Abstract

MULTIPLE TRANSLATION FACTOR eIF4G (IFG-1) ISOFORMS ARE REQUIRED FOR THE APOPTOSOME-DEPENDENT ACTIVATION OF GERM CELL APOPTOSIS

by Vince Contreras

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Apoptosis is a naturally occurring process during animal development required for the programmed killing and removal of injured cells. Cellular insult induces a switch in translation that allows for the rapid synthesis of proteins that quickly destroy the unhealthy cell and remove it from undamaged tissue. In mammalian cells, this selective modulation of translation has been attributed to modifications of the protein synthesis machinery by apoptotic caspases. Specifically the initiation complex scaffold protein eIF4G becomes cleaved to disrupt cap-dependent translation and induce the IRES-dependent synthesis of apoptogenic mRNAs like Apaf-1. While the biochemistry of these cleavage events has been well characterized, how disruptions in translation mechanism affect apoptotic signaling still remains largely unknown. The nematode *Caenorhabditis elegans* was used to study how the balance between cap-dependent and –independent translation mechanisms mediate the translation of pro-apoptotic Apaf-1/CED-4 to alter the fate of healthy germ cells. Two major eIF4G (IFG-1) isoforms, derived from a single gene, were found to be broadly expressed throughout all stages of development. IFG-1 p170 and p130 appeared to both associate with ribosomes yet differed in their N-termini. Analysis of mRNA cap complexes showed that only IFG-1 p170 associates with *C. elegans* eIF4Es (IFEs) and participates in cap-dependent translation. IFG-1 p130, like its human paralog p97, was not

retained in bound complexes and likely participates in cap-independent translation. We further showed that disruption of the balance between the *C. elegans* eIF4G cap-dependent (IFG-1 p170) and cap-independent (IFG-1 p130) isoforms increased apoptosis in developing oocytes. This induction of germ cell apoptosis coincided with the upregulated synthesis of the Apaf-1 homolog, CED-4. In addition, IFG-1 p170, like mammalian eIF4GI, is a substrate for human caspase-3 and its worm homolog, CED-3. Site-directed mutagenesis of the cleavage site indicated that CED-3 processes IFG-1 at a non-canonical caspase motif, $TTTD^{456}$. This caspase recognition site is located 65 amino acids downstream of the IFG-1 p130 start site indicating that this truncated form may be required to support cap-independent initiation in the germline. Furthermore, genetic analysis involving ced-3/caspase-3 and ced-4/Apaf-1 mutants confirmed that apoptosis induced by loss of IFG-1 p170 is caspase and apoptosome dependent. These findings provide the first demonstration that modal changes in protein synthesis are required to initiate the cell death signal, rather than merely downstream consequences of the apoptotic event.

# MULTIPLE TRANSLATION FACTOR eIF4G (IFG-1) ISOFORMS ARE REQUIRED FOR THE APOPTOSOME-DEPENDENT ACTIVATION OF GERM CELL APOPTOSIS

A DISSERTATION

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by

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TABLE OF CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AMPK | 5’ adenosine monophosphate-activated protein kinase |
| Apaf | apoptotic protease activating factor |
| ATF | activating transcriptionfactor |
| ATP | adenosine-5’-triphosphate |
| eIF | eukaryotic translation factor |
| 4E-BP | eIF4E binding protein |
| Bcl | B-cell lymphoma |
| BiP | binding immunoglobulin protein |
| BSA | bovine serum albumin |
| caspase | cysteine aspartate directed protease |
| CED | cell death defective |
| Cdk | cyclin-dependent kinase |
| cDNA | complementary DNA |
| CPE | cytoplasmic polyadenylation element |
| CPEB | cytoplasmic polyadenylation element binding protein |
| CPSF | cleavage and polyadenylation specificity factor |
| CrPV | cricket paralysis virus |
| DAPI | 4',6-diamidino-2-phenylindole |
| DED | death effector domain |
| DIC | differential interference contrast |
| DISC | death inducing signaling complex |
| EMCV | encephalomyocarditis virus |
| ER | endoplasmic reticulum |
| EST | expressed sequence tag |
| FADD | fas-associated protein with death domain |
| Fas | fibroblast associated antigen |
| FGF | fibroblast growth factor |
| FITC | fluorescein isothiocyanate |
| GCN2 | general control non-derepressible -2 |
| GFP | green fluorescence protein |
| GST | glutathione-S-transferase |
| HCV | hepatitis C virus |
| HRI | haem-regulated inhibitor |
| IBC | inflammatory breast cancer |
| IRES | internal ribosome entry site |
| ITAF | IRES trans-acting factor |
| Mdm | murine double minute |
| MIF4G | middle fragment of eIF4G |
| miRNA | micro RNA |
| mRNA | messenger RNA |

| | |
|---|---|
| mTor | mammalian target of rapamycin |
| NGM | nematode growth medium |
| ODC | ornithine decarboxylase |
| PABP | poly(A) binding protein |
| PARP | poly ADP ribose polymerase |
| PAS | poly(A) site |
| PCR | polymerase chain reaction |
| PERK | pancreatic endoplasmic reticulum eIF2 kinase |
| PKR | protein kinase activated by double-stranded RNA |
| PTB | polypyrimidine tract-binding protein |
| PVDF | polyvinylidene fluoride membrane |
| RISC | RNA-induced silencing complex |
| RNAi | RNA interference |
| SCC | squamous cell carcinoma |
| SDS | sodium dodecyl sulfate |
| SL | splice leader |
| TAP | tobacco acid pyrophosphatase |
| TST | tris buffered saline with tween |
| uORF | upstream open reading frame |
| UTR | untranslated region |
| UV | ultraviolet |
| VEGF | vascular endothelial growth factor |
| XIAP | X-linked inhibitor of apoptosis |

LIST OF FIGURES

# CHAPTER 1: LITERATURE REVIEW

The specification of a cell to a particular fate depends heavily on the production of proteins. These proteins can commit the cell to changes in its biochemistry and structure that will ultimately lead to changes in appearance and function. For example, differentiated epithelial cells form interdigital web-like structures at the terminal ends of fetal limbs (Mori, Nakamura et al. 1995). Pre-determined signals program cells separating limb buