotic translation initiation factor eIF4E is essential for the regulation of cap-dependent protein synthesis. eIF4E binds the 7-methylguanosine 5'-cap of mRNAs and associates with translation initiation factor eIF4G to recruit these mRNAs to form the 48S translation initiation complex, the rate-limiting step for protein synthesis initiation (Dinkova et al., 2005). eIF4E-binding proteins (4EBPs) associate with the dorsal side of eIF4E and inhibit its ability to bind to eIF4G, thus inhibiting cap-dependent protein synthesis, but do not prevent mRNA cap binding (Fukuyo et al., 2011; Liu et al., 2011). In mammalian cells the eIF4E-eIF4G association is highly regulated in response to mitogenic, nutrient and insulin signaling activities in the PI3K and Ras/Raf/Erk pathways, which subsequently activate mTOR kinase to alleviate eIF4E repression. mTOR directly phosphorylates 4EBP1, causing its dissociation from eIF4E, thus restoring association with eIF4G and cap-dependent protein synthesis.

Regulation of eIF4E by 4EBP-type complexes in the germ line has been shown in multiple species (detailed in Chapter 1). Verified physical protein complexes include: eIF4E1-maskin-CPEB in *Xenopus*; eIF4E-Cup1-Bruno in *Drosophila*; PGL-1-IFE-1 in *C. elegans* (isoform specific); and IFET-1-IFE-3 in *C. elegans* (isoform specific). In the case of eIF4E-maskin-CPEB, the functional consequences of this cap-dependent regulation for *Xenopus* oocyte maturation have been clearly described. In frogs, oocyte maturation (G2-M) is arrested in meiotic prophase II by the translational suppression of cyclin B and c-mos mRNAs. Each mRNA contains a cytoplasmic polyadenylation element (CPE) in the 3'-untranslated region (3'UTR)

(Cao and Richter, 2002). Suppression occurs when the eIF4E-maskin-CPEB (CPE binding factor) complex forms on the CPE at the 3' end of each mRNA. Maskin acts as a specialized 4EBP by binding to eIF4E and simultaneously associating with CPEB (Figure 4.1A). This inhibits eIF4E's ability to recruit the bound mRNA to the translation initiation complex. Maskin bound to CPEB also represses translation of the mRNA. To resume the meiotic cell cycle, progesterone stimulation activates aurora kinase (Cao and Richter, 2002; Mendez and Richter, 2001), which phosphorylates CPEB to promote recruitment of CPSF (polyadenylation specificity factor), and subsequently PAP (poly(A) polymerase), to the 3'–end of the mRNA (Mendez et al., 2000). As PAP catalyzes polyadenylation, PABPs (poly(A)-binding proteins) stack end-to-end on the lengthening poly(A) tail and associate with eIF4G (Stebbins-Boaz et al., 1996; Cao and Richter, 2002). Based in part on its association with the 3' end of the mRNA via PABP, eIF4G is then able to displace maskin and binds to eIF4E. The rearrangement of this 5'-3' end complex thus switches its activity to translation initiation on cyclin B mRNA and its recruitment to ribosomes. Poly(A) elongated mRNAs move into "residence" in active translation initiation, and re-initiation, complexes (Figure 4.1B). Identifying alternative eIF4E-4EBP complexes in other species will provide a further mechanistic understanding of how mRNA translational control comes about and is responsible for distinct developmental events.

Most species encode several eIF4E isoforms, adding to the complexity of sequestered eIF4E in the germ line. Often these are co-expressed in germ cells (Hernandez et al., 2005). *C. elegans* produce five isoforms of eIF4E (IFE1-5) (Keiper et al., 2000). One difficulty in deriving mono-specific antibodies to study these proteins separately is their high level of homology (e.g. IFE-1 and IFE-5 are ~80% homologous). Yet evidence from our lab over the last 15 years

Figure 4.1

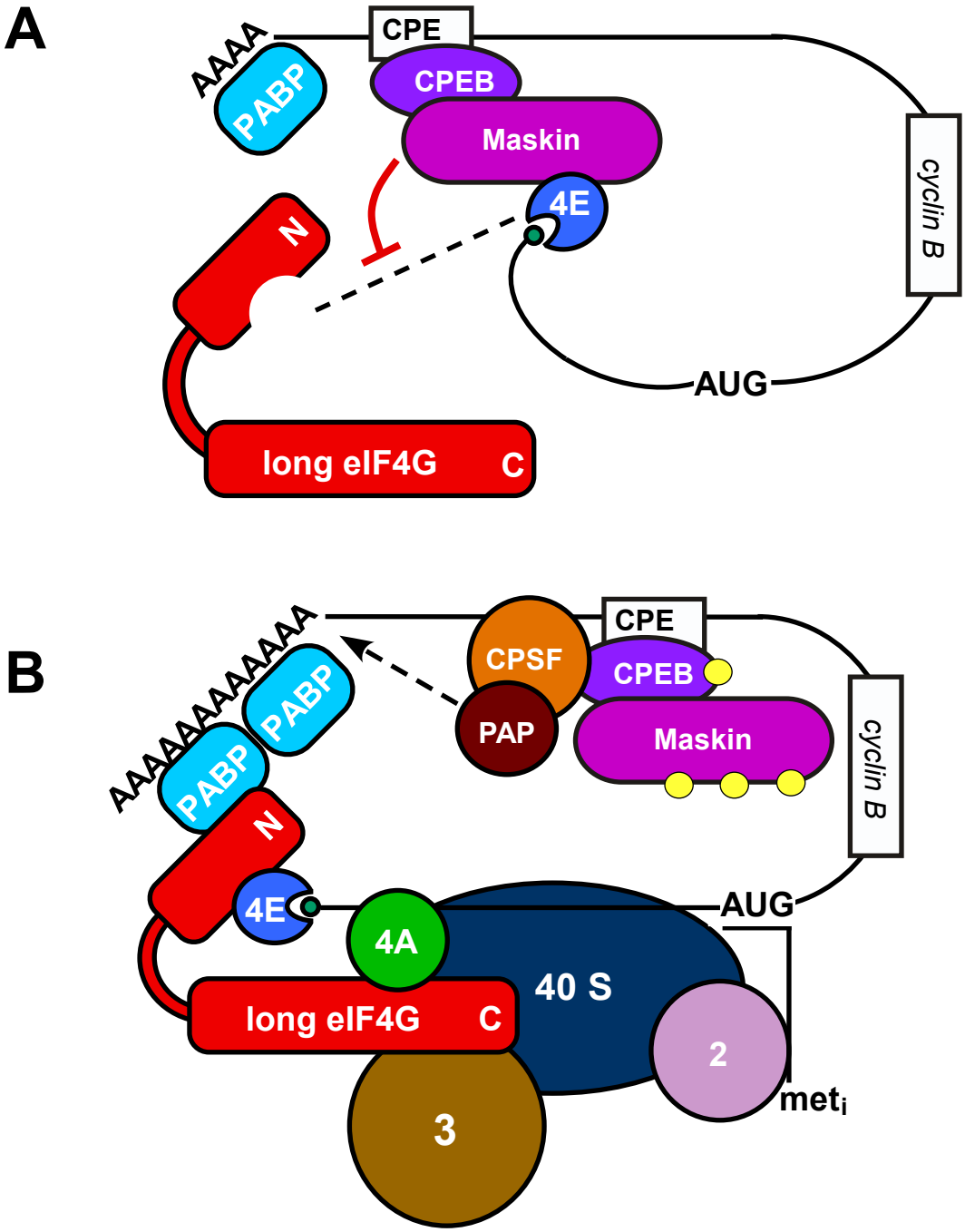

A
AAAA
PABP
CPE
CPEB
Maskin
4E
N
cyclin B
AUG
long eIF4G
C
B
AAAAAAAAAA
PABP
PABP
CPSF
CPE
CPEB
PAP
Maskin
cyclin B
N
4E
4A
AUG
40 S
long eIF4G
C
2
3
met$_i$

Figure 4.1. Maskin-eIF4E inhibitory complex. In *Xenopus* oocytes, maturation is arrested at meiotic prophase II by the translational suppression of cyclin B and c-mos mRNAs. Cytoplasmic polyadenylation element (CPE) binding protein (CPEB) binds the CPE in the mRNA's 3'UTR. The 4EBP maskin binds and sequesters eIF4E in an eIF4E-maskin-CPEB repressive complex. This complex inhibits eIF4E from recruiting the mRNA cap to eIF4G and the translation initiation complex (A). Progesterone stimulation activates aurora kinase, which phosphorylates CPEB (depicted by yellow dots). CPEB phosphorylation promotes the recruitment of polyadenylation specificity factor, CPSF, and poly(A) polymerase (PAP). PABP accumulation on the poly(A)-tail and maskin displacement allows for the recruitment of mRNA cap-bound eIF4E to eIF4G (B). This rearrangement of the mRNP complex allows for cyclin B mRNA to be actively translated, thus promoting meiotic resumption.

suggests that individual eIF4E isoforms regulate mRNA translation in a tissue-specific and sequence-specific manner. Although IFE-2 and IFE-4 are both expressed in somatic tissue, they regulate different developmental events and recruit different mRNAs (Syntichaki et al., 2007; Dinkova et al., 2005). IFE-2 regulates aging, and has a role in meiotic recombination, while IFE-4 regulates nerve and muscle tissue development (Dinkova et al., 2005; Syntichaki et al., 2007; Song et al., 2010). IFE isoforms 1, 3, and 5 (and to a lesser extent IFE-2) are expressed in the germ line (Keiper et al., 2000). Of these germ line-expressed eIF4Es, only IFE-1 is localized in P granules in association with PGL-1. P granules (called germ granules or nuage in other species) are germ line-specific ribonucleoprotein complexes that contain stored mRNAs. Stored mRNAs are used in germ cells during a period of transcriptional silencing coincident with chromosomal condensation during meiosis (detailed in Chapter 1). (Amiri et al., 2001; Updike et al., 2011; Seydoux et al., 1996). For many years, P granules and their associated proteins (e.g., PGL-1, PIE-1, MEX-1, MEX-5, OMA-1, IFE-1, IFF-1 and POS-1) and mRNA cargo were thought to define the germ cell lineage in developing embryos following fertilization. Recent studies, however, showed that P granules are not required for germ line specification; rather they maintain germ cell identity by antagonizing a somatic cell fate (Updike et al., 2014). Where their influence is fully lost, germ cells may spuriously differentiate into neurons and muscle cells, even while still residing in the gonad (Ciosk et al., 2006). IFE-1's unique P granule localization among worm eIF4Es suggests that it plays a regulatory role in the translational recruitment of maternal mRNAs stored in these granules during meiotic progression and even into early embryogenesis.

Beyond localization, the basis for sequence-specific mRNA binding by an eIF4E isoform remains unclear. The mRNA-cap binding cleft has low binding affinity and is not likely to attain sequence recognition beyond the first two mRNA nucleotides (Marcotrigiano et al., 1997b; Miyoshi et al., 2002). Never the less, IFE-1 and IFE-2 were shown to recruit different subsets of mRNAs even in oocytes (Henderson et al., 2009; Song et al., 2010). The specificity for unique mRNA populations is therefore likely to come from differential mRNP interactions of the various eIF4Es. RBPs have a greater capacity to bind mRNAs in a sequence specific manner. Several RBPs also associate with the dorsal face of eIF4E, suppressing initiation. Our current approach is to determine if RNP complex remodeling during germ cell development allows pre-bound IFEs to accompany the mRNAs directly to ribosomes for translation initiation recruitment as depicted in Figure 4.1. Our efforts have resulted in the development of transgenic worm strains that express Flag-tagged-IFE constructs for use in future experiments to identify IFE-associated RNP complexes.

We further proposed that isolation of transgenic Flag-IFE-1 would allow us to identify bound 4EBPs and associated proteins, perhaps allowing a partial characterization of P granule components associated with mRNA 5'-caps. In addition, we will identify mRNAs bound to IFE-1 mRNP complexes. This would allow comparison of IFE-1 mRNP-complexed messages to those found to be translational regulated by IFE-1 via polysomal TSAAs. Original attempts to develop IFE-1 transgenic strains resulted in unstable extrachromosomal arrays that were quickly silenced in subsequent generations (data not shown). These strains also showed mosaic transgene expression throughout the worms and very little germ line expression. In an attempt to express stable Flag-IFE-1 in the germ line, transgenes containing Flag-tagged transgenic constructs of IFE-1 and IFE-5 expressed from the germ line specific promoter, *pie-1*, were re-injected and

subsequently integrated by UV irradiation (transgenesis carried out by M. Henderson and J. Subash). The Flag-tag was fused in frame on plasmids with the corresponding *ife* and encoded an 11 amino acid FLAG peptide. The transgenic array contained a dominant *rol-6* marker gene that causes worms to roll over when swimming.

Results

Isolation of expressed Flag::IFE-1 and characterization of PGL-1 association

For clarity in this chapter, *ife-1* (lowercase italics) is the correct nomenclature for the *C. elegans* homozygous null *ife-1*(bn127) mutant, unless otherwise stated. Likewise, in C. elegans nomenclature, the term *flag::ife-1* refers to the fusion transgene that expresses Flag-tagged protein (Flag::IFE-1), and is not a mutant allele of *ife-1*. The co-injected dominant *rol-6* transgene results in worms that change their locomotion pattern from a sine curve to a circular rolling pattern. This phenotype was used as a visual selection marker to track coinjected *flag::ife-1* transgene, since injected DNAs become concatenated into large propagated arrays.

The purpose of transgenic Flag-IFE isolation experiments was to biochemically identify regulatory proteins and other initiation factors bound to IFE-1 in mRNP complexes. Populations of *flag::ife-1* and *flag::ife-5* worms were grown on a large scale, lysed, and centrifuged to remove debris. Clarified lysates were incubated with anti-Flag antibody-linked agarose beads (Sigma-Aldrich) in batch binding experiments for three hours at 4°C. Unbound proteins were washed from the beads under modestly stringent conditions (0.5 M NaCl, 0.2% Tween20). Bound proteins were eluted with SDS-PAGE loading buffer and subjected to western blot analysis. Using a FLAG epitope antibody (ANTI-FLAG M2, Sigma), Flag::IFE-1 and Flag::IFE-5 were each detected (Figure 4.2). Unfortunately, these tagged proteins could not be

Figure 4.2

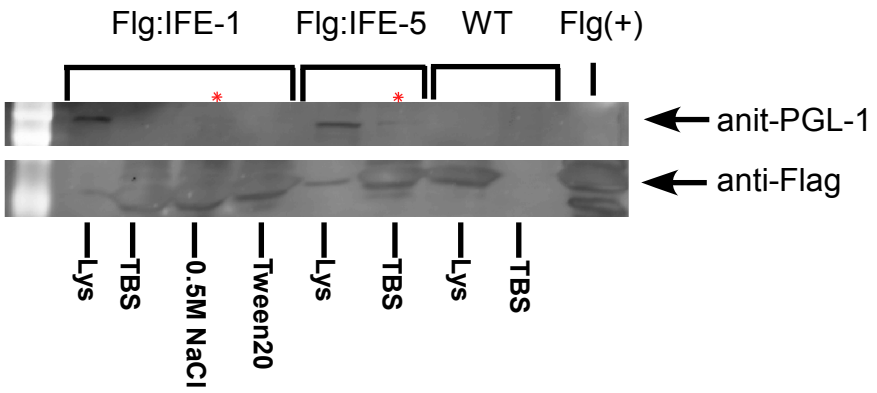

Flg:IFE-1
Flg:IFE-5
WT
Flg(+)
anit-PGL-1
anti-Flag
Lys
TBS
0.5M NaCl
Tween20
Lys
TBS
Lys
TBS

Figure 4.2. Isolation of Flag-tagged IFEs and Associated PGL-1. Western blot of whole worm lysates for worm lines expressing transgenic Flag-tagged IFE-1 or IFE-5. Lysates were bound to anti-Flag antibody beads, washed and eluted. Lys = untreated whole worm lysates; TBS = elutions under standard protocol conditions; 0.5M NaCl = elutions under increased salt conditions; Tween 20 = elutions with a 0.2% addition of a mild detergent. (Bottom) Anti-flag antibody shows the presence of Flag-tagged IFE-1 or IFE-5 in elutions. (Top) Anti-PGL-1 antibody shows the presence of PGL-1 (*) in Flag::IFE-1 0.5M NaCl elution and Flag::IFE-5 TBS elution. WT is a non-transgenic, wild type (N2) worm strain.

detected in total worm extracts without prior enrichment (data not shown). Blotting with a primary antibody for PGL-1, a known IFE-1 binding protein, yielded detectable levels of PGL-1 present in the batch-bound elutions. The low detection of PGL-1suggested that IFE-associated PGL-1 was mostly lost during washes, or that PGL-1 only associated with a small proportion of Flag-IFE. PGL-1 was detected in both the Flag-IFE-1 and Flag-IFE-5 batch-bound elutions. This provided preliminary evidence that PGL-1 directly associated with Flag-tagged recombinant IFE proteins. This was unexpected in the case of Flag-IFE-5, as IFE-5 was not localized to P granules and would not likely be associated with a constitutive P granule component such as PGL-1. PGL-1 was primarily localized to P granules. However, association with Flag-IFE-5 suggested that there were low levels of cytoplasmic PGL-1 free to bind IFEs other than IFE-1.

The applicability of these tagged transgenic strains to IFE-1 complex characterization remains in question. Preliminary batch binding experiments proved that we could capture and detect flag-tagged IFEs. Although there was very faint detection of an associated 4EBP (PGL-1) it was unclear from these preliminary results whether its association was specific, or if these strains would allow the identification of other proteins in native complexes. The current transgenic strains containing UV integrated arrays had very low levels of detectable flag-tagged IFE-1 and -5 protein and even lower levels of bound PGL-1. Therefore, we sought to enhance our detection by increasing the proportion of expressed Flag::IFE-1 to the endogenous IFE-1, and thereby drive the tagged protein into native complexes. To do this, we attempted to cross the *flag::ife-1* transgene into worms with an *ife-1* null background (Table 4.1). Based on Mendelian segregation of the alleles (assuming that *flag::ife-1* was not integrated into chromosome III, where *ife-1* resides), offspring should be heterogeneous, containing a single *ife-1* allele and may or may not inherit the dominant *rol-6::flag::ife-1* transgene. To sort them out, and homozygous

Table 4.1

ife-1 males

flag::ife-1 (rol-6) hermaphrodites

	r i	r i
R I	R r I i	R r I i
r I	r r I i	r r I i

Table 4.1. Table of *flag::ife-1* cross with *ife-1* null worms. This table represents the Mendelian genetics for the *flag::ife-1* cross with *ife-1* null background. R+ represents the *rol-6* transgene that was our phenotypic marker for the *flag::ife-1* transgene. I+ indicates the wild type *ife-1* allele and I- the *ife-1* deletion. Heterozygote progeny (F1) were clonally isolated to self-cross (blue dashes).

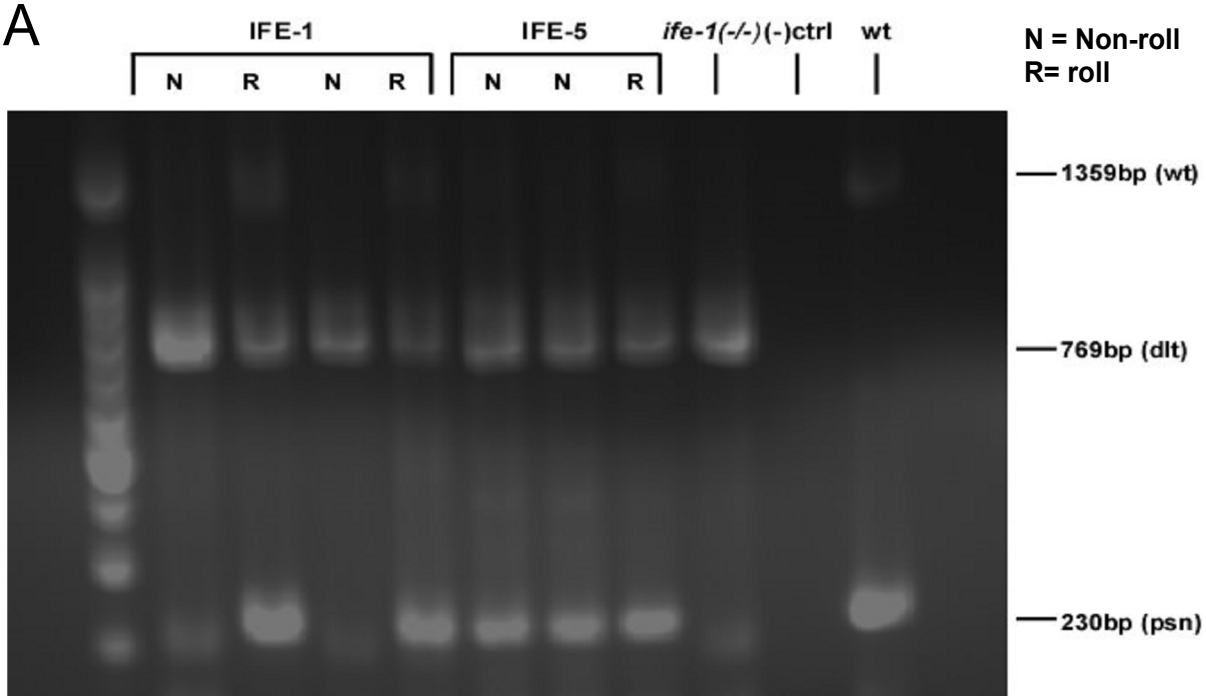
the *ife-1* deficiency, heterozygous F1 progeny with the dominant *rol-6* transgenic marker were clonally isolated and blindly self-crossed. F2 progeny were also cloned and phenotypically scored (rolling worms) for the presence of the Flag-tagged transgene. F2 clones were subsequently analyzed for the presence of the *ife-1*(bn127) deletion by genomic PCR. By PCR analysis, we determined that roll-positive worms were *ife-1(+/-)* heterozygotes (Figure 4.3). None of the transgenic worms were ever found to be homozygous for the *ife-1* deletion, even upon subsequent self-crossing. Unfortunately, the fact that worm lines expressing recombinant *flag::ife-1* could not be isolated in a homozygous *ife-1* background suggested that the ectopically expressed Flag::IFE-1 may worsen, rather than rescue, fertility. All isolates produced broods of mixed wild type and transgenic (*rol-6*) locomotive phenotypes (non-rollers and rollers, respectively). Interestingly, PCR analysis of the non-rolling offspring did confirm the reappearance of homozygous *ife-1* (-/-) worms, but only in the absence of the transgene. This finding was again suggestive that Flag::IFE-1 was incompatible with the loss of endogenous IFE-1, even at the permissive temperature (20°C) (Figure 4.3). Another strategy of *flag::ife* transgenesis was required.

Development of MOS-SCI flag::ife-1 Strains

A new transgenesis approach in *C. elegans* was recently developed that might provide a more "natural" Flag::IFE-1 transgenic strain to address these shortcomings. We decided to use the MOS SCI (single copy insertion) transposon technique developed by the Jorgensen Lab (Frokjaer-Jensen et al., 2008). This was a preferable technique for the development of transgenic worm strains as has been reported to: 1) insert a single transgene copy into a defined MOS transposon site on a known chromosome, 2) express the transgene within the germ line exclusively using the pie-1 promoter, and 3) stably express the transgene where most *C. elegans*

Figure 4.3

A


IFE-1
IFE-5
ife-1(-/-)(-)ctrl
wt
N R N R
N N R
N = Non-roll
R= roll
1359bp (wt)
769bp (dlt)
230bp (psn)

B

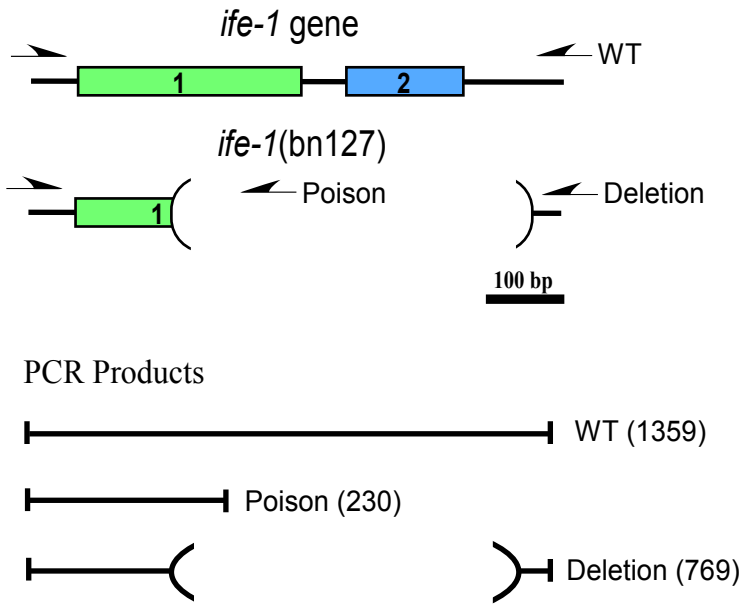

ife-1 gene
WT
1
2
ife-1(bn127)
Poison
Deletion
1
100 bp
PCR Products
WT (1359)
Poison (230)
Deletion (769)

Figure 4.3. *flag::ife-1* Strains are Not *ife-1* Homozygous null. (A) PCR around the *ife-1* gene with a third internal primer within the *ife-1*(bn127) deletion. Products are as follows: 1359bp = full length *ife-1* gene present; 769bp = *ife-1*(bn127) deletion; 230bp = *ife-1* gene present. We tested both N = non-*rol-6* and R = *rol-6* worms for *flag::ife-1* and *flag::ife-5* strains and discovered *ife-1* heterozygosity not easily detected in preliminary trials. (B) Primer diagram for wild type, *ife-1* deletion, and poison primers against the *ife-1*(bn127) deletion mutant.

germline transgenic arrays were prone to being silenced. We used three-way Gateway cloning (Invitrogen) to create *flag::ife-1* and *flag::ife-5* destination vectors. These vectors contain a *pie-1* promoter inserted in the 5' sequence flanking *flag::ife* and the *unc-54* 3'UTR in the 3' sequence downstream. Destination plasmids also contain *mos* transposon recombination sites that allow for recombination into a single chromosome IV target site. Additionally, these plasmids carried an *unc-119*(+) rescue gene for simple visual assessment of transgenics. Briefly, when injected into recipient *unc-119* worms, which swim very poorly ("uncoordinated"), the unc-119(+)-containing transgene allows stable transgenics to be sorted simply by their wild type locomotion. The *flag::ife-1* and *flag::ife-5* plasmid mixes were microinjected into worm gonads. After multiple generations, progeny were screened for wild type swimmers and against negative selection markers that identified lines maintaining extrachromosomal arrays.

These efforts resulted in the development of at least one new flag-tagged IFE-1 transgenic line that finally expressed Flag::IFE-1 that was readily detectible even by western blot from total protein lysates (Figure 4.4). One potential Flag::IFE-5 line was also characterized, but its expression remains in question. This new *flag::ife-1* worm strain will be used as a tool to isolate and identify IFE-1-bound RNP proteins that could explain IFE-1's mRNA selectivity.

Figure 4.4

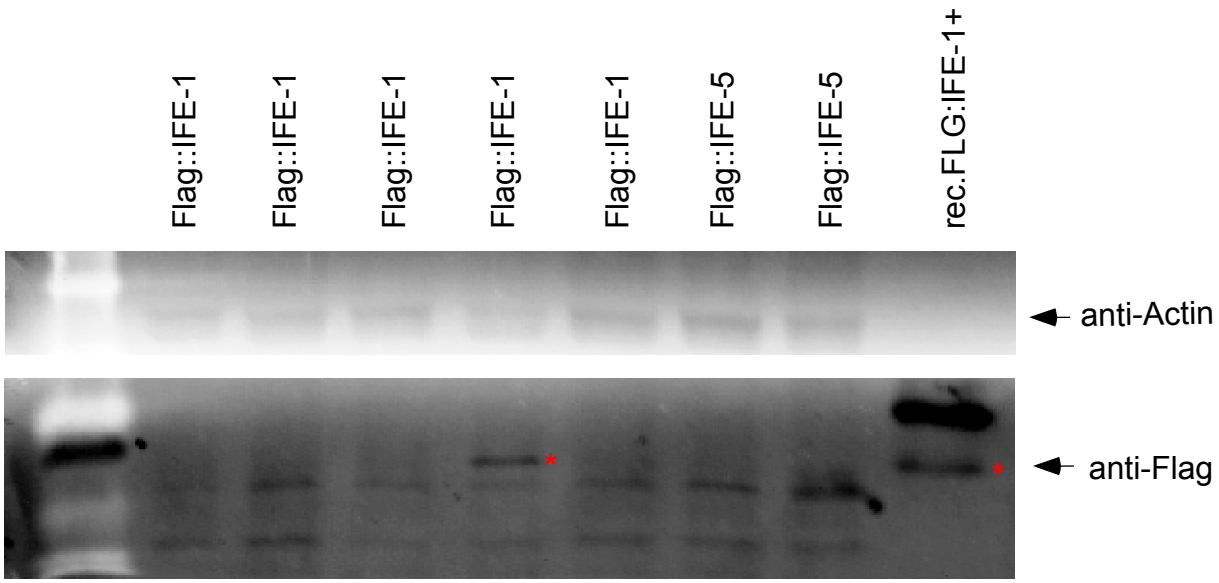

Flag::IFE-1
Flag::IFE-1
Flag::IFE-1
Flag::IFE-1
Flag::IFE-1
Flag::IFE-5
Flag::IFE-5
rec.FLG:IFE-1+
anti-Actin
anti-Flag

Figure 4.4. Western blot Identification of Flag::IFE-1 in MOS SCI Transgenic Strain. MOS SCI standard transgenic and microinjection techniques were performed to develop worm strains expressing Flag::IFE-1 and Flag::IFE-5 at stable levels in the germ line. Whole worm lysates were resolved on by 12% SDS-PAGE. Flag::IFE-1 was detected (*) in a *flag::ife-1* transgenic strain. Faint, but not considered detectable, bands for Flag::tagged proteins may be Enriched by column purification or subsequent batch-binding experiments.

Future Directions

As described in detail in Chapter 1, there are several well-documented examples of mRNP complexes that regulate cell growth and development. Some involve mRNP-eIF4E complexes that are hypothesized to set up a negative-to-positive translational control transition. The dynamics of that transition are thought to govern germ cell fate decisions (Mendez and Richter, 2001). Identifying mRNP proteins associated with translation initiation machinery in *C. elegans* would be a significant advance in understanding the molecular characteristics of protein synthesis regulation in developing oocytes. To that end, we developed transgenic worms that express a flag-tagged eIF4E (Flag::IFE-1) in the germ line. This will be used as a tool for re-isolating IFE-1-bound mRNPs and identifying proteins that might influence the IFE-1-dependent mRNA recruitment steps. We also expect to find Flag::IFE-1 associated with the translation initiation machinery (e.g. eIF4G and eIF4A). It is noteworthy that PGL-1 is required for IFE-1 to associate with P granules. In *pgl-1* null worms, IFE-1 dissociated from P granules and dispersed into the cytoplasm (Amiri et al., 2001). A complex of IFE-1 with IFG-1 p170 must also exist to support rapid translation initiation upon de-repression of such mRNPs. It is unclear, however, whether the "inactive" IFE-1-PGL-1 and "active" IFE-1-IFG-1 complexes are fully distinct. It may be that recruitment involves stepwise remodeling of a conglomerate mRNP. It will be of interest to use biochemistry and genetics to dissect intermediate transition states in the mRNA recruitment pathway. Other P granule proteins (PIE-1, MEX-1, MEX-5, POS-1, etc.) that are required for germ cell development and embryogenesis may also be identified in IFE-1 mRNPs. These proteins, too, may be involved in distinct or coordinated mRNP complexes with IFE-1. Based on their RNA sequence specificity, they may regulate IFE-1's ability to recruit specific mRNAs for translation upon RNP complex remodeling. Other regulatory protein (such as IFET-

1/OMA-1) are slowly being ruled out based on their preferential binding to other eIF4E isoforms. IFET-1 and OMA-1 have shown preferential association with isoform IFE-3 (Spike et al., 2014).

The Flag::IFE recapture experiments will also provide a powerful resource to identify IFE-1-bound mRNAs. It remains to be determined what overlap is found between mRNAs recaptured from a Flag::IFE-1 mRNP and those demonstrated to be translationally regulated by IFE-1 by other experimental means [TSAA of *ife-1*(bn127), Polysome gradient analysis, 3'UTR reporter analysis]. For example, it is possible that one mRNA may be bound in an mRNP complex that is IFE-isoform specific for de-repression. By this mechanism the mRNA may be bound in an IFE-1 mRNP complex that is not remodeled and thus remains in a repressed state. The same mRNA, however, may be just as likely to associate with an IFE-3 mRNP complex that is signaled for remodeling. That mRNA eventually becomes de-repressed; and is translationally recruited by the alternative, IFE-3, isoform. Identifying IFE-1-bound proteins and mRNAs, as well as those bound to other IFEs, will be useful in understanding the mechanisms by which specific mRNAs are positively regulated in developing germ cells. This may serve to contrast the repressive role of RBPs in germ cells with the positive isoform-specific translational role of IFEs upon mRNP remodeling.

Chapter 5: Conclusions

The work described in this dissertation demonstrates unique functional roles for translation factor eIF4 isoforms in the regulation of germ cell protein synthesis. The translational regulation of pre-existing mRNAs in germ cells has been traditionally attributed solely to repression by RBPs. My findings have shown that subtypes of eIF4E and eIF4G, themselves, selectively recruit certain mRNAs, either in spite of, or in conjunction with, RBPs. These findings also demonstrated that their recruitment occurs at temporally defined stages of gamete development.

This dissertation focuses on the regulation of selective cap-dependent mRNA recruitment for translation in germ cells. As outlined in Chapter 1, translational control of gene expression is vital to germ cells as a means to produce new proteins at a time when new gene transcription is unavailable. We conclude that regulation of mRNA translation during *C. elegans* germ cell development involves the coordinated de-repression of stored mRNAs and their coincident recruitment to ribosomes by IFE-1 and IFG-1. Results from my work have outlined how IFE-1 influences germ cell fate decisions. In addition, my work reveals how the obligatory partner in the initiation complex, IFG-1 p170, promotes germ cell survival. My findings make it clear that the mRNA translation factors IFE-1 and IFG-1 exert their own regulation on the selective protein expression in gamete development.

Protein synthesis regulation is particularly important during germ cell development. Germ line stem cells possess the ability to self-replicate and differentiate into gametes which transmit genetic information to future generations. Germ cells rely on controlled *de novo* protein

synthesis to guide successful differentiation into viable sperm and eggs. The mechanisms regulating mRNA translation are particularly important for germ cells while they are experiencing periods of transcriptional silencing. For instance, even early in *C. elegans* embryogenesis, the germ line is established in blastomeres that are defined through a series of asymmetric cell divisions. Enriched in the nuclei of germ line blastomeres, the zinc finger protein, PIE-1, functions as a transcriptional repressor for the "P" (germ) lineage, and is required to maintain a germ line fate (Mello et al., 1996; Seydoux et al., 1996). As the worm matures, germ cells accumulate mRNAs and translational machinery in ribonucleoprotein particles that are used during transcriptional silencing to provide developing gametes with new proteins required for development and maturation.

1. Translation of germ granule mRNAs

The storage of mRNA for germ cells to utilize during development has been a focus of study for over one-hundred years (Onohara and Yokota, 2012). In numerous animal germ cell types, an electron-dense perinuclear structure called "nuage" was shown to be integral to mRNA translational silencing (Eddy, 1974). Similar regulatory structures have been reported across multiple species (e.g. polar granules in *Drosophila*, germinal granules in *Xenopus*, and P granules in *C. elegans*). In *Drosophila*, polar granule-component Vasa (eIF4A-like helicase) acts in a complex with eIF5B to exert mRNA translational control (e.g. of gurken mRNA). Vasa-deficient females develop oocyte tumors and accumulate undifferentiated nurse cells in their egg chambers (Styhler et al., 1998). P granules in *C. elegans* are likewise perinuclear mRNPs partitioned to the germ line (Reese et al., 2000; Updike et al., 2011; Seydoux et al., 1996). As

germ cells develop into gametes, these become a main focus for modulating protein synthesis via RBPs. P granules maintain their perinuclear localization during early oocyte development. They are subsequently dispersed through the cytoplasm in late stage oocytes and segregate exclusively to the germ cell lineage beginning at the two-cell stage and continue a discrete localization throughout embryogenesis (Figure 5.1). Surprisingly, however, the partitioning of P granules to the germ lineage is not essential for its specification and early gamete development (Gallo et al., 2010). Rather, P granules have been implicated in maintaining germ line identity by repressing somatic factors that would otherwise drive neuronal or muscle differentiation in the germ line (Updike et al., 2014). While P granules are required for maintaining germ line identity, P granule-components, such as translation factors (IFE-1 and IFG-1), promote the timed translational control of stored mRNAs that subsequently regulate developmental events in gametogenesis (Figure 5.1).

The *C. elegans* eIF4E isoform IFE-1, unique among eIF4E types, is localized within P granules where it is proposed to facilitate the eventual recruitment of stored mRNAs to the ribosome (Amiri et al., 2001). In support of this hypothesis, several P granule-associated mRNAs (*gld-1, pos-1, mex-1*) were shown in this dissertation and previous publications to be translationally regulated by IFE-1 (Friday et al., 2015; Henderson et al., 2009). Our lab identified these mRNAs by biochemical polysome fractionation from wild type and *ife-1* worms. Each mRNA showed a greater proportion in medium-to-heavy polysome fractions in the presence of IFE-1. In mutant worms that are devoid of IFE-1, their translation was much less efficient. This suggests that IFE-1 preferentially recruits several P granule mRNAs for translation initiation upon RBP de-repression.

Figure 5.1

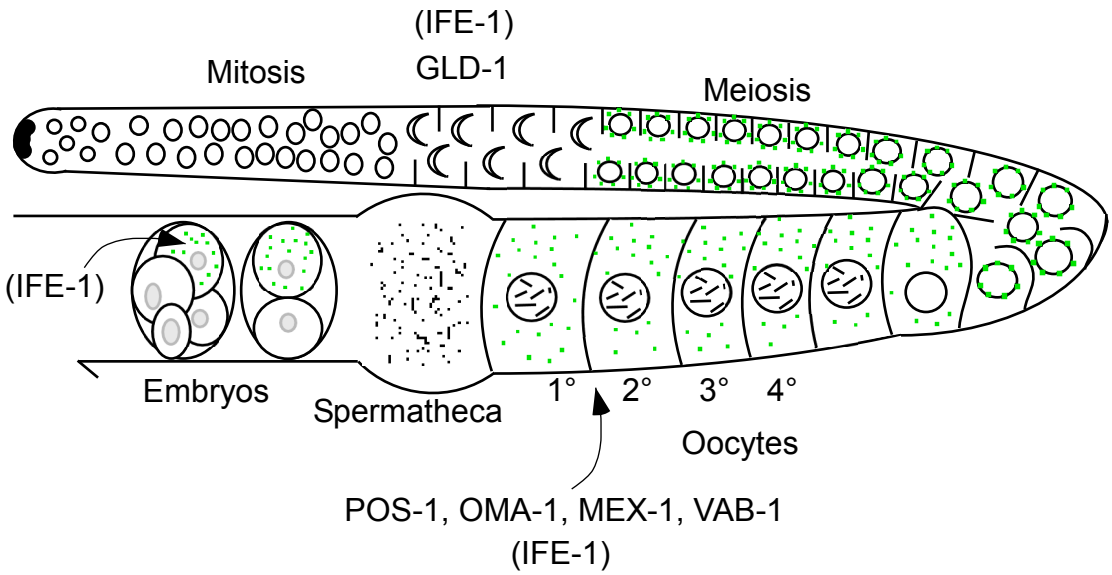

(IFE-1)
GLD-1
Mitosis
Meiosis
(IFE-1)
Embryos
Spermatheca
1°
2°
3°
4°
Oocytes
POS-1, OMA-1, MEX-1, VAB-1
(IFE-1)

Figure 5.1. IFE-1 P granule localization. IFE-1 is localized to P granules (depicted in green) throughout the *C. elegans* germ line. P granules are located perinuclear in young oocytes in syncytium. Following the bend in the gonad, P granules are dispersed as cytoplasmic granules that are sequestered to the P lineage post-fertilization. Association with P granules allows IFE-1 to recruit stored mRNAs upon their de-repression to regulate developmental events throughout the germ line.

2. Early and late mRNA recruitment during oogenesis

We have shown that mRNAs recruited either in immature germ cells (*gld-1*) or in maturing oocytes (*pos-1, mex-1*) utilize IFE-1 for efficient translation. The difference in the timing of these translational control events merits some discussion, as their protein products have very different roles in the development of an oocyte. GLD-1 promotes the transition to meiosis and suppresses mitosis in immature oocytes. IFE-1-mediated recruitment of *gld-1* mRNA for translation shows that IFE-1 is present and active early to support the transition of immature oocytes into the meiotic differentiation program. Notably, this supports the idea that a population of IFE-1 that is likely free from P granules in order to initiate productive mRNA translation events. At later points in development, IFE-1-mediated recruitment of *pos-1* and *mex-1* mRNAs suggests that the same cap-binding factor supports late stage oocyte protein synthesis that prepares the cells for fertilization and proper early embryonic asymmetric divisions. It did not escape our attention that some *pos-1, gld-1,* and *mex-1* mRNA is translated in the absence of IFE-1. This means that other eIF4E isoforms present in the germ line (e.g. IFE-2, IFE-3 or IFE-5) are able to support some translational recruitment of these mRNAs, but with poor efficiency.

While these results show the prioritization of overall mRNA translational efficiencies in whole worm lysates, we sought to prove that IFE-1 promotes the translation of specific mRNAs at specific stages in oocyte development within individual germ cells. To do this we used an mRNA 3' UTR reporter assay that showed GFP expression following mRNA recruitment. We observed that IFE-1 preferentially recruited *gld-1* and *pos-1* 3'UTR reporter mRNAs at very different times (but consistent with their endogenous regulation) within individual developing cells *in vivo.* These data support a mechanism by which P granule-associated mRNAs *gld-1* and *pos-1* are under positive translational control by IFE-1 as they are natively de-repressed from

RNPs in immature oocytes or late stage maturing oocytes respectively. This implicates IFE-1 as being "available" for such recruitment events both early and late in the developmental progression of these cells. Polysome analysis also identified non-P granule mRNAs translationally regulated by IFE-1. This finding too supports there being both a soluble cytoplasmic fraction as well as a P granule bound fraction of IFE-1. Indeed, early localizations with an IFE-1::GFP reporter showed that, while most of IFE-1 was localized to P granules, there was still a proportion of cytoplasmic IFE-1 available to contribute to mRNA translation (Amiri et al., 2001). The IFE-1-mediated translation of *vab-1* mRNA showed that IFE-1 had a role in regulating the very late step of oocyte maturation through the recruitment of an mRNA that did not associate with P granules.

It was logical that some mRNAs have no need of IFE-1 for translation, given that three other isoforms of eIF4E are present in germ cells (IFEs 2, 3 and 5). Housekeeping gene transcripts *gpd-3* and *tbb-2* showed no change in translational activity in the presence or absence of IFE-1. This might be expected because neither of these mRNAs are translationally repressed in germ cells; IFE-1 seems to preferentially utilize mRNAs under temporal control or storage. Interestingly, a 3'UTR reporter mRNA for the integral P granule protein PGL-1 showed increased translation in the *absence* of IFE-1. This suggests that another IFE recruits *pgl-1* mRNA to ribosomes. More to the point, IFE-1 translational events may be competing with those other germ line IFEs for mRNPs or translational machinery (e.g. IFG-1 p170) that happen modulate PGL-1 synthesis. These data suggest three classes of mRNAs regulated by IFE-1. There are mRNAs that uniquely require IFE-1 for efficient and controlled recruitment, mRNAs that do not require IFE-1 at all, and mRNAs translate more efficiently via other germ line IFEs when IFE-1 is not active.

3. RBPs regulate eIF4E recruitment of mRNAs

Our results using an *in vivo* 3'UTR reporter assay (Figure 2.8) support a model in which IFE-1 selectively recruits mRNAs for translation initiation upon their de-repression. Sequence specificity in the corresponding 3'UTRs likely comes from RBP complexes that bind and repress such mRNAs, and may also associate with IFE-1. Remodeling of this complex would allow IFE-1 to initiate the de-repressed mRNA while still bound to its cap structure. There are several paradigms for the regulation of germ cell mRNAs that support this model. For example, in *Drosophila* germ granules, *oskar* mRNA is translationally repressed, stored and localized. The Bruno protein binds *oskar* mRNA's 3'UTR during translocation to oocyte cytoplasmic granules to keep it from associating with initiation complexes and ribosomes. Bruno also binds the 4E-BP, Cup, which in turn associates with eIF4E at the *oskar* 5' mRNA cap. This forms an eIF4E-Cup-Bruno circularized repression complex on *oskar* mRNA that prevents bound eIF4E from interacting with eIF4G (Figure 5.2) (Nakamura et al., 2004). This mechanism guarantees the translational repression of *oskar* mRNA during its transport to the oocyte's posterior pole (Ephrussi et al., 1991). Mutations that disrupt the formation of this mRNP complex result in precocious *oskar* translation that subsequently cause defects in embryonic polarity necessary for asymmetrical divisions following fertilization, and even body patterning in the embryo (Nakamura et al., 2004). This is one example of how RBPs and translation initiation factors coordinate negative and positive translational control to drive carefully timed cellular development. Similar circularized repression of mRNAs through eIF4E-4EBP complexes has been identified in multiple species including: eIF4E1-maskin-CPEB in *Xenopus*; PGL-1-IFE-1 in *C. elegans* (isoform specific); and OMA-1-IFET-1-IFE-3 in *C. elegans* (isoform specific) (Figure 5.2). These diverse complexes serve to bind and repress mRNA 3'UTRs with sequence

Figure 5.2

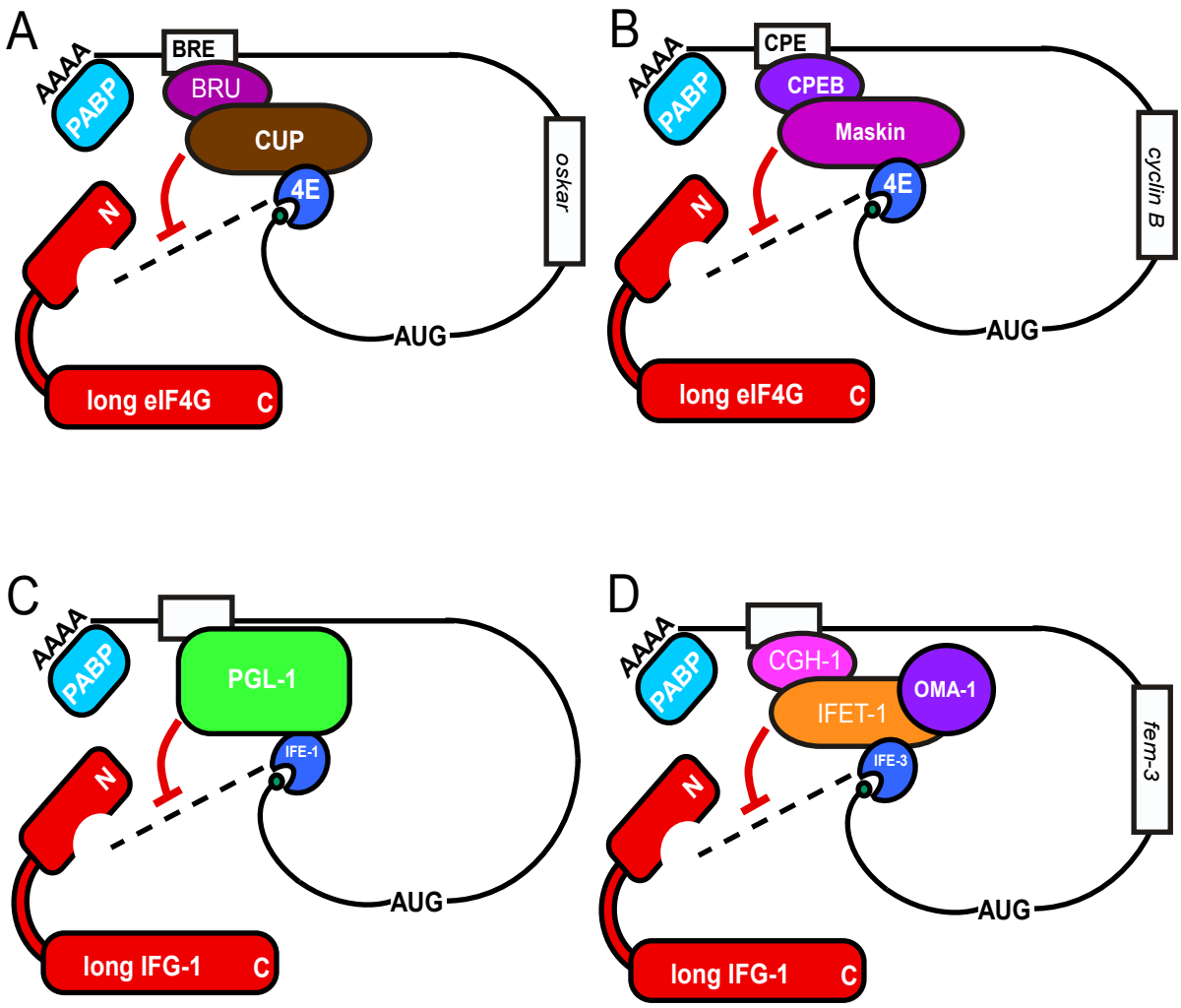

A
AAAA
PABP
BRE
BRU
CUP
4E
N
oskar
AUG
long eIF4G
C
B
AAAA
PABP
CPE
CPEB
Maskin
4E
N
cyclin B
AUG
long eIF4G
C
C
AAAA
PABP
PGL-1
IFE-1
N
AUG
long IFG-1
C
D
AAAA
PABP
CGH-1
IFET-1
OMA-1
IFE-3
N
fem-3
AUG
long IFG-1
C

Figure 5.2. eIF4E mRNP regulatory complexes. These diagrams represent known and predicted mRNP complexes relevant to eIF4E and mRNA repression. (A) In *Drosophila* germ granules *oskar* mRNA is translationally repressed. This repression occurs through an eIF4E-Cup-Bruno complex that circularizes *oskar* mRNA and prevents bound eIF4E from interacting with eIF4G. (B) A similar complex regulates cyclin B mRNA in *Xenopus*. An mRNP complex is formed between CPEB-maskin-eIF4E and cyclin B mRNA 3' UTR and 5' cap. This mRNP complex represses cyclin B in arrested meiotic oocytes (detailed in Chapter 4). (C) In *C. elegans* constitutive P granule protein, PGL-1, is known to bind eIF4E isoform IFE-1. It is predicted that PGL-1 forms an mRNP complex with IFE-1 to regulate mRNA recruitment for translation. (D) Preliminary evidence suggests that another eIF4E isoform present in the *C. elegans* germ line, IFE-3, may interact with the IFET-1-CGH-1 complex. This interaction is predicted to sequester IFE-3 in an mRNP complex that represses *fem-3* mRNA. These represent several known and predicted mRNP complexes that can repress mRNAs. Remodeling of these complexes can result in eIF4E being readily available to recruit bound-mRNA for translation initiation.

specificity. At the same time, they sequester eIF4E from association with eIF4G, and subsequently set up eIF4E to readily recruit bound mRNAs for translation upon mRNP remodeling. These paradigms make it clear that negative and positive mRNA translational control may not be separable events, but rather necessarily work together.

4. Differing roles for each eIF4E (IFE)

Over many years the Keiper lab's publications have cumulatively shown that each eIF4E isoform in *C. elegans* recruits a unique set of mRNAs to promote different developmental events. For instance, IFE-2 and IFE-4 are both expressed in somatic tissue. IFE-2 regulates aging in somatic tissue (Syntichaki et al., 2007). IFE-4 is also expressed in somatic tissue, but predominantly regulates muscle and neuron development by promoting the translation of a specific sub-set of mRNAs (e.g. *egl-3, egl-12, daf-12, kin-29*) (Dinkova et al., 2005; Syntichaki et al., 2007). IFE isoforms 1, 3, and 5 (and to a lesser extent IFE-2) are all expressed in the germ line, but mutational analysis shows that each has a unique role. While worms lacking IFE-1 show defects in late spermatocyte and oocyte maturation, IFE-3-deficient worms, by contrast, are unable to complete the sperm-to-oocyte switch much earlier in larval germ cell development, such that hermaphrodites become sexually masculinized (Friday et al., 2015; Henderson et al., 2009). Loss of still another eIF4E isoform (IFE-2), which is expressed at low levels in germ cells, leads to a defect in chromosome crossover due to IFE-2-mediated recruitment of key mRNAs (*msh-4/him-14, msh-5*) (Song et al., 2010). Unique defects observed for each isoform-specific mutant implies differences in their biological functions, despite the fact that biochemically, each binds mRNA caps (Keiper et al., 2000). The fact that their molecular

function (mRNA recruitment to the translation initiation complex) is universal among isoforms suggests that divergent phenotypes come from specificity in the unique populations of mRNAs with which they associate (Keiper et al., 2000). Above-named examples of distinct roles for the nematode eIF4Es support a paradigm in which the translational machinery selectively recruits important mRNAs for protein synthesis to drive developmental events. In this dissertation I have identified the population of mRNAs preferentially recruited by one of three germ cell IFE isoforms, IFE-1 (Friday et al., 2015; Henderson et al., 2009).

5. IFE-1 recruits a unique set of mRNAs for translation

By taking a bioinformatics approach to germline translational regulation by IFE-1, and taking advantage of biochemical fractionation and genetic tools, we were able to identify 77 mRNAs that specifically require IFE-1 for efficient translation. The identified mRNAs are involved in a range of cellular activities that are essential to gametogenesis and early embryogenesis. These include *ran-1* and *rab-7,* which are involved in vesicle trafficking, used both in oocyte maturation and establishment of embryo polarity. *ran-1* encodes a Ran GTPase that promotes VAB-1 trafficking to the endocytic-recycling vesicles of maturing oocytes in the absence of sperm. *rab-7,* on the other hand, is predicted to promote VAB-1 trafficking for lysosomal degradation. The VAB-1 protein encodes a major sperm protein receptor that suppresses MAP kinase activity required for late stage oocyte maturation. Additionally, *vab-1* mRNA was itself recruited by IFE-1. These data suggest that IFE-1 promotes the translation of a functionally interrelated set of mRNAs that together regulate oocyte meiotic maturation. Similarly, other data also showed that IFE-1 mediates the efficient translation of pro-meiotic *gld-*

1 and pro-mitotic *glp-1* mRNAs. These two protein products are antagonistic regulators of the mitosis/meiosis switch. It is not clear why IFE-1 would promote synthesis for two opposing activities, except perhaps that both are occurring in a defined region of the gonad or time of germ cell development. Perhaps IFE-1 supports a balance of these opposing factors for the proper timing of the transition from mitosis to meiosis. GLP-1 is a Notch receptor homolog that promotes mitosis in the earliest GSC pool (Austin and Kimble, 1987). As germ cells progress through the transition zone of the gonad, *gld-1* mRNA becomes de-repressed and GLD-1 protein begins to accumulate. GLD-1 encodes a STAR/Quaking homolog that is a sequence-specific RBP to repress translation of several target mRNAs (Francis et al., 1995; Jan et al., 1999; Saccomanno et al., 1999). The buildup of GLD-1 progressively represses *glp-1* mRNA translation to promote meiosis. Depletion of IFE-1 apparently does not dramatically alter this balance. Germ lines lacking IFE-1 still show progression into meiosis in the early germ line. It is likely that suppressed levels of both GLP-1 and GLD-1 are still sufficient to counteract one another's activity. Together, these defined instances show that IFE-1-mediated cap-dependent translation initiation drives the selective recruitment of mRNAs that are important for both early and late stage oogenesis.

6. Translation initiation without any eIF4E (IFE) activity

Regulation of germ line protein synthesis is not limited to the mRNA cap-binding eIF4E isoforms. A significant contribution to germ cell mRNA translational control comes from eIF4G isoforms (IFG-1 p170 and p130). The long *C. elegans* eIF4G (IFG-1 p170) catalyzes cap-dependent recruitment of mRNA to the ribosome by providing the scaffold framework to

coordinate other initiation factors, including mRNA 5' cap-binding factor eIF4E and mRNA helicase eIF4A, with the mRNA and the 40S ribosomal subunit (Keiper et al., 1999). The IFG-1 p170-dependent mode of translation promotes germ cell proliferation and oocyte differentiation and suppresses germ cell apoptosis (Contreras et al., 2008). The short eIF4G (IFG-1 p130) isoform maintains the ability to bind eIF4A, eIF3, and mRNA 5'UTR, but does not encode an eIF4E binding domain. IFG-1 p130 supports cap-independent translation of mRNAs, including stress related and pro- and anti-apoptotic mRNAs during natural germ cell apoptotic events (Chapter 3).

Apoptosis- Our lab developed a biological system (series of mutant and reporter worm strains) in which we could induce physiological germ cell apoptosis either by direct manipulation of the cell death (ced) signaling pathway, or indirectly, through protein synthesis mechanisms. These tools were useful in eventual studies that functionally identified pro- and anti-apoptotic mRNAs under strict cap-dependent or cap-independent translational regulation during apoptosis. My contribution to this effort was to develop a conditional mutant worm strain with temperature-regulated induction of apoptosis and germ cell corpses that could be visually monitored. I created a Bcl-2 homolog knockdown (*ced-9ts*) strain that demonstrated the *in vivo* cleavage of IFG-1 p170 and the simultaneous sustained increase of physiological apoptotic germ cell corpses. We also developed a conditional "cap-independent translation" worm strain (*ifg-1:mos*) in which a Mos transposon insertion in the *ifg-1* gene was sufficient to significantly deplete IFG-1 p170 but leave p130 intact. This strain, like the Bcl-2 strain, showed an inducible increase in germ cell corpses within the gonad by 24 hr. These strains were used in parallel to show that disruption of anti-apoptotic Bcl-2 or the direct genetic disruption of cap-dependent translation was sufficient to physiologically induce germ cell apoptosis by reducing the IFG-1 p170 to IFG-1 p130

representation (Contreras et al., 2011). These strains were used by Dr. Morrison to address the translational efficiency of individual pro- and anti-apoptotic mRNAs following genetic (IFG-1 p170 splicing defect) and physiological (*ced-9* inactivation) depletion of IFG-1 p170. mRNAs encoding the stress-induced BiP homolog, *hsp-3*, and the Bcl-2 homolog itself, *ced-9*, were shown to translate even more efficiently upon IFG-1 p170 depletion. These data showed that a small subset of mRNAs respond positively under protein synthetic stress conditions by taking advantage of cap-independent translation (Morrison et al., 2014). Our lab favors a model in which the cap-independent translation machinery also selects specific anti-apoptotic mRNAs to provide the cell with an initial recovery phase during times of stress.

7. Bioinformatic analysis of cap-dependent and cap-independent mRNAs in worms

We took a similar bioinformatics approach to separately identify mRNAs that show the greatest preference for either cap-dependent or cap-independent initiation (see Figure 1.1). In mammalian cells committed to programmed cell death, there is an increase in the translational efficiency of pro-apoptotic mRNAs via the cap-independent mechanism ; Holcik, 2005 #600}. To discover the corresponding mRNAs in *C. elegans*, our lab performed an RNA-Seq analysis to identify all worm mRNAs that have altered translational efficiency under cap-independent conditions. This was an unbiased genome-wide approach to identify mRNA candidates whose translation may promote survival or death. Such a bioinformatics approach was made possible by the conditional *ifg-1::mos* strains described above, together with our ability to biochemically fractionate worm polysomes in large scale. My contribution to this project was to assist the sucrose gradient resolution of worm polysomes. Additionally, I was able to adapt the bioinformatics computation tools (multi-functional Excel workbook templates) that I had

previously developed for IFE-1-dependent translation status array analysis (TSAA) to the similar RNA-Seq applications. Dr. Keiper prepared samples from our gradients that were analyzed by Beckman Coulter Genomics (BCG) by RNA deep sequencing. Preliminary results identified 144 known mRNAs that were highly cap-dependent; these are involved in various cell proliferation, signaling, or RNA metabolism processes. These included factors involved in cell cycle regulation [cyclin-dependent kinases (*cdc-7* and *-14, cdk-12*)], signal transduction kinases [Erk and protein kinase C isoforms (*sek-5, pkc-1*)], RNA metabolism [capping and decapping enzymes, CPEBs (*cpb-1, cel-1, dcap-2*)], DNA damage repair [Rb protein, homologous recombination factors (*lin-35, rad-54*)], and meiosis/mitosis [recombination proteins, chromatin deacetylases, SET proteins (*mre-11, kle-2, sir2.1, mes-2 and -4*). The same analysis also identified 55 mRNAs that translated more efficiently under cap-independent conditions (depleted of IFG-1 p170). Most of these encoded ribosomal protein mRNAs, but other notable cap-independent mRNAs are involved in cell cycle arrest and apoptosis [CDK phosphatase (*cdc-25.4*), caspase inhibitor (*cps-6*)].

Results from this RNA-Seq analysis will need to be validated by qPCR across fully resolved gradients and protein analysis. Further analysis will also be performed by comparing the RNA-Seq results with the IFE-1 TSAA data. We have completed the TSAA and determined the population of mRNAs translationally regulated by IFE-1. We can now compare the population of IFE-1-dependent mRNAs with IFG-1 p170-dependent mRNAs. Overlap between populations is expected as IFE-1 recruits mRNAs for translation initiation via IFG-1 p170 association. mRNAs identified as IFG-1 p170-dependent, but not IFE-1 dependent, are likely recruited by one of the four remaining eIF4E isoforms. Comparison of changes in total mRNA levels can also be analyzed for both analyses to determine which mRNAs become destabilized following loss of

IFE-1 or IFG-1 p170. Based on the currently known identities of just these handful of mRNA candidates, we suspect that cap-dependent and cap-independent translation will have pivotal roles in cell cycle progression and recovery from cellular insult, respectively.

8. The connections between IFE isoforms and IFG-1 isoforms in development

Translation initiation factor isoforms (IFEs and IFG-1s) are integral to the remodeling of mRNP complexes that support distinct protein synthetic events likely and drive cell fate decisions. Depletion of IFE isoforms results in a broad array of developmental defects. Knockdown of IFE-4 caused a decrease in translation of mRNAs involved in neuronal and muscle development (Dinkova et al., 2005). Depleting animals of IFE-3 caused a direct change in cell fate (oocytes to sperm) (unpublished, J. Subash). Loss of IFE-1, rather than re-defining the whole identity of developing germ cells, instead influenced events after gamete identity was established, both in sperm and oocytes. We know the most about the role of IFE-1. For example, spermatogenesis occurs at the proper time and for the correct population of gametes in *ife-1* mutants, but these cells have a cytokinetic defect that yields non-viable multinucleated sperm. As opposed to *ife-3* worms, loss of *ife-1* does not cause a drastic change in gamete identity during oogenesis. Depletion of IFE-1 simply reduces the translational efficiency of inter-related mRNAs that support various mitotic, meiotic and post-meiotic events (e.g. late stage oocyte maturation, spermatocyte division, and cell cycle resumption). The wide range of isoform-specific effects and the degree to which they affect cell fates are subject to some limited functional redundancies when multiple isoforms are present. While IFE isoforms promote the translation of unique sets of mRNAs, all of the IFEs promote mRNA translation through the functionality of initiation factor IFG-1 p170. In fact, IFG-1 p170 probably has the most basal level of mRNA selectivity for cap-

dependent protein synthesis. Our model postulates that, in *C. elegans* germ cells containing multiple IFEs, mRNAs all apparently rely on a single IFG-1 isoform, despite the fact that each IFE selectively recruit unique sets of mRNAs (see Figure 1.2). Within this second level of mRNA selectivity is the contribution of RBPs that repress both mRNAs and IFEs. The coordinated remodeling of such mRNPs permits the associated mRNA-bound IFE isoform to successfully join with IFG-1 p170 for recruitment to ribosomes. Therefore, dynamic regulatory complexes allow for the translational selectivity necessary to drive the many cell fate decisions in gametogenesis.

The conclusions drawn from my studies support a shift in the traditional paradigm of stored mRNAs being fully regulated by RBP repression in germ cells. The emerging evidence suggests a coordinated effort in which RBPs repress mRNAs and provide the sequence specificity for complex formation. Isoforms of eIF4E become part of these RBP complexes exclusive of eIF4G binding. The pre-associated translation initiation factors then rearrange to positively recruit mRNAs to eIF4G and to ribosomes. This coordinated remodeling of mRNP complexes leads to specific induction of new protein synthesis in germ cells. Ultimately, the *de novo* protein synthesis drives distinct germ cell developmental events.

9. Future Directions

There are still many avenues of exploration to be followed in the future of the projects I described here. First and foremost is the characterization of IFE-1 mRNP complexes by specific isolation using the Flag-tagged IFE transgenic lines I prepared and presented in Chapter 4. Recently we were able to develop transgenic worms that stably express Flag::IFE-1 using MOS SCI transgenesis techniques, following several unsuccessful attempts at expression by other

by other transgenic technologies. Importantly, this chromosome-integrated transgene produced Flag::IFE-1 that was easily detectible by western blot analysis from whole worm lysates. Previous transgenic approaches (using extrachromosomal or UV-integrated arrays), while successfully introducing the fusion gene, yielded poor tagged-protein expression that required biochemical enrichment steps before detection was possible. With more stable/robust expression of Flag::IFE-1 in the germ line, these transgenic worms may provide the tool for identifying IFE-1 regulatory complexes. Specifically, anti-Flag antibody beads can be used in batch-binding experiments to immunoprecipitate Flag::IFE-1 and associated proteins. By western blot analysis we expect to find other translation factors (e.g. IFG-1, eIF4A), in productive initiation complexes, and P granule proteins (e.g. PGL-1, PIE-1, MEX-1, MEX-5, POS-1), perhaps indicative of inactive mRNP structures, as detailed in Chapter 4. This approach is unbiased, however, and has the potential to identify proteins associated with Flag::IFE-1 that are outside of the range of our predictions. Proteins isolated by the anti-Flag antibody beads can also be separated on SDS-PAGE gels, and additional bands can then be excised and analyzed/identified by mass-spectrometry. In parallel, Flag::IFE-1 complex isolation will allow for the identification of specific mRNAs that reside in IFE-1 complexes. The same anti-Flag batch bound elutions can be analyzed for mRNAs by qPCR. The Flag::IFE-1-bound mRNA population can be directly compared to those identified as being translationally regulated by IFE-1 using our TSAA results. Enrichment of these mRNAs in Flag::IFE-1 elutions compared to control (other Flag::IFEs) would show a more direct association of Flag::IFE-1 with the mRNAs it regulates.

Another experiment that I think would be interesting to perform with the Flag::IFE-1 transgenic worms is isolation of Flag::IFE-1 across a fractionated sucrose gradient. For instance, polysomes from transgenic Flag::IFE-1 expressing worms could be resolved via sucrose gradient

fractionation. Flag::IFE-1 could then be isolated from each resulting fraction by the same anti-Flag batch binding procedure. This could prove useful to determine which proteins are brought along with Flag::IFE-1 into initial ribosomal, and even polysomal structures. For example, western blot analysis of Flag::IFE-1 elutions will likely identify IFG-1 in ribosome-containing f fractions. A significant caveat to interpreting these experiments is that the proportion of Flag::IFE-1 associating with polysomes is likely to be small (substoichiometric to ribosomes), since it is expected to cycle in, and back out, of ribosomal complexes once initiation events are complete. Control experiments using EDTA to release ribosomes will also be necessary to confirm that IFG-1 (or other RBP) association with Flag::IFE-1 is not due to a heavy mRNP complex. Indeed, such a complex containing IFE-3, IFG-1 and OMA-1 has been identified by another lab (Spike et al., 2014), and this finding gives credence to the likely diversity of mRNP complexes in germ cells. The simplest interpretation for localization of IFG-1 in polysome fractions is that IFE-1 is involved in a closed-loop translation re-initiation complex that has been proposed to coordinate the 5' cap and poly(A) tail (Keiper et al., 1999; Hentze, 1997). This cap-to-tail closed-loop re-initiation model is widely accepted, but its interactions with 3' UTR RBP complexes is still only postulated (Figure 4.1). Recent studies in live yeast have shown compelling evidence in support of this model (Archer et al., 2015). The experiment proposed here would support the model that IFE-1 takes part in a closed-loop translation re-initiation complex with IFG-1, and allows for the identification of other RBPs which are also bound. There are substantial technical issues with this approach, in that the amount of material recovered from each fraction is limited by the resolution capacity of sucrose gradients. In experiments partnered with Dr. Morrison, we tested range of starting worm lysate amounts loaded onto a sucrose gradient from 2 worm pellets (estimated at 0.1 ml packed worms), up to 16 worm pellets. We

showed that lysate from 8 pellets (less than 0.5 ml packed worms) was the maximal amount that still allowed clean resolution of individual polysomes, as evidenced by absorbance profiles. It is unclear whether this would be sufficient material for detection of proteins via western blot analysis following fractionation and anti-Flag precipitations. However, our lab has detected PGL-1 by western from individual sucrose gradient fractions, demonstrating that P granules do not comigrate with polysomes in sucrose gradients (data not shown). The approach is therefore a worthwhile opportunity to obtain evidence supporting a coordinated closed-loop re-initiation complex mediated by IFE-1 and IFG-1.

Given the severe, temperature-sensitive spermatogenic defect caused by loss of IFE-1, it would be interesting to determine the mRNAs it regulates specifically in males. The *ife-1* deletion causes temperature sensitive sterility due to the production of multinucleated sperm (Henderson et al., 2009). The mRNAs translationally regulated by IFE-1 that could be responsible for this phenotype have proven difficult to identify because oocyte mRNAs are much more abundant in hermaphrodite populations, and male-only populations cannot be propagated. Recently, however, our lab acquired a set of size exclusion screens that may provide a means of experimentally identifying these mRNAs. For this experiment one would perform a TSAA similar to the one detailed in Chapter 2, but rather than comparing hermaphrodite worm populations, adult males (wild type vs *ife-1*) would be sorted prior to polysome fractionation. The increased proportion of sperm-related mRNA in this TSAA may allow for identification of sperm-specific mRNAs translationally regulated by IFE-1 that were underrepresented in the original TSAA. Briefly, from large scale growth plates of wild type and *ife-1* worms, populations could be developmentally synchronized and adult males could be isolated with a size exclusion mesh screen (hermaphrodites = ~1mm, males = ~0.7mm), washed and pelleted. On a small scale

cross plates between males and hermaphrodites, offspring are roughly 50% male. On a larger scale, however, after multiple generations, the proportion of males produced becomes diluted to less than 1%. The proportion of males could be enhanced genetically to about 30% by introducing a *him-5* mutation into populations (Singson et al., 1999). Alternatively, beginning with enough small scale cross plates (I would estimate 30 per worm strain), and limited expansion of the population, enough males could be sorted to load a single gradient. Each gradient yields more than enough RNA for a deep sequencing experiment, so that three such sortings would provide the triplicate samples necessary. This method could identify novel, spermatocyte-specific mRNAs that are IFE-1-dependent, and help explain the *ife-1* sperm maturation defect.

The experiments I have proposed in this "Future Directions" section are approaches that I consider the most practical, interesting, and of direct relevance to the outcomes of my projects. I hope to find one day that the ideas presented herein benefit the Keiper lab and the overall impact on germ cell mRNA translational control. To any future graduate students who may read this, I wish you good luck.

Chapter 6: Experimental Procedures

Strains. The *C. elegans* strains used were Bristol, N2 wild type strain, *Caenorhabditis* Genetics Center (CGC) strains AZ212 *unc-119*(ed3), ruls32 [P*pie-1*:GFP:H2B:*tbb-2* 3'UTR +*unc-119*(+)],CZ337 *vab-1* (dx31) II, DH245 *fem-2*(b245) III, JH2060 *unc-119*(ed3) III; axls 1498 [P*pie-1*:GFP:*gld-1* ORF:*gld-1* 3'UTR +*unc-119*(+)], JH2320 *unc-119*(ed3) III; axls 1677 [P*pie-1*:GFP:H2B:*pgl-1* 3'UTR +*unc-119*(+)], JH2427 *unc-119*(ed3) III; axls 1751 [P*pie-1*:GFP:H2B:*pos-1* 3'UTR +*unc-119*(+)], JH2223 *unc-119*(ed3) III; axls 1611 [P*pie-1*:GFP:H2B:*daz-1* 3'UTR +*unc-119*(+)], JH2252 *unc-119*(ed3) III; axls 1640 [P*pie-1*:GFP:H2B:*glp-1* 3'UTR +*unc-119*(+)], JH2200 *unc-119*(ed3) III; axls 1577 [P*pie-1*:GFP:H2B:*nos-3* 3'UTR +*unc-119*(+)], JH2236 *unc-119*(ed3) III; axls 1624 [P*pie-1*:GFP:H2B:*pal1* 3'UTR +*unc-119*(+)], JH2623 *unc-119*(ed3) III; axls 1851 [P*pie-1*:GFP:H2B:*rad-1* 3'UTR +*unc-119*(+)], JH2107 *unc-119*(ed3) III; axls 1492 [P*pie-1*:GFP:*tbb-2* ORF: *tbb-2* 3'UTR +*unc-119*(+)], JH2647 axls 1928 [*par-6::mCherry*]; axls 1182 [*par-2::gfp*], EG6703 *unc-119*(ed3) III; cxTi10816 IV; oxEx1582; MT3970 [*ced-9* (n1653); *mab-5*(mv114)] III; KX54 *ifg-1:mos-1* (cxP9279)II; bcTs39v[Plim-7 *ced-1::gfp +lin-15(+)*]; MD701 bcls39 [P*lim-7 ced-1::gfp +lin-15(+)*]; KX110 *ced-9* (n163 *mab-5*(mv114) III, bcls 39V [P*lim-7 ced-1::gfp + lin-15(+)*], KX111 *ced-9* (n163 *mab-5*(mv114) III, bcls 39V [P*lim-7 ced-1::gfp + lin-15(+)*]. SS712 [*ife-1*(bn-127)] was obtained from Dr. Susan Strome. All strains were maintained on normal growth medium (NGM) plates with *E. coli* strain OP50 at 15 ˚C. For sucrose gradient fractionation and polysomal analysis, worms were grown on chicken egg yolk OP50 plates at 20 ˚C. As previously described, mix-staged worm populations were isolated in M9 buffer, sucrose floated, and pelleted in liquid nitrogen in the presence of RNase inhibitors (Dinkova et al., 2005).

Worm culture and general manipulations. As standard procedure, worms were grown at 20˚C for experimentation, and strains maintained at 15˚C unless otherwise stated. Dissections were done in a Petri dish containing 5 ml of dissection buffer (0.75x M9, 5 mM levamisole, 0.1% Tween 20).Worms were decapitated using a #15 Bard-Parker carbon steel surgical blade. Embryos were dissected by cutting worms in half at the vulva. When required in large quantities, worms were grown on chicken egg plates. Egg plates were prepared as follows. 90mL of 2xYT broth was heated to 60°C. In a separate, sterile beaker, 8 egg yolks were isolated (pre-rinse shells in EtOH to prevent additional chance of bacterial contamination). Yolks ant 2xYT broth were mixed and returned to 60°C for 10min. Mixture was then cooled to 30°C and added to 90mL of OP50 bacterial culture that had been grown over the previous night at 37°C. New mixture was then incubated at 37°C for 1 hour. 5mL of yolk-bacterial mixture was dispensed onto 10cm 5x Peptone plates (or 10mL per 15cm plate) that had been dried at 25°C over the previous night. Seeded peptone plates were incubated at 37°C overnight with lids tilted open. (3-10cm plates and 1-15cm plate yielded 7mL of concentrated worms. This translated to roughly 2.5 15mL falcon tubes of frozen worm pellets.)

Microscopy and immunostaining. Worm gonads were prepared for microscopy as previously described (Henderson et al., 2009), with the following exceptions: Following dissections, gonads were fixed in formaldehyde (3% formaldehyde, 1x PBS) for 30 minutes, washed with PTW (1x PBS, 0.1% Tween 20), and stored at -20 ˚C in methanol. To prepare gonads for immunostaining, samples were reconstituted and washed with PTW. Gonads were blocked overnight in PTWB (PTW + 0.5% BSA) at 4˚C. The anti-MAP kinase (activated) mouse monoclonal antibody (Sigma) was diluted 1:200 in PTWB and incubated with samples at room temperature for 2 hours. Following another PTWB wash, goat anti-mouse Alexa Fluor 488

(Invitrogen) at 1:400 was used as the secondary antibody. Samples were washed in PTWB and mounted on 1.2% agarose pads with 5 ul Vectashield (Vector Laboratories) with DAPI. Gonads were imaged on an Axiovert 200M inverted microscope (Carl Zeiss) using DIC, FITC, Texas Red, and DAPI filter cubes and analyzers. Images were analyzed with Axiovision 4.8 software (Carl Zeiss).

RNAi Feeding Conditions. Bacterial transformation and feeding techniques were conducted exactly as described in Contreras V., et. al. 2008, with the following exceptions. The *ife-1*-specific dsRNA-expressing plasmid (pT72i1d) was constructed by subcloning 153bp (nt 589-741) of *ife-1* cDNA sequence into plasmid pL4440. Double stranded RNA was expressed in *E. coli* strain HT115(DE3) in culture containing tetracycline and ampicillin and were induced with IPTG. Induced cultures were grown on NGM agar media plates supplemented with tetracycline, ampicillin, and IPTG. Wild type, AZ212, CZ337, JH2060, JH2320, and JH2427 strains were synchronized and L3/L4 hermaphrodites were transferred onto seeded RNAi plates at 22˚C for 36-40 hrs. Hermaphrodites were transferred to seeded RNAi plates, with a limit of 3 F0 adults per plate, and incubated at 25 ˚C for 24hrs. F0 adults were then removed. The F1 generation was incubated at 25 ˚C for another 24hrs. Progeny were dissected or directly utilized for microscopy.

Live Worm Fluorescence Imaging and Quantification. Adult AZ212, JH2060, JH2320, JH2427, JH2223, JH2252, JH1999, JH2200, JH2236, JH2636, JH2647, and JH2107 hermaphrodites were transferred onto agarose pads with 5uL Vectashield mounting medium with DAPI for microscopy. AxioVision V4.8.2 was used to identify optimal exposure time (OET) for linear fluorescence output. Images were acquired along a time gradient of -40% OET, -20% OET, OET, +20% OET, and +40% OET. A measurement for regions of interest (ROI) were

defined manually for each image. The densiometric mean was within each ROI and assembled on an Excel spreadsheet. Background fluorescence was defined from the Y-intercept of the linear regression derived by plotting densiometric mean versus %OET. Mean fluorescence at OET minus background fluorescence was divided by OET. Multiple values of mean fluorescence per exposure time were used to derive average values and standard deviations presented in the graphs in Figure 3. ROIs were defined for the distal region of immature oocytes (*gld-1* 3'UTR reporter) or for expression in the nucleus of individual oocytes (*tbb-2, pos-1, and pgl-1*).

Worm lysis and sucrose gradient centrifugation to resolve polysomes. We resolved polysomes mixed population whole worm lysates over 11 mL 10-45% sucrose gradients. By systematically varying mass loaded onto the gradients, we determined that 8-10 pellets of densely packed worms per gradient resulted in a clearly resolved polysome profile with enough material per fraction to do RNA and protein quantitation.

Making sucrose gradients. Sucrose gradients are made the day before sucrose gradient centrifugation. We made 35mL of 45% sucrose (15.75g sucrose, 7mL 5xPolysome Profile Buffer, 35uL 1M DTT, and DEPC H2O) and 10% sucrose (3.5g sucrose, 7mL 5xPolysome Profile Buffer, 35uL 1M DTT, and DEPC H2O) solutions and let them equilibrate to 4°C. (5x Polysome Profile Buffer consists of 1.5M KCl, 250mM HEPES, 10mM MgCl2). SW-41 plastic 11mL tubes (Beckman #331372) were marked at a 5mL line. Tubes were first filled with 6mL of the 10% sucrose solution. 45% sucrose solution was then added directly to the bottom of the tube using a needle and syringe, until the 5mL line was reached. Long black caps were placed on top of the tubes. Tubes were rotated on a Seton (Radiant) Gradient Maker (time = 4.06min, angle = 80 degrees, speed = 8) and then stored overnight at 4°C.

Worm lysis and sucrose gradient centrifugation and fractionation. 8-10 pellets of densely packed frozen worm pellets were ground with a mortar and pestle with the 1:1 addition of 2xLysis Buffer [400uL 5xLysis Buffer Stock (250mM Tris-HCl pH 8, 1500 mM NaCl, 50mM MgCl2, 5mM EGTA) in 3mL total DEPC buffer with 0.4mg/mL heparin, 800U/mL RNasin, 4mM Vanadyl RNC, 5mM PMSF, 0.4mg/mL CHX, 2mM DTT, 1% Triton X]. Ground lysates were thawed on ice and centrifuged at 10k rpm at 4°C for 15min. 0.5 mL was removed from the top of stored gradient tubes and lysated were layered evenly on top of the gradients. Sucrose gradients were spun in a pre-chilled Beckman centrifuge (vacuum < 200u, time = 1hr 45min, speed = 38,000, temp = 4°C, decal = 0). Following centrifugation, sucrose gradients were fractionated using the Teledyne Foxy Jr. fraction collector. Sample was pushed through the fractionator with 70% sucrose in DEPC. Absorbance was continually monitored by an ISCO UA-6 UV-detector chart recorder (speed = 150, sensitivity = 2, peak separator = "off"). Eleven 1 mL fractions were collected for each sucrose gradient.

RNA isolation and qPCR. Sucrose gradient fractionation was performed on wild type and *ife-1*(bn127) worms as previously described with the exception that gradient fractions were isolated in 4 volumes Trizol (Henderson et al., 2009). RNA was isolated from one half of each 1 ml gradient fraction. 0.25ug of RNA from each fraction was synthesized into cDNA using Verso cDNA synthesis kit (Thermo Scientific). Real-time PCR was performed on biological duplicates using Sso Fast Evagreen Super mix (BioRad) in an iCycler iQ5 Real-time PCR machine, following the manufacturer's protocol. Primer sequences used were: *gpd-3* (forward 5'-GATCTCAGCTGGGTCTCTT-3', reverse 5'-TCCAGTACGATTCCACTCAC-3'); *rnp-3* (forward 5'-CGATCTATGTGAACAATCTCAA-3', reverse 5'-ACCGAATTGTGTGAAAACC-3'); *rab-7* (forward 5'-GCTCGGAGTCGCTTTTTATC-3',

reverse 5'-GGACAAACGGGAAATGGTCT-3'); *vpr-1* (forward 5'-ACGAGGATAGTTTTGCTTCTT-3', reverse 5'-ACTGTCGATTTCAACGATTT-3'); *gld-1* (forward 5'-TTCAGGTCCAGTTTTGATGT-3', reverse 5'-GACGTTAGATCCGAGAAGGT-3'); *vab-1* (forward 5'-AAGAATATTGGACGGTTGG-3', reverse 5'-GTCGCATATTCGGTAGTAAA-3'); *ran-1* (forward 5'-ACTTGTCTTCCACACCAATC-3', reverse 5'-GAGCGGTAACATCGAACA-3'). Real-time quantification of each mRNA was normalized to total RNA content.

Translational State Array Analysis. Three independent sets of sucrose gradients were prepared for wild type (w) and *ife-1*(bn127) worms as previously described by Henderson et al. (Henderson et al., 2009). Following RNA isolation from individual fractions, non-polysomal (NP) fractions 1-4 and polysomal (P) fractions 6-11 were pooled separately. Fraction 5 contained the 80S peak. These twelve samples were purified and hybridized to Affymetrix *C. elegans* Arrays (UNC Functional Genomics Core Facility). We created formulas in Excel to compare raw microarray hybridization signal values with ratios that were normalized to total non-polysomal and polysomal mRNA signals. [Pw/(NPw+Pw)]=Rw, [Pi/(NPi+Pi)]=Ri, [Rw/Ri]= Relative Fold Change in R (MFR). Summing the non-polysomal and polysomal signals (NP+P) from single replicates, we also accounted for the mean fold change in total mRNA (MFT). Affymetrix returned a probe signal output value for each of 22,625 genes. Each gene was tagged as being statistically "present" or "absent" for each of 4 samples (Pw, NPw, Pi, and NPi). Signals flagged as "absent" by Affymetrix in 3 or more of the 4 samples for a single replicate were removed. This analysis was performed in three biological replicates. Any mRNAs that did not pass the "present"/"absent" test in all three replicates were removed from the data set, leaving a representative group of 13,372 mRNAs. To derive mRNAs translationally regulated by IFE-1

only, mRNAs with an MFR greater than 1.5 fold and an MFR-S.D.>1 were considered. This yielded a prioritized list of 77 mRNAs with decreased translational efficiency in the absence of IFE-1. mRNAs were identified by cross-referencing information provided by Affymetrix with WormBase and GeneBank online resources.

Western blot analysis and anti-Flag batch binding Western blots were performed using anti-actin antibody produced in rabbit (Sigma A2066), anti-PGL-1 affinity purified from rabbit, and anti-FLAG (DYKDDDDK) from rabbit (Thermo Scientific) as described previously(Contreras et al., 2008). 100ug of protein lysates isolated from whole worms was resolved on an 8% polyacrylamide gel (29:1) at 70-100 V. Protein transfer was conducted overnight at 70 mA. Blot imaging was performed using ECL+ Detection Sytem (GE Health Care) on a Typhoon 9410 with ImageQuant TL 2003 imaging software. Flag capture by batch binding was done with ANTI-FLAG M2-Agarose (Sigma A2220). Whole worm lysates ground in CL+ buffer were incubated on ANTI-FLAG resin at 4°C for 3hours. Resin was washed under three separate conditions: 1) 1x TBS 2) 1xTBS +0.5 M NaCl 3) 1xTBS + 0.2% Tween20. Protein was eluted with 0.1M glycine pH 3.5.

MosSCI injection plasmid construction. DONR Flag::IFE-1 and DONR Flag::IFE-5 plasmid construction. PCR was performed on Flag::*ife*-1dx and Flag::*ife*-5dx plasmids using gateway primers GWFlag-Start, GWFlg-ife-1a, and GWFlg-ife-5a with Expand Long Template PCR amplification (Roche). These products were added to BP reactions as per the manufacturer's instructions, creating the pDONR 221 *flg ife-1* and pDONR 221 *flg ife-5* plasmids.

Destination Flag::ife-1 and Flag::ife-5 plasmid construction. pCFJ212 Destination vector, pCM5.37 *unc-54* DONR vector, *pie-1* promoter PODNRP4P1R (pCG142) vector were combined with the pDONR 221 *flg ife-1* and pDONR 221 *flg ife-5* plasmids om the LR clonase recombination reaction as per Invitrogen's "Experienced Users Guide" instructions. This combines the plasmids into a final destination vector containing all three elements added: 1) the *pie-1* promoter. 2) the *Flag:ife-1* or *Flag:ife-5* sequence. 3) the *unc-54* 3'UTR. These destination vectors were defined as *ife-1* pCFJ212 Chromosome IV and *ife-5* pCFJ212 Chromosome IV. Each destination vector contained *mos* transposon recombination sites that allow for site-directed recombination onto *C. elegans* exon IV.

Injection plasmid miniprep. Destination plasmids were transformed into TOP10 cells. Clones were picked and grown in 2mL of LB (100ug/uL amp). 1mL was centrifuged at 8k rpm for 1min and pellet was collected. Pellet was resuspended in Buffer P1 (250uL of 50mM Tris-HCl pH 8, 10mM EDTA). 250uL of Buffer P2 (0.2M NaOH, 1% SDS) was subsequently added. The sample was then suspended in 350uL of Buffer N3 (4M Guanidine Hydrochloride, 0.5M Potassium Acetate pH4.2). This was centrifuged for 10min at 12k rpm. Supernatant was spun through a filter (EconoSpin Spin Column for DNA, 1920-250) for 1min at 12k, washed with 1x Buffer PE (25mM NaCl, 2.5mM Trish-HCl pH7.5, in EtOH), and spun an additional time to dry. DNA was eluted in 50uL H2O in a 1min 12k rpm spin.

Microinjection protocol. Purified destination plasmids *ife-1* pCFJ212 Chromosome IV and *ife-5* pCFJ212 Chromosome IV were injected into the EG6703 strain with *mos-1* transposase plasmid (pCFJ601) and co-injected negative selection markers (*Prab-3::mCHerry::unc-54UTR* (pGH8), *Pmyo-2::mCherry::unc-54UTR* (pCFJ90) and *Pmyo-3::mCherry::unc-54UTR* (pCFJ104)) at both high and low concentrations recommended by the Jorgensen Lab (Frokjaer-

Jensen et al., 2012; Frokjaer-Jensen et al., 2008). The high concentration injection mix consisted of: pCFJ601 (50 ng/uL), pGH8 (5-10 ng/uL), pCFJ90 (2-2.5 ng/uL), pCFJ104 (5-10 ng/uL) and destination vector (10-50 ng/uL). The Jorgensen Lab protocol was followed with the exception that we omitted the negative selection plasmid pMA122 from the injection cocktail. This plasmid expresses *peel-1* which kills worms at an elevated temperature. After initial injection trials, it was evident by the few visible markers for extrachromosomal material, that the highly stringent negative selection plasmid (*peel-1*) was not necessary.

REFERENCES

Akkaraju, G.R., Hansen, L.J., and Jagus, R. (1991). Increase in eukaryotic initiation factor 2B activity following fertilization reflects changes in redox potential. J Biol Chem *266*, 24451-24459.

Allende, C.C., Hinrichs, M.V., Santos, E., and Allende, J.E. (1988). Oncogenic ras protein induces meiotic maturation of amphibian oocytes in the presence of protein synthesis inhibitors. FEBS Lett *234*, 426-430.

Alves, V.S., Motta, F.L., Roffe, M., Delamano, A., Pesquero, J.B., and Castilho, B.A. (2009). GCN2 activation and eIF2alpha phosphorylation in the maturation of mouse oocytes. Biochem Biophys Res Commun *378*, 41-44.

Amiri, A., Keiper, B.D., Kawasaki, I., Fan, Y., Kohara, Y., Rhoads, R.E., and Strome, S. (2001). An isoform of eIF4E is a component of germ granules and is required for spermatogenesis in C. elegans. Development *128*, 3899-3912.

Archer, S.K., Shirokikh, N.E., Hallwirth, C.V., Beilharz, T.H., and Preiss, T. (2015). "Probing the closed-loop model of mRNA translation in living cells". RNA Biol *12*, 248-254.

Austin, J., and Kimble, J. (1987). glp-1 is required in the germ line for regulation of the decision between mitosis and meiosis in C. elegans. Cell *51*, 589-599.

Badura, M., Braunstein, S., Zavadil, J., and Schneider, R.J. (2012). DNA damage and eIF4G1 in breast cancer cells reprogram translation for survival and DNA repair mRNAs. Proc Natl Acad Sci U S A *109*, 18767-18772.

Baker, C.C., and Fuller, M.T. (2007). Translational control of meiotic cell cycle progression and spermatid differentiation in male germ cells by a novel eIF4G homolog. Development *134*, 2863-2869.

Barnard, D.C., Cao, Q., and Richter, J.D. (2005). Differential phosphorylation controls Maskin association with eukaryotic translation initiation factor 4E and localization on the mitotic apparatus. Mol Cell Biol *25*, 7605-7615.

Bauer, C., Brass, N., Diesinger, I., Kayser, K., Grasser, F.A., and Meese, E. (2002). Overexpression of the eukaryotic translation initiation factor 4G (eIF4G-1) in squamous cell lung carcinoma. Int J Cancer *98*, 181-185.

Berry, L.W., Westlund, B., and Schedl, T. (1997). Germ-line tumor formation caused by activation of glp-1, a Caenorhabditis elegans member of the Notch family of receptors. Development *124*, 925-936.

Boag, P.R., Atalay, A., Robida, S., Reinke, V., and Blackwell, T.K. (2008). Protection of specific maternal messenger RNAs by the P body protein CGH-1 (Dhh1/RCK) during Caenorhabditis elegans oogenesis. J Cell Biol *182*, 543-557.

Borden, K.L. (2011). Targeting the oncogene eIF4E in cancer: From the bench to clinical trials. Clin Invest Med *34*, E315.

Braunstein, S., Karpisheva, K., Pola, C., Goldberg, J., Hochman, T., Yee, H., Cangiarella, J., Arju, R., Formenti, S.C., and Schneider, R.J. (2007). A hypoxia-controlled cap-dependent to cap-independent translation switch in breast cancer. Mol Cell *28*, 501-512.

Browning, K.S. (1996). The plant translational apparatus. Plant Mol Biol *32*, 107-144.

Bushell, M., McKendrick, L., Janicke, R.U., Clemens, M.J., and Morley, S.J. (1999). Caspase-3 is necessary and sufficient for cleavage of protein synthesis eukaryotic initiation factor 4G during apoptosis. FEBS Lett *451*, 332-336.

Cao, Q., and Richter, J.D. (2002). Dissolution of the maskin-eIF4E complex by cytoplasmic polyadenylation and poly(A)-binding protein controls cyclin B1 mRNA translation and oocyte maturation. EMBO J *21*, 3852-3862.

Carvallo, P., Mateu, M.G., Sierra, J.M., and Allende, J.E. (1988). Characterization of protein synthesis initiation factor 2 from Xenopus laevis oocytes. Biochimie *70*, 237-243.

Castro, A., Peter, M., Magnaghi-Jaulin, L., Vigneron, S., Galas, S., Lorca, T., and Labbe, J.C. (2001). Cyclin B/cdc2 induces c-Mos stability by direct phosphorylation in Xenopus oocytes. Mol Biol Cell *12*, 2660-2671.

Cha, D.S., Datla, U.S., Hollis, S.E., Kimble, J., and Lee, M.H. (2012). The Ras-ERK MAPK regulatory network controls dedifferentiation in Caenorhabditis elegans germline. Biochim Biophys Acta *1823*, 1847-1855.

Chappell, V.A., Busada, J.T., Keiper, B.D., and Geyer, C.B. (2013). Translational activation of developmental messenger RNAs during neonatal mouse testis development. Biol Reprod *89*, 61.

Cheng, H., Govindan, J.A., and Greenstein, D. (2008). Regulated trafficking of the MSP/Eph receptor during oocyte meiotic maturation in C. elegans. Curr Biol *18*, 705-714.

Ciosk, R., DePalma, M., and Priess, J.R. (2006). Translational regulators maintain totipotency in the Caenorhabditis elegans germline. Science *311*, 851-853.

Collier, B., Gorgoni, B., Loveridge, C., Cooke, H.J., and Gray, N.K. (2005). The DAZL family proteins are PABP-binding proteins that regulate translation in germ cells. EMBO J *24*, 2656-2666.

Contreras, V., Friday, A.J., Morrison, J.K., Hao, E., and Keiper, B.D. (2011). Cap-independent translation promotes C. elegans germ cell apoptosis through Apaf-1/CED-4 in a caspase-dependent mechanism. PLoS ONE *6*, e24444.

Contreras, V., Richardson, M.A., Hao, E., and Keiper, B.D. (2008). Depletion of the cap-associated isoform of translation factor eIF4G induces germline apoptosis in C. elegans. Cell Death Differ *15*, 1232-1242.

Culjkovic, B., Topisirovic, I., and Borden, K.L. (2007). Controlling gene expression through RNA regulons: the role of the eukaryotic translation initiation factor eIF4E. Cell Cycle *6*, 65-69.

Dahanukar, A., Walker, J.A., and Wharton, R.P. (1999). Smaug, a novel RNA-binding protein that operates a translational switch in *Drosophila*. Mol Cell *4*, 209-218.

Datla, U.S., Scovill, N.C., Brokamp, A.J., Kim, E., Asch, A.S., and Lee, M.H. (2014). Role of PUF-8/PUF protein in stem cell control, sperm-oocyte decision and cell fate reprogramming. J Cell Physiol *229*, 1306-1311.

Datta, S.R., Katsov, A., Hu, L., Petros, A., Fesik, S.W., Yaffe, M.B., and Greenberg, M.E. (2000). 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Mol Cell *6*, 41-51.

De Benedetti, A., and Graff, J.R. (2004). eIF-4E expression and its role in malignancies and metastases. Oncogene *23*, 3189-3199.

De Benedetti, A., and Rhoads, R.E. (1990). Overexpression of eukaryotic protein synthesis initiation factor 4E in HeLa cells results in aberrant growth and morphology. Proc Natl Acad Sci USA *87*, 8212-8216.

Derry, M.C., Yanagiya, A., Martineau, Y., and Sonenberg, N. (2006). Regulation of poly(A)-binding protein through PABP-interacting proteins. Cold Spring Harb Symp Quant Biol *71*, 537-543.

Detwiler, M.R., Reuben, M., Li, X., Rogers, E., and Lin, R. (2001). Two zinc finger proteins, OMA-1 and OMA-2, are redundantly required for oocyte maturation in C. elegans. Dev Cell *1*, 187-199.

Dinkova, T.D., Keiper, B.D., Korneeva, N.L., Aamodt, E.J., and Rhoads, R.E. (2005). Translation of a small subset of Caenorhabditis elegans mRNAs is dependent on a specific eukaryotic translation initiation factor 4E isoform. Mol Cell Biol *25*, 100-113.

Dworkin, M.B., and Dworkin-Rastl, E. (1990). Functions of maternal mRNA in early development. Molecular Reproduction and Development *26*, 261-297.

Eberhart, C.G., Maines, J.Z., and Wasserman, S.A. (1996). Meiotic cell cycle requirement for a fly homologue of human Deleted in Azoospermia. Nature *381*, 783-785.

Eckmann, C.R., Crittenden, S.L., Suh, N., and Kimble, J. (2004). GLD-3 and control of the mitosis/meiosis decision in the germline of Caenorhabditis elegans. Genetics *168*, 147-160.

Eddy, E.M. (1974). Fine structural observations on the form and distribution of nuage in germ cells of the rat. Anat Rec *178*, 731-757.

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

Forbes, A., and Lehmann, R. (1998). Nanos and Pumilio have critical roles in the development and function of *Drosophila* germline stem cells. Development *125*, 679-690.

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

Franklin-Dumont, T.M., Chatterjee, C., Wasserman, S.A., and Dinardo, S. (2007). A novel eIF4G homolog, Off-schedule, couples translational control to meiosis and differentiation in Drosophila spermatocytes. Development *134*, 2851-2861.

Friday, A.J., Morrison, J.K., and Keiper, B.D. (2015). Spatial and Temporal Translational Control of Germ Cell mRNAs by an eIF4E Isoform, IFE-1. Journal of Cell Science *Under Review*.

Frokjaer-Jensen, C., Davis, M.W., Ailion, M., and Jorgensen, E.M. (2012). Improved Mos1-mediated transgenesis in C. elegans. Nat Methods *9*, 117-118.

Frokjaer-Jensen, C., Davis, M.W., Hopkins, C.E., Newman, B.J., Thummel, J.M., Olesen, S.P., Grunnet, M., and Jorgensen, E.M. (2008). Single-copy insertion of transgenes in Caenorhabditis elegans. Nat Genet *40*, 1375-1383.

Fukuyo, A., In, Y., Ishida, T., and Tomoo, K. (2011). Structural scaffold for eIF4E binding selectivity of 4E-BP isoforms: crystal structure of eIF4E binding region of 4E-BP2 and its comparison with that of 4E-BP1. J Pept Sci *17*, 650-657.

Furic, L., Rong, L., Larsson, O., Koumakpayi, I.H., Yoshida, K., Brueschke, A., Petroulakis, E., Robichaud, N., Pollak, M., Gaboury, L.A., *et al.* (2010). eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression. Proc Natl Acad Sci U S A *107*, 14134-14139.

Gallo, C.M., Wang, J.T., Motegi, F., and Seydoux, G. (2010). Cytoplasmic partitioning of P granule components is not required to specify the germline in C. elegans. Science *330*, 1685-1689.

Gebauer, F., and Hentze, M.W. (2004). Molecular mechanisms of translational control. Nat Rev Mol Cell Biol *5*, 827-835.

Gingras, A.-C., Raught, B., and Sonenberg, N. (1999). eIF4 initiation factors: Effectors of mRNA recruitment to ribosomes and regulators of translation. Annual Reviews in Biochemistry *68*, 913-963.

Goehring, N.W., Hoege, C., Grill, S.W., and Hyman, A.A. (2011). PAR proteins diffuse freely across the anterior-posterior boundary in polarized C. elegans embryos. J Cell Biol *193*, 583-594.

Goldstein, B., and Macara, I.G. (2007). The PAR proteins: fundamental players in animal cell polarization. Dev Cell *13*, 609-622.

Goodwin, E.B., and Evans, T.C. (1997). Translational control of development in *C. elegans*. Semin Cell Dev Biol *8*, 551-559.

Goodwin, E.B., Okkema, P.G., Evans, T.C., and Kimble, J. (1993). Translational regulation of tra-2 by its 3' untranslated region controls sexual identity in C. elegans. Cell *75*, 329-339.

Graber, T.E., and Holcik, M. (2007). Cap-independent regulation of gene expression in apoptosis. Mol Biosyst *3*, 825-834.

Graff, J.R., Konicek, B.W., Carter, J.H., and Marcusson, E.G. (2008). Targeting the eukaryotic translation initiation factor 4E for cancer therapy. Cancer Res *68*, 631-634.

Griffin, E.E., Odde, D.J., and Seydoux, G. (2011). Regulation of the MEX-5 gradient by a spatially segregated kinase/phosphatase cycle. Cell *146*, 955-968.

Groisman, I., Huang, Y.S., Mendez, R., Cao, Q., Theurkauf, W., and Richter, J.D. (2000). CPEB, maskin, and cyclin B1 mRNA at the mitotic apparatus: implications for local translational control of cell division. Cell *103*, 435-447.

Gumienny, T.L., Lambie, E., Hartwieg, E., Horvitz, H.R., and Hengartner, M.O. (1999). Genetic control of programmed cell death in the Caenorhabditis elegans hermaphrodite germline. Development *126*, 1011-1022.

Hansen, D., Hubbard, E.J., and Schedl, T. (2004a). Multi-pathway control of the proliferation versus meiotic development decision in the Caenorhabditis elegans germline. Dev Biol *268*, 342-357.

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

Harris, T.E., Chi, A., Shabanowitz, J., Hunt, D.F., Rhoads, R.E., and Lawrence, J.C. (2006). mTOR-dependent stimulation of the association of eIF4G and eIF3 by insulin. EMBO J.

Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. Genes Dev *18*, 1926-1945.

Henderson, M.A., Cronland, E., Dunkelbarger, S., Contreras, V., Strome, S., and Keiper, B.D. (2009). A germline-specific isoform of eIF4E (IFE-1) is required for efficient translation of stored mRNAs and maturation of both oocytes and sperm. J Cell Sci *122*, 1529-1539.

Hentze, M.W. (1997). eIF4G: a multipurpose ribosome adaptor? Science *275*, 500-501.

Hernandez, G., Altmann, M., Sierra, J.M., Urlaub, H., del Corral, R.D., Schwartz, P., and Rivera-Pomar, R. (2005). Functional analysis of seven genes encoding eight translation initiation factor 4E (eIF4E) isoforms in Drosophila. Mech Dev *122*, 529-543.

Hodgkin, J. (1986). Sex determination in the nematode C. elegans: analysis of tra-3 suppressors and characterization of fem genes. Genetics *114*, 15-52.

Holcik, M., and Sonenberg, N. (2005). Translational control in stress and apoptosis. Nat Rev Mol Cell Biol *6*, 318-327.

Hsu, Y.C., and Fuchs, E. (2012). A family business: stem cell progeny join the niche to regulate homeostasis. Nat Rev Mol Cell Biol *13*, 103-114.

Igreja, C., Peter, D., Weiler, C., and Izaurralde, E. (2014). 4E-BPs require non-canonical 4E-binding motifs and a lateral surface of eIF4E to repress translation. Nat Commun *5*, 4790.

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

Jankowska-Anyszka, M., Lamphear, B.J., Aamodt, E.J., Harrington, T., Darzynkiewicz, E., Stolarski, R., and Rhoads, R.E. (1998). Multiple isoforms of eukaryotic protein synthesis initiation factor 4E in *Caenorhabditis elegans* can distinguish between, mono- and trimethylated mRNA cap structures. J Biol Chem *273*, 10538-10542.

Jeong, J., Verheyden, J.M., and Kimble, J. (2011). Cyclin E and Cdk2 control GLD-1, the mitosis/meiosis decision, and germline stem cells in Caenorhabditis elegans. PLoS Genet *7*, e1001348.

Jones, A.R., Francis, R., and Schedl, T. (1996). GLD-1, a cytoplasmic protein essential for oocyte differentiation, shows stage- and sex-specific expression during Caenorhabditis elegans germline development. Dev Biol *180*, 165-183.

Joshi, B., Cameron, A., and Jagus, R. (2004). Characterization of mammalian eIF4E-family members. Eur J Biochem *271*, 2189-2203.

Kawasaki, I., Jeong, M.H., and Shim, Y.H. (2011). Regulation of sperm-specific proteins by IFE-1, a germline-specific homolog of eIF4E, in C. elegans. Mol Cells *31*, 191-197.

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

Keiper, B.D., Gan, W., and Rhoads, R.E. (1999). Protein synthesis initiation factor 4G. International Journal of Biochemistry & Cell Biology *31*, 37-41.

Keiper, B.D., Lamphear, B.J., Deshpande, A.M., Jankowska-Anyszka, M., Aamodt, E.J., Blumenthal, T., and Rhoads, R.E. (2000). Functional characterization of five eIF4E isoforms in Caenorhabditis elegans. J Biol Chem *275*, 10590-10596.

Keiper, B.D., and Rhoads, R.E. (1997). Cap-independent translation initiation in *Xenopus* oocytes. Nucleic Acids Res *25*, 395-403.

Keiper, B.D., and Rhoads, R.E. (1999). Translational recruitment of *Xenopus* maternal mRNAs in response to poly(A) elongation requires initiation factor eIF4G-1. Dev Biol *206*, 1-14.

Kelly, W.G., and Fire, A. (1998). Chromatin silencing and the maintenance of a functional germline in *Caenorhabditis elegans*. Development *125*, 2451-2456.

Kimble, J., and Crittenden, S. (2006). Control of Germline Stem Cells, Entry into Meiosis, and the Sperm/Oocyte Decision in Caenorhabditis elegans. Annu Rev Cell Dev Biol.

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

Ko, S.Y., Guo, H., Barengo, N., and Naora, H. (2009). Inhibition of ovarian cancer growth by a tumor-targeting peptide that binds eukaryotic translation initiation factor 4E. Clin Cancer Res *15*, 4336-4347.

Kouvaraki, M.A., Liakou, C., Paraschi, A., Dimas, K., Patsouris, E., Tseleni-Balafouta, S., Rassidakis, G.Z., and Moraitis, D. (2011). Activation of mTOR signaling in medullary and aggressive papillary thyroid carcinomas. Surgery *150*, 1258-1265.

Kraemer, B., Crittenden, S., Gallegos, M., Moulder, G., Barstead, R., Kimble, J., and Wickens, M. (1999). NANOS-3 and FBF proteins physically interact to control the sperm-oocyte switch in *Caenorhabditis elegans*. Curr Biol *9*, 1009-1018.

Kyrpides, N.C., and Woese, C.R. (1998). Universally conserved translation initiation factors. Proc Natl Acad Sci U S A *95*, 224-228.

Lamphear, B.J., Kirchweger, R., Skern, T., and Rhoads, R.E. (1995). Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. Implications for cap-dependent and cap-independent translational initiation. J Biol Chem *270*, 21975-21983.

Lee, M.H., Hook, B., Pan, G., Kershner, A.M., Merritt, C., Seydoux, G., Thomson, J.A., Wickens, M., and Kimble, J. (2007). Conserved regulation of MAP kinase expression by PUF RNA-binding proteins. PLoS Genet *3*, e233.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell *91*, 479-489.

Li, Y., and Zhao, X. (2014). Concise review: Fragile X proteins in stem cell maintenance and differentiation. Stem Cells *32*, 1724-1733.

Liu, W., Jankowska-Anyszka, M., Piecyk, K., Dickson, L., Wallace, A., Niedzwiecka, A., Stepinski, J., Stolarski, R., Darzynkiewicz, E., Kieft, J., *et al.* (2011). Structural basis for nematode eIF4E binding an m(2,2,7)G-Cap and its implications for translation initiation. Nucleic Acids Research *39*, 8820-8832.

Liu, Y., Kuersten, S., Huang, T., Larsen, A., MacMorris, M., and Blumenthal, T. (2003). An uncapped RNA suggests a model for Caenorhabditis elegans polycistronic pre-mRNA processing. RNA *9*, 677-687.

Lodish, H.F. (1976). Translational control of protein synthesis. Annual Reviews in Biochemistry *45*, 39-72.

Long, X., Muller, F., Avruch, J., Spycher, C., Han, Z.S., Rose, A.M., Hara, K., Maruki, Y., Yoshino, K., Oshiro, N., *et al.* (2004). TOR action in mammalian cells and in Caenorhabditis elegans

TOR deficiency in C. elegans causes developmental arrest and intestinal atrophy by inhibition of mRNA translation

Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Curr Top Microbiol Immunol *279*, 115-138.

Lu, C., Srayko, M., and Mains, P.E. (2004). The Caenorhabditis elegans microtubule-severing complex MEI-1/MEI-2 katanin interacts differently with two superficially redundant beta-tubulin isotypes. Mol Biol Cell *15*, 142-150.

Macdonald, P.M., and Smibert, C.A. (1996). Translational regulation of maternal mRNAs. Current Opinions in Genetics and Development *6*, 403-407.

Marcotrigiano, J., Gingras, A.-C., Sonenberg, N., and Burley, S.K. (1997a). Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell *89*, 951-961.

Marcotrigiano, J., Gingras, A.C., Sonenberg, N., and Burley, S.K. (1997b). X-ray studies of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. Nucleic Acids Symp Ser, 8-11.

Marin, V.A., and Evans, T.C. (2003). Translational repression of a C. elegans Notch mRNA by the STAR/KH domain protein GLD-1. Development *130*, 2623-2632.

Marin, V.A., Evans, T.C., Barbee, S.A., and Lublin, A.L. (2003). Translational repression of a C. elegans Notch mRNA by the STAR/KH domain protein GLD-1; A novel function for the Sm proteins in germ granule localization during C. elegans embryogenesis. Development *130*, 2623-2632.

McCall, K. (1998). Requirement for DCP-1 Caspase During Drosophila Oogenesis. Science *279*, 230-234.

McCarter, J., Bartlett, B., Dang, T., and Schedl, T. (1999). On the control of oocyte meiotic maturation and ovulation in Caenorhabditis elegans. Dev Biol *205*, 111-128.

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

Mendez, R., Murthy, K.G., Ryan, K., Manley, J.L., and Richter, J.D. (2000). Phosphorylation of CPEB by Eg2 mediates the recruitment of CPSF into an active cytoplasmic polyadenylation complex. Mol Cell *6*, 1253-1259.

Mendez, R., and Richter, J.D. (2001). Translational control by CPEB: a means to the end. Nat Rev Mol Cell Biol *2*, 521-529.

Merritt, C., Rasoloson, D., Ko, D., and Seydoux, G. (2008). 3' UTRs are the primary regulators of gene expression in the C. elegans germline. Curr Biol *18*, 1476-1482.

Miller, M.A., Ruest, P.J., Kosinski, M., Hanks, S.K., and Greenstein, D. (2003). An Eph receptor sperm-sensing control mechanism for oocyte meiotic maturation in Caenorhabditis elegans. Genes Dev *17*, 187-200.

Minshall, N., Reiter, M.H., Weil, D., and Standart, N. (2007). CPEB interacts with an ovary-specific eIF4E and 4E-T in early Xenopus oocytes. J Biol Chem *282*, 37389-37401.

Miyoshi, H., Dwyer, D.S., Keiper, B.D., Jankowska-Anyszka, M., Darzynkiewicz, E., and Rhoads, R.E. (2002). Discrimination between mono- and trimethylated cap structures by two isoforms of *Caenorhabditis elegans* eIF4E. EMBO J *21*, 4680-4690.

Mori, C., Nakamura, N., Kimura, S., Irie, H., Takigawa, T., and Shiota, K. (1995). Programmed cell death in the interdigital tissue of the fetal mouse limb is apoptosis with DNA fragmentation. Anat Rec *242*, 103-110.

Morrison, J.K., Friday, A.J., and Keiper, B.D. (2014). Induction of cap-independent BiP (*hsp-3*) and Bcl-2 (*ced-9*) translation in response to eIF4G (IFG-1) depletion in *C. elegans.* Translation *2*.

Mosquera, L., Forristall, C., Zhou, Y., and King, M.L. (1993). A mRNA localized to the vegetal cortex of Xenopus oocytes encodes a protein with a nanos-like zinc finger domain. Development *117*, 377-386.

Munro, E., and Bowerman, B. (2009). Cellular symmetry breaking during Caenorhabditis elegans development. Cold Spring Harb Perspect Biol *1*, a003400.

Munro, E., Nance, J., and Priess, J.R. (2004). Cortical flows powered by asymmetrical contraction transport PAR proteins to establish and maintain anterior-posterior polarity in the early C. elegans embryo. Dev Cell *7*, 413-424.

Nakamura, A., Sato, K., and Hanyu-Nakamura, K. (2004). Drosophila cup is an eIF4E binding protein that associates with Bruno and regulates oskar mRNA translation in oogenesis. Dev Cell *6*, 69-78.

Nance, J. (2005). PAR proteins and the establishment of cell polarity during C. elegans development. BioEssays *27*, 126-135.

Nevins, T.A., Harder, Z.M., Korneluk, R.G., and Holcik, M. (2003). Distinct regulation of internal ribosome entry site-mediated translation following cellular stress is mediated by apoptotic fragments of eIF4G translation initiation factor family members eIF4GI and p97/DAP5/NAT1. J Biol Chem *278*, 3572-3579.

Newport, J.W., and Kirschner, M.W. (1984). Regulation of the cell cycle during early Xenopus development. Cell *37*, 731-742.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., *et al.* (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature *376*, 37-43.

Nousch, M., and Eckmann, C.R. (2013). Translational control in the Caenorhabditis elegans germ line. Adv Exp Med Biol *757*, 205-247.

Onohara, Y., and Yokota, S. (2012). Expression of DDX25 in nuage components of mammalian spermatogenic cells: immunofluorescence and immunoelectron microscopic study. Histochem Cell Biol *137*, 37-51.

Parisi, M., and Lin, H. (2000). Translational repression: a duet of Nanos and Pumilio. Curr Biol *10*, R81-83.

Patrick, T.D., Lewer, C.E., and Pain, V.M. (1989). Preparation and characterization of cell-free protein synthesis systems from oocytes and eggs of Xenopus laevis. Development *106*, 1-9.

Pei, H., Zhang, L., Luo, K., Qin, Y., Chesi, M., Fei, F., Bergsagel, P.L., Wang, L., You, Z., and Lou, Z. (2011). MMSET regulates histone H4K20 methylation and 53BP1 accumulation at DNA damage sites. Nature *470*, 124-128.

Pelletier, J., and Sonenberg, N. (1988). Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature *334*, 320-325.

Pestova, T.V., Hellen, C.U., and Shatsky, I.N. (1996). Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. Mol Cell Biol *16*, 6859-6869.

Pique, M., Lopez, J.M., Foissac, S., Guigo, R., and Mendez, R. (2008). A combinatorial code for CPE-mediated translational control. Cell *132*, 434-448.

Preiss, T. (2000). The End in Sight: Poly(A), Translation and mRNA Stability in Eukaryotes. Madame Curie Bioscience Database.

Prevot, D., Darlix, J.L., and Ohlmann, T. (2003). Conducting the initiation of protein synthesis: the role of eIF4G. Biol Cell *95*, 141-156.

Proud, C.G. (2006). Regulation of protein synthesis by insulin. Biochem Soc Trans *34*, 213-216.

Reese, K.J., Dunn, M.A., Waddle, J.A., and Seydoux, G. (2000). Asymmetric segregation of PIE-1 in C. elegans is mediated by two complementary mechanisms that act through separate PIE-1 protein domains. Mol Cell *6*, 445-455.

Rhoads, R.E. (1991). Protein synthesis, cell growth, and oncogenesis. Current Opinions in Cell Biology *3*, 1019-1024.

Rinker-Schaeffer, C.W., Graff, J.R., DeBenedetti, A., Zimmer, S.G., and Rhoads, R.E. (1993). Decreasing the level of translation initiation factor 4E with antisense RNA causes reversal of ras-mediated transformation and tumorigenesis of cloned rat embryo fibroblasts. Int J Cancer *55*, 841-847.

Robalino, J., Joshi, B., Fahrenkrug, S.C., and Jagus, R. (2004). Two zebrafish eIF4E family members are differentially expressed and functionally divergent. J Biol Chem *279*, 10532-10541.

Roy, L.M., Singh, B., Gautier, J., Arlinghaus, R.B., Nordeen, S.K., and Maller, J.L. (1990). The cyclin B2 component of MPF is a substrate for the c-*mos*^{xe} proto-oncogene product. Cell *61*, 825-831.

Ruggiu, M., Speed, R., Taggart, M., McKay, S.J., Kilanowski, F., Saunders, P., Dorin, J., and Cooke, H.J. (1997). The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis. Nature *389*, 73-77.

Saccomanno, L., Loushin, C., Jan, E., Punkay, E., Artzt, K., and Goodwin, E.B. (1999). The STAR protein QKI-6 is a translational repressor. Proc Natl Acad Sci (USA) *96*, 12605-12610.

Saldi, T., Wilusz, C., MacMorris, M., and Blumenthal, T. (2007). Functional redundancy of worm spliceosomal proteins U1A and U2B". Proc Natl Acad Sci U S A *104*, 9753-9757.

Salehi, Z., and Mashayekhi, F. (2006). Expression of the eukaryotic translation initiation factor 4E (eIF4E) and 4E-BP1 in esophageal cancer. Clin Biochem *39*, 404-409.

Sattler, M., Liang, H., Nettesheim, D., Meadows, R.P., Harlan, J.E., Eberstadt, M., Yoon, H.S., Shuker, S.B., Chang, B.S., Minn, A.J., *et al.* (1997). Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. Science *275*, 983-986.

Scheper, G.C., van der Knaap, M.S., and Proud, C.G. (2007). Translation matters: protein synthesis defects in inherited disease. Nat Rev Genet *8*, 711-723.

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

Schumacher, B., Hanazawa, M., Lee, M.H., Nayak, S., Volkmann, K., Hofmann, E.R., Hengartner, M., Schedl, T., and Gartner, A. (2005). Translational repression of C. elegans p53 by GLD-1 regulates DNA damage-induced apoptosis. Cell *120*, 357-368.

Sengupta, M.S., and Boag, P.R. (2012). Germ granules and the control of mRNA translation. IUBMB Life *64*, 586-594.

Sengupta, M.S., Low, W.Y., Patterson, J.R., Kim, H.M., Traven, A., Beilharz, T.H., Colaiacovo, M.P., Schisa, J.A., and Boag, P.R. (2013). ifet-1 is a broad-scale translational repressor required for normal P granule formation in C. elegans. J Cell Sci *126*, 850-859.

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

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

Shen, Q., He, B., Lu, N., Conradt, B., Grant, B.D., and Zhou, Z. (2013). Phagocytic receptor signaling regulates clathrin and epsin-mediated cytoskeletal remodeling during apoptotic cell engulfment in C. elegans. Development *140*, 3230-3243.

Shen, R., Weng, C., Yu, J., and Xie, T. (2009). eIF4A controls germline stem cell self-renewal by directly inhibiting BAM function in the Drosophila ovary. Proc Natl Acad Sci U S A *106*, 11623-11628.

Sherrill, K.W., Byrd, M.P., Van Eden, M.E., and Lloyd, R.E. (2004). BCL-2 translation is mediated via internal ribosome entry during cell stress. J Biol Chem *279*, 29066-29074.

Shyh-Chang, N., and Daley, G.Q. (2013). Lin28: primal regulator of growth and metabolism in stem cells. Cell Stem Cell *12*, 395-406.

Silvera, D., Arju, R., Darvishian, F., Levine, P.H., Zolfaghari, L., Goldberg, J., Hochman, T., Formenti, S.C., and Schneider, R.J. (2009). Essential role for eIF4GI overexpression in the pathogenesis of inflammatory breast cancer. Nat Cell Biol *11*, 903-908.

Silvera, D., and Schneider, R.J. (2009). Inflammatory breast cancer cells are constitutively adapted to hypoxia. Cell Cycle *8*, 3091-3096.

Singson, A., Hill, K.L., and L'Hernault, S.W. (1999). Sperm competition in the absence of fertilization in Caenorhabditis elegans. Genetics *152*, 201-208.

Sonenberg, N., and Gingras, A.-C. (1998). The mRNA 5' cap-binding protein eIF4E and control of cell growth. Current Opinions in Cell Biology *10*, 268-275.

Song, A., Labella, S., Korneeva, N.L., Keiper, B.D., Aamodt, E.J., Zetka, M., and Rhoads, R.E. (2010). A C. elegans eIF4E-family member upregulates translation at elevated temperatures of mRNAs encoding MSH-5 and other meiotic crossover proteins. J Cell Sci *123*, 2228-2237.

Song, Y., and Lu, B. (2011). Regulation of cell growth by Notch signaling and its differential requirement in normal vs. tumor-forming stem cells in Drosophila. Genes Dev *25*, 2644-2658.

Spassov, D., and Jurecic, R. (2003). The PUF Family of RNA-binding Proteins: Does Evolutionalrily Conserved Structure Equal Conserved Function? IUBMB Life *55*, 359-366.

Spike, C.A., Coetzee, D., Eichten, C., Wang, X., Hansen, D., and Greenstein, D.I. (2014). The TRIM-NHL Protein LIN-41 and the OMA RNA-Binding Proteins Antagonistically Control the Prophase-to-Metaphase Transition and Growth of Caenorhabditis elegans Oocytes. Genetics *2014*, 168831 (epub ahead of print).

Stebbins-Boaz, B., Cao, Q., de Moor, C.H., Mendez, R., and Richter, J.D. (1999). Maskin is a CPEB-associated factor that transiently interacts with elF-4E. Mol Cell *4*, 1017-1027.

Stebbins-Boaz, B., Hake, L.E., and Richter, J.D. (1996). CPEB controls the cytoplasmic polyadenylation of cyclin, Cdk2 and c-*mos* mRNAs and is necessary for oocyte maturation in Xenopus. EMBO J *15*, 2582-2592.

Styhler, S., Nakamura, A., Swan, A., Suter, B., and Lasko, P. (1998). *vasa* is required for Gurken accumulation in the oocyte, and is involved in oocyte differentiation and germline cyst development. Development *125*, 1569-1578.

Sun, F., Palmer, K., and Handel, M.A. (2010). Mutation of Eif4g3, encoding a eukaryotic translation initiation factor, causes male infertility and meiotic arrest of mouse spermatocytes. Development *137*, 1699-1707.

Syntichaki, P., Troulinaki, K., and Tavernarakis, N. (2007). eIF4E function in somatic cells modulates ageing in Caenorhabditis elegans. Nature *445*, 922-926.

Szalai, G., Krishnamurthy, R., and Hajnoczky, G. (1999). Apoptosis driven by IP(3)-linked mitochondrial calcium signals. EMBO J *18*, 6349-6361.

Tadros, W., and Lipshitz, H.D. (2005). Setting the stage for development: mRNA translation and stability during oocyte maturation and egg activation in Drosophila. Dev Dyn *232*, 593-608.

Tarun, S.Z., Jr., and Sachs, A.B. (1995). A common function for mRNA 5' and 3' ends in translation initiation in yeast. Genes Dev *9*, 2997-3007.

Tavernarakis, N. (2007). Protein synthesis and aging: eIF4E and the soma vs. germline distinction. Cell Cycle *6*, 1168-1171.

Tocchini, C., Keusch, J.J., Miller, S.B., Finger, S., Gut, H., Stadler, M.B., and Ciosk, R. (2014). The TRIM-NHL protein LIN-41 controls the onset of developmental plasticity in Caenorhabditis elegans. PLoS Genet *10*, e1004533 (epub ahead of print).

Tomancak, P., Guichet, A., Zavorszky, P., and Ephrussi, A. (1998). Oocyte polarity depends on regulation of gurken by Vasa. Development *125*, 1723-1732.

Tomek, W., and Wollenhaupt, K. (2012). The "closed loop model" in controlling mRNA translation during development. Anim Reprod Sci *134*, 2-8.

Updike, D.L., Hachey, S.J., Kreher, J., and Strome, S. (2011). P granules extend the nuclear pore complex environment in the C. elegans germ line. J Cell Biol *192*, 939-948.

Updike, D.L., Knutson, A.K., Egelhofer, T.A., Campbell, A.C., and Strome, S. (2014). Germ-granule components prevent somatic development in the C. elegans germline. Curr Biol *24*, 970-975.

Wakiyama, M., Saigoh, M., Shiokawa, K., and Miura, K. (1995). mRNA encoding the translation initiation factor eIF-4E is expressed early in Xenopus embryogenesis. FEBS Lett *360*, 191-193.

Wakiyama, M., Suzuki, A., Saigoh, M., Sakai, N., Miyoshi, H., Kojima, S., and Miura, K. (2001). Analysis of the isoform of Xenopus euakryotic translation initiation factor 4E. Biosci Biotechnol Biochem *65*, 232-235.

Wilhelm, J.E., Hilton, M., Amos, Q., and Henzel, W.J. (2003). Cup is an eIF4E binding protein required for both the translational repression of oskar and the recruitment of Barentsz. J Cell Biol *163*, 1197-1204.

Winkler, M.M., Nelson, E.M., Lashbrook, C., and Hershey, J.W.B. (1985). Multiple levels of regulation of protein synthesis at fertilization in sea urchin eggs. Dev Biol *107*, 290-300.

Wormington, M. (1993). Poly(A) and translation: developmental control. Current Opinions in Cell Biology *5*, 950-954.

Wright, J.E., and Ciosk, R. (2013). RNA-based regulation of pluripotency. Trends Genet *29*, 99-107.

Yoon, S., Kawasaki, I., and Shim, Y.H. (2012). CDC-25.1 controls the rate of germline mitotic cell cycle by counteracting WEE-1.3 and by positively regulating CDK-1 in Caenorhabditis elegans. Cell Cycle *11*, 1354-1363.

Zanin, E., Pacquelet, A., Scheckel, C., Ciosk, R., and Gotta, M. (2010). LARP-1 promotes oogenesis by repressing fem-3 in the C. elegans germline. J Cell Sci *123*, 2717-2724.

Zonies, S., Motegi, F., Hao, Y., and Seydoux, G. (2010). Symmetry breaking and polarization of the C. elegans zygote by the polarity protein PAR-2. Development *137*, 1669-1677.

Appendix A: Analysis of transgenic GFP::reporter strains following IFE-1 depletion

This Appendix represents a screening of transgenic fluorescent reporter worm strains with two aims. The first aim was to determine if IFE-1 is involved in early embryo polarity fates that involve PAR protein localization. The second aim was to screen a larger battery of reporter strains with an mRNA 3'UTR GFP transgenes for dependence on IFE-1 for expression.

Introduction

Potential role for IFE-1 in establishment of early embryonic polarity

Among the mRNAs that were found by TSAA to be IFE-1 dependent were several involved in establishing embryo polarity following fertilization. Therefore, we sought to determine if IFE-1 had a role in establishing early embryonic polarity through PAR protein segregation. Our TSAA suggested that IFE-1 initiates *spat-3* (E3 ubiquitin ligase subunit), *plk-1* (polo kinase), and *nos-3* (translational repressor) mRNAs. Each cognate protein is involved in the segregation of PAR (abnormal embryonic PARtitioning of cytoplasm) proteins into their proper anterior-posterior domains that are required for establishing early embryonic polarity. Following fertilization, establishment of polarity is an essential process that occurs in *C. elegans* during the 17 minutes prior to the first division (Munro and Bowerman, 2009). When polarity is disrupted, the embryos are non-viable and arrested in early divisions. Interestingly, such arrested embryos are prevalent among *ife-1* offspring, even at the permissive temperature, which allows sperm production and fertilization (Henderson et al., 2009). During the establishment of

polarity, various PAR proteins segregate to anterior and posterior cortical domains (Nance, 2005; Zonies et al.). The anterior PAR domain contains PAR-3, PAR-6, and PKC-3, while the posterior domain contains PAR-1 and PAR-2 (Munro et al., 2004; Nance, 2005). Through a mechanism that is not yet understood, these PAR proteins remain in their respective domains while diffusing laterally along the membrane (Goehring et al.; Goldstein and Macara, 2007). Once the PAR domains are established, they begin recruiting other proteins to either pole of the cell via affinity and phosphorylation events (Griffin et al.; Zonies et al.). One important protein localized by the PAR complex is MEX-5 (muscle excess protein). MEX-5 is localized it to the anterior cortex of the embryo, and its position is important for embryonic viability and differentiation of the somatic and germ line lineages (Schubert et al., 2000). MEX-5 segregation is dually important because it also serves as an RBP that likely recruits and localizes mRNAs as well as ribosomal/translation complexes in early embryogenesis (G. Seydoux, personal communication). Despite these many circumstantial links, data shown below demonstrate no IFE-1-dependence for PAR protein localization.

Regulation of germ line mRNAs by 3'UTR repression

Both germ line and embryonic development require *de novo* synthesis of essential proteins for cell proliferation, differentiation, maturation, and survival. During early oogenesis, stored mRNAs are utilized when transcription is largely silenced due to chromosomal condensation (Kelly and Fire, 1998). As these mRNAs become de-repressed, available ribosomes translate them to provide proteins necessary for development and maturation (Dworkin and Dworkin-Rastl, 1990; Goodwin and Evans, 1997; Macdonald and Smibert, 1996; Mendez and Richter, 2001; Wormington, 1993). The coordination of repression and de-repression of stored mRNAs relies, in part, on RBPs. There are defined instances where a

translation initiation factor is also directly involved in these regulatory mRNP complexes. Examples in various species are detailed in Chapter 5 (Figure 5.2). In this thesis, I have shown that a single eIF4E isoform (IFE-1) is able to preferentially recruit mRNAs as they are de-repressed in germ cells, using a degree of selectivity that requires sequence-specific recognition in the mRNA 3'UTR. The results under Aim 2 of this Appendix catalog other 3'UTR reporters that were tested upon *ife-1*(RNAi), but showed no consistent IFE-1-dependence.

Results and Relevance

The initial experiments were designed to determine if IFE-1 contributed to the localization of PAR proteins in the single cell embryo. To address this goal, we purchased a worm strain that stably expressed the transgenes *par-6::mcherry* and *par-2::gfp*. In these strains, we used RNAi to knockdown IFE-1 exactly as was done in Chapter 2 (see Figure 2.8). The *par-6::mCherry par-2::gfp* strain under control conditions showed the expected anterior localization of PAR-6::mCherry (Figure A.1A). The posterior localization of PAR-2::GFP was as not well defined, but was faintly visible in the single cell embryos. Following *ife-1*(RNAi) depletion, there was no substantial difference in PAR-6::mCherry or PAR-2::GPF localization. These data suggested that depletion of IFE-1 was not sufficient to disrupt the segregation of PAR proteins into their proper anterior and posterior cortical domains, even though IFE-1 may translationally regulate several genes that support proper partitioning of PAR proteins. A further interpretation of these findings is that the high incidence of embryonic arrest upon loss of IFE-1 may have nothing to do with dysregulation of PAR protein segregation.

Figure A.1

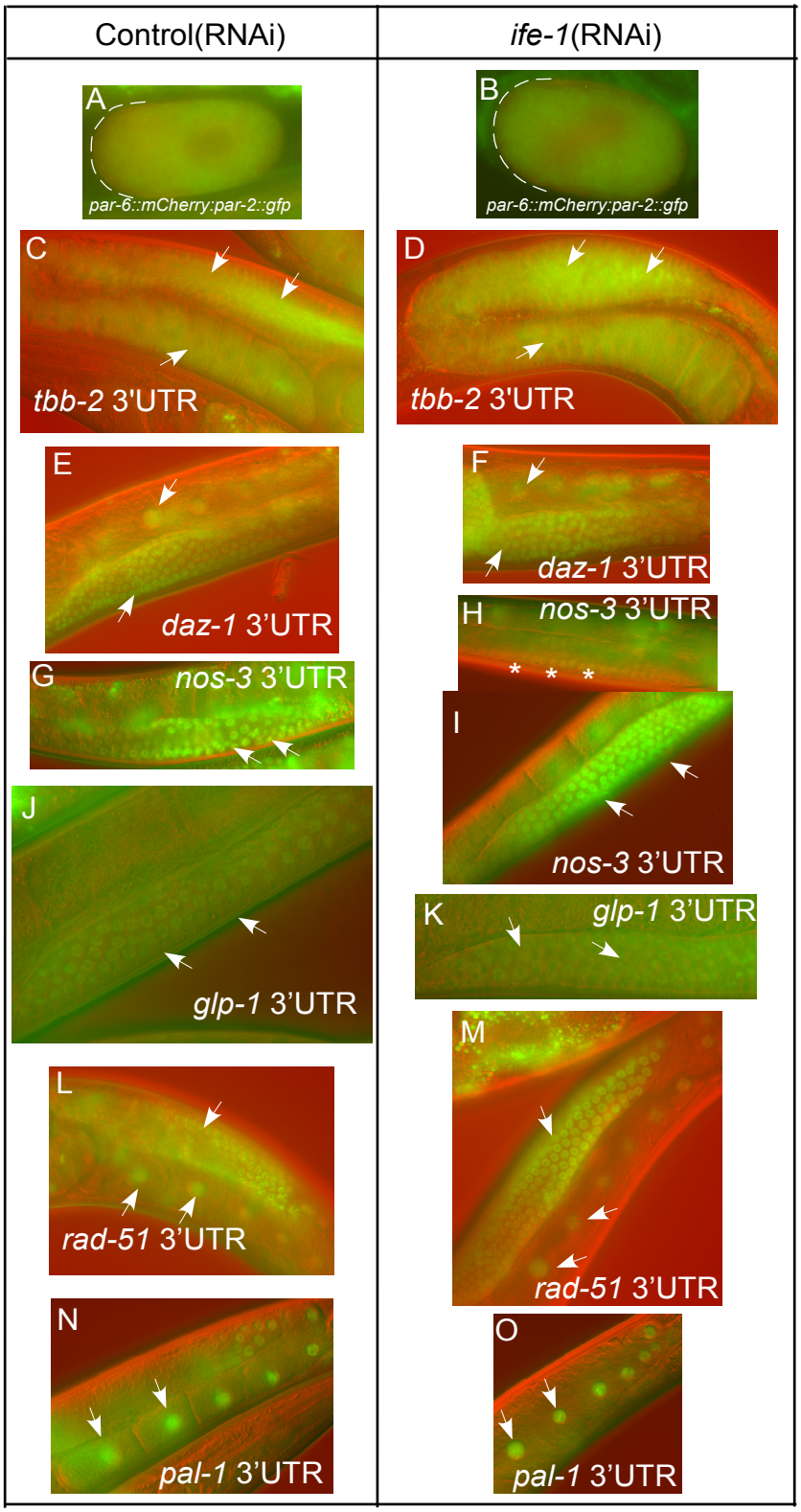

Control(RNAi)
ife-1(RNAi)
A
par-6::mCherry:par-2::gfp
B
par-6::mCherry:par-2::gfp
C
tbb-2 3'UTR
D
tbb-2 3'UTR
E
daz-1 3'UTR
F
daz-1 3'UTR
H
nos-3 3'UTR
G
nos-3 3'UTR
I
nos-3 3'UTR
J
glp-1 3'UTR
K
glp-1 3'UTR
L
rad-51 3'UTR
M
rad-51 3'UTR
N
pal-1 3'UTR
O
pal-1 3'UTR

Figure A.1. Analysis of transgenic GFP::reporter strains following IFE-1 depletion. Transgenic worms were grown at 25°C and fed control dsRNA or *ife-1* dsRNA on bacterial lawns. *par-6::mcherry:par-2::gfp* worms were dissected at the vulva to expose embryos and images were captured sequentially for GFP and mCherry fluorescence (n=15). Under both control (A) and *ife-1*(RNAi) (B) conditions, PAR-6::mCherry was visibly localized to the anterior domain of single cell embryos. Anterior PAR-6::mCherry was highlighted by white dashes. Microscopy of *in vivo* 3'UTR reporter constructs for *tbb-2::ORF* (C,D), *daz-1::H2B* (E,F), *nos-3::H2B* (G,H,I), *glp-1::H2B* (J,K), *rad-51::H2B* (L,M), and *pal-1::H2B* (N,O). Arrows indicate cells or regions in which reporter constructs were de-repressed and GFP was detected. In panel H, "*" denotes a region where *nos-3::H2B::gfp* expression was not detected. (n=20)

The second set of experiments here demonstrate a screening of transgenic worm strains with a 3'UTR reporter assay to determine if IFE-1 has a role in recruiting their cognate mRNAs for translation. For each strain I visually monitored the expression of GFP-fused transgene mRNAs for changes when IFE-1 was depleted by RNAi. Many of these mRNAs are known to become translationally repressed during some phase of oogenesis. As the reporter 3' UTR becomes de-repressed, GFP should be expressed in appropriate staged oocytes. If regulation of these mRNAs is mediated solely by de-repression of 3' UTR binding, GFP expression will be unaffected by the loss of any one eIF4E isoform. However, if only IFE-1 is able to recruit these mRNAs as they become de-repressed, GFP expression from the reporter will not occur. As seen in Chapter 2 (Figure 2.8), some mRNAs demonstrated clear preferential recruitment by IFE-1 for translation upon de-repression (e.g. *gld-1, pos-1*). We found that these examples were exceptional cases when compared to the panel of 10 transgenic 3'UTR reporter strains assayed in our screen (Figure A.1, Table A.1).

Two tubulin (*tbb-2* 3'UTR) reporter strains were assayed. Because *tbb-2* is not a translationally repressed mRNA in germ cells (Merritt et al., 2008), these were used as control reporters for general mRNA translation levels during early and late oogenesis. The first reporter control contained a histone H2B fusion to GFP under the control of the *tbb-2* 3'UTR and was already shown in Figure 2.8E. The second *tbb-2* contained the tubulin ORF directly fused to GFP, and controlled by its own 3'UTR (Figure A.1C, D). Expression from this strain was cytoplasmic throughout the gonad, and also indicated that the assay was altered by nuclear vs. cytoplasmic localization of the product. Neither *tbb-2* reporter showed any loss of expression upon IFE-1 depletion, indicating that *tbb-2* mRNA does not rely on IFE-1 for translational regulation. Several other 3'UTR reporter strains for mRNAs known to be translationally

Table A.1

3'UTR mRNA	Description	GFP +/-	
		Control RNAi	*ife-1* RNAi
daz-1	RBP, promotes oocyte meiosis	++	++
gld-1	RBP, promotes oocyte meiosis, suppresses mitosis	+	-
glp-1	promotes mitosis and supresses meiosis	+	+
nos-3	NANOS homolog; promotes sperm to oocyte switch	+	+/-
pal-1	embryonic cell polarity determination	++	++
pgl-1	constitutive P granule component	+	++
pos-1	embryonic cell polarity determination	+	-
rad-51	meiotic recombination	++	++
tbb-2 H2B	beta-tubulin	+	+
tbb-2 ORF	beta-tubulin	+	+

Table A.1. Screening of *in vivo* 3'UTR reporter constructs. Ten transgenic *gfp::3'UTR* worm strains were screened for dependence on IFE-1-dependence for expression in the germ line. Worms were treated with control and *ife-1*(RNAi). "+" indicated that GFP was detected and the appropriate expression pattern was observed. "-" indicated that there was a significant decrease in GFP expression. "++" signified there was an increase in GFP detected. "+/-" was reported in one case where four out of five worms showed full GFP expression, while one had no detectable GFP. (n=20)

controlled in the germ line were assayed as well (*daz-1, nos-3, glp-1, rad-51, pal-1*) (Figure A.1 E-O). With the exception of the *nos-3* 3'UTR reporter strain, there was no discernable difference in GFP expression levels or expression patterns when IFE-1 was depleted. The *nos-3* 3'UTR strain would inconsistently (roughly 1 out of 5 worms) show a loss of GFP expression following IFE-1 knockdown, even within a single RNAi experiment (Figure A.1 H vs I). It is not likely that the variable loss of GFP::*nos-3* 3'UTR demonstrated true IFE-1-dependence. It is possible, however, that the transgene was unstable in some subpopulation of worms in this strain. It is also conceivable that the *nos-3* transgene had a propensity to be silenced, which may happen after many generations of growth, though it rarely shows this type of heterogeneity in the population.

The *glp-1* and *pal-1* mRNAs have previously been shown by our lab to be translationally regulated by IFE-1 (Henderson et al., 2009). These findings were obtained by polysome resolution, and both mRNAs show rather complex distributions among mRNPs and polysomes of various sizes. Neither mRNA becomes efficiently translated, even when all IFEs are present. In our *gfp::3'UTR* assays, neither the *glp-1* nor the *pal-1* reporter showed a demonstrable dependence upon IFE-1 for translation. It is noteworthy that *gfp::glp-1* 3'UTR recapitulated the known expression pattern of endogenous *glp-1* mRNA; that is, active translation in early germ cells followed by stable repression in subsequent meiotic stages. It is possible that other IFEs present in the germ line supported modest translation of *glp-1* reporter sufficiently that any noticeable to changes in GFP expression were masked. On the other hand, the *gfp::pal-1, gfp::daz-1, and gfp::rad-51* did not recapitulate the endogenous mRNA regulation, which should have been repressed through most of oogenesis rather than actively translated at all stages (Figure A.1 E,F,L,M,N,O). It is therefore likely that some of these *gfp::3'UTR* constructs are not valid indicators of translational control. In summary, we have demonstrated in single live worms

that several mRNAs (*gld-1*, *pos-1*) are dependent upon IFE-1 for efficient translation upon de-repression. However, it is clear that many other mRNA constructs we tested do not. This suggests a system in which the translation regulation of some mRNAs is very selective and isoform specific. Many other mRNAs are able to be recruited for translation by other, or perhaps multiple, IFE isoforms.

Appendix B: GO Analysis of TSAA results.

Results and Relevance

Our initial analysis of the *ife-1* polysome translation state analysis (TSAA) identified 77 mRNAs recruited by IFE-1 for translation initiation (Chapter 2). These mRNA identities were cross-referenced with the online *C. elegans* database, WormBase, to determine the functional role of each protein product, where it had been documented. By this method we were able to classify most of these mRNAs as involved in late stage oocyte maturation, meiotic progression, and embryonic development. To further analyze potential group functions, using objective software analysis, we performed a gene ontology analyses using both GOrilla and SPELL online software. GOrilla, Gene Ontology enrichment analysis and visuaLizAtoin tool, is an online resource for identifying and visualizing enriched GO terms in ranked list of genes. Our first attempt compared two unranked lists of genes (target against background). We used the 77 mRNAs identified in our TSAA as the "target" list and compared it to the 13,372 probes classified as present as the "background". This method was insufficient for the GOrilla software to identify GO terms that were statistically enriched in the target list compared to background. The second GOrilla analysis used a single ranked list of mRNAs. We sorted the 13,372 probes by MFR value so those with the greatest MFR (most IFE-1 dependence) were at the top of the list. GOrilla software then searched for GO terms that appear preferentially at the top of the IFE-1-ranked list. This iteration returned developmental functions statistically enriched near the top of our ranked list, but the results were not wholly useful as they were not limited to mRNAs

translationally regulated by IFE-1. Relevant GO terms included: positive regulation of RNA biosynthetic process; embryo development; protein localization; protein transport; larval development; and post-embryonic development.

Finally, to narrow our results into a list more relevant to IFE-1-regulated mRNAs, we turned to the *C. elegans*-specific SPELL software developed by Caltech. SPELL, Serial Pattern of Expression Levels Locator, is a query-driven search engine for microarray and RNASeq datasets. Given a small set of genes, SPELL compares them to 278 datasets to identify which datasets are most informative, other genes that have the most similar expression profiles, and finally which GO terms are statistically correlated with the set of genes provided. SPELL analysis returned a list of relevant GO terms weighted towards: reproductive system development; sex differentiation; embryo development; and larval development (Table B1). The benefit of this analysis was that it groups the genes we submitted into the program with other related genes identified in the compared datasets that had correlative patterns of expression levels. The genes annotated by the SPELL software may prove of further interest in future studies to discover other IFE-1-regulated mRNAs and discern IFE-1's potential role in distinct developmental processes. Overall these data confirm what we had discovered by TSAA and polysome profiling in that IFE-1 translationally regulates a set of mRNAs that are involved in germ cell development.

Table B.1

GOTerm	P-value	% query	% genome	Annotated Genes
multicellular organismal development(biological_process)	1.18E-18	32 of 47	4979 of 49552	CYC-2.2, TOFU-5, B0238.11, CKS-1, F25H2.4, CUTC-1, ASH-2, PIR-1, LIN-7, RFC-4, LIN-37, T04A8.8, RAB-7,VAB-1, VPR-1, ABI-1, LIN-26, ZC434.4, CCNK-1, K04F10.3, RAB-5, HIS-2, PAS-7, PQN-96, CLPP-1, F21D5.6,CDK-1, LET-49, RNP-3, SWD-2.2, AOS-1, TOMM-20
developmental process(biological_process)	1.84E-18	33 of 47	5553 of 49552	CYC-2.2, TOFU-5, B0238.11, CKS-1, F25H2.4, CUTC-1, ASH-2, PIR-1, LIN-7, RFC-4, LIN-37, T04A8.8, RAB-7,DMD-11, VAB-1, VPR-1, ABI-1, LIN-26, ZC434.4, CCNK-1, K04F10.3, RAB-5, HIS-2, PAS-7, PQN-96, CLPP-1, F21D5.6, CDK-1, LET-49, RNP-3, SWD-2.2, AOS-1, TOMM-20
anatomical structure development(biological_process)	1.49E-17	30 of 47	4454 of 49552	CYC-2.2, TOFU-5, B0238.11, CKS-1, F25H2.4, CUTC-1, ASH-2, PIR-1, LIN-7, RFC-4, LIN-37, T04A8.8, RAB-7,VAB-1, VPR-1, ABI-1, LIN-26, ZC434.4, CCNK-1, K04F10.3, RAB-5, HIS-2, PAS-7, PQN-96, CLPP-1, CDK-1,LET-49, SWD-2.2, AOS-1, TOMM-20

Table B.1

single-organism developmental process (biological_process)	2.65E-17	32 of 47	5516 of 49552	CYC-2.2, TOFU-5, B0238.11, CKS-1, F25H2.4, CUTC-1, ASH-2, PIR-1, LIN-7, RFC-4, LIN-37, T04A8.8, RAB-7,VAB-1, VPR-1, ABI-1, LIN-26, ZC434.4, CCNK-1, K04F10.3, RAB-5, HIS-2, PAS-7, PQN-96, CLPP-1, F21D5.6,CDK-1, LET-49, RNP-3, SWD-2.2, AOS-1, TOMM-20
nematode larval development(biological_process)	3.12E-17	24 of 47	2290 of 49552	B0238.11, F25H2.4, CUTC-1, ASH-2, PIR-1, LIN-7, LIN-37, RAB-7, VAB-1, VPR-1, ABI-1, LIN-26, ZC434.4,RAB-5, HIS-2, PAS-7, PQN-96, CLPP-1, F21D5.6, LET-49, RNP-3, SWD-2.2, AOS-1, TOMM-20
larval development(biological_process)	3.19E-17	24 of 47	2292 of 49552	B0238.11, F25H2.4, CUTC-1, ASH-2, PIR-1, LIN-7, LIN-37, RAB-7, VAB-1, VPR-1, ABI-1, LIN-26, ZC434.4,RAB-5, HIS-2, PAS-7, PQN-96, CLPP-1, F21D5.6, LET-49, RNP-3, SWD-2.2, AOS-1, TOMM-20
post-embryonic development(biological_process)	3.78E-17	24 of 47	2309 of 49552	B0238.11, F25H2.4, CUTC-1, ASH-2, PIR-1, LIN-7, LIN-37, RAB-7, VAB-1, VPR-1, ABI-1, LIN-26, ZC434.4,RAB-5, HIS-2, PAS-7, PQN-96, CLPP-1, F21D5.6, LET-49, RNP-3, SWD-2.2, AOS-1, TOMM-20
embryo development ending in birth or egg hatching (biological_process)	1.79E-16	26 of 47	3165 of 49552	CYC-2.2, TOFU-5, CKS-1, F25H2.4, ASH-2, PIR-1, RFC-4, LIN-37, T04A8.8, RAB-7, VAB-1, VPR-1, ABI-1,LIN-26, ZC434.4, K04F10.3, RAB-5, HIS-2, PAS-7, PQN-96, CLPP-1, CDK-1, LET-49, SWD-2.2, AOS-1,TOMM-20

Table B.1

reproduction (biological_process)	3.15E-16	25 of 47	2873 of 49552	TOFU-5, B0238.11, C30A5.3, F25H2.4, CUTC-1, LIN-7, RFC-4, LIN-37, RAB-7, VAB-1, VPR-1, ABI-1, LIN-26,ZC434.4, CCNK-1, RAB-5, HIS-2, PAS-7, CLPP-1, CDK-1, LET-49, HMG-5, SWD-2.2, AOS-1, TOMM-20
embryo development(biological_process)	3.44E-16	26 of 47	3250 of 49552	CYC-2.2, TOFU-5, CKS-1, F25H2.4, ASH-2, PIR-1, RFC-4, LIN-37, T04A8.8, RAB-7, VAB-1, VPR-1, ABI-1,LIN-26, ZC434.4, K04F10.3, RAB-5, HIS-2, PAS-7, PQN-96, CLPP-1, CDK-1, LET-49, SWD-2.2, AOS-1,TOMM-20
single-multicellular organism process(biological_process)	1.06E-15	32 of 47	6235 of 49552	CYC-2.2, TOFU-5, B0238.11, CKS-1, F25H2.4, CUTC-1, ASH-2, PIR-1, LIN-7, RFC-4, LIN-37, T04A8.8, RAB-7,VAB-1, VPR-1, ABI-1, LIN-26, ZC434.4, CCNK-1, K04F10.3, RAB-5, HIS-2, PAS-7, PQN-96, CLPP-1, F21D5.6,CDK-1, LET-49, RNP-3, SWD-2.2, AOS-1, TOMM-20
multicellular organismal process(biological_process)	2.09E-15	32 of 47	6377 of 49552	CYC-2.2, TOFU-5, B0238.11, CKS-1, F25H2.4, CUTC-1, ASH-2, PIR-1, LIN-7, RFC-4, LIN-37, T04A8.8, RAB-7,VAB-1, VPR-1, ABI-1, LIN-26, ZC434.4, CCNK-1, K04F10.3, RAB-5, HIS-2, PAS-7, PQN-96, CLPP-1, F21D5.6,CDK-1, LET-49, RNP-3, SWD-2.2, AOS-1, TOMM-20
reproductive process(biological_process)	1.79E-10	17 of 47	1785 of 49552	B0238.11, F25H2.4, CUTC-1, ASH-2, LIN-7, RAB-7, DMD-11, VAB-1, ABI-1, LIN-26, ZC434.4, CCNK-1,CLPP-1, CDK-1, LET-49, SWD-2.2, AOS-1
developmental process involved in reproduction (biological_process)	9.06E-10	15 of 47	1374 of 49552	B0238.11, F25H2.4, ASH-2, RAB-7, DMD-11, VAB-1, ABI-1, LIN-26, ZC434.4, CCNK-1, CLPP-1, CDK-1,LET-49, SWD-2.2, AOS-1
organ development(biological_process)	4.96E-09	14 of 47	1266 of 49552	B0238.11, F25H2.4, CUTC-1, ASH-2, LIN-7, LIN-37, ABI-1, LIN-26, ZC434.4, CCNK-1, CLPP-1, CDK-1, LET-49, SWD-2.2
system development(biological_process)	6.43E-09	15 of 47	1578 of 49552	B0238.11, F25H2.4, CUTC-1, ASH-2, LIN-7, LIN-37, VAB-1, ABI-1, LIN-26, ZC434.4, CCNK-1, CLPP-1, CDK-1, LET-49, SWD-2.2

Table B.1

single organism reproductive process(biological_process)	1.97E-08	14 of 47	1405 of 49552	B0238.11, F25H2.4, ASH-2, RAB-7, VAB-1, ABI-1, LIN-26, ZC434.4, CCNK-1, CLPP-1, CDK-1, LET-49, SWD-2.2, AOS-1
sex differentiation (biological_process)	1.8E-07	12 of 47	1075 of 49552	B0238.11, F25H2.4, ASH-2, DMD-11, ABI-1, LIN-26, ZC434.4, CCNK-1, CLPP-1, CDK-1, LET-49, SWD-2.2
gonad development(biological_process)	1.14E-06	11 of 47	987 of 49552	B0238.11, F25H2.4, ASH-2, ABI-1, LIN-26, ZC434.4, CCNK-1, CLPP-1, CDK-1, LET-49, SWD-2.2
development of primary sexual characteristics (biological_process)	1.14E-06	11 of 47	987 of 49552	B0238.11, F25H2.4, ASH-2, ABI-1, LIN-26, ZC434.4, CCNK-1, CLPP-1, CDK-1, LET-49, SWD-2.2
reproductive system development(biological_process)	1.28E-06	11 of 47	998 of 49552	B0238.11, F25H2.4, ASH-2, ABI-1, LIN-26, ZC434.4, CCNK-1, CLPP-1, CDK-1, LET-49, SWD-2.2
reproductive structure development(biological_process)	1.28E-06	11 of 47	998 of 49552	B0238.11, F25H2.4, ASH-2, ABI-1, LIN-26, ZC434.4, CCNK-1, CLPP-1, CDK-1, LET-49, SWD-2.2
hermaphrodite genitalia development(biological_process)	1.29E-06	10 of 47	752 of 49552	B0238.11, F25H2.4, ASH-2, ABI-1, LIN-26, CCNK-1, CLPP-1, CDK-1, LET-49, SWD-2.2
genitalia development(biological_process)	1.41E-06	10 of 47	759 of 49552	B0238.11, F25H2.4, ASH-2, ABI-1, LIN-26, CCNK-1, CLPP-1, CDK-1, LET-49, SWD-2.2
locomotion (biological_process)	7.23E-05	12 of 47	1845 of 49552	F25H2.4, ASH-2, RAB-7, VAB-1, VPR-1, ABI-1, CCNK-1, TTR-39, HIS-2, PQN-96, CDK-1, LET-49
multi-organism process(biological_process)	7.73E-05	10 of 47	1162 of 49552	CUTC-1, LIN-7, RAB-7, VAB-1, ABI-1, ZC434.4, DKF-2, CDK-1, LET-49, AOS-1
cell differentiation (biological_process)	7.85E-05	10 of 47	1164 of 49552	CUTC-1, ASH-2, RAB-7, VAB-1, ABI-1, LIN-26, ZC434.4, CDK-1, LET-49, AOS-1

Table B.1. SPELL Analysis of TSAA mRNAs. 77 mRNAs were identified in the TSAA as being translationally regulated by IFE-1. The identities of these mRNAs were submitted for analysis by SPELL, Serial Pattern of Expression Levels Locator, software developed at the California Technology Institute. SPELL is a free-to-use analysis software that compares a set of given genes to 278 datasets to identify genes of similar expression profiles and statistically relevant GO terms. Of the 77 mRNAs we input, SPELL software was able to match 47 of them for analysis. SPELL returned GO terms with a statistically significant p-value and associated annotated gene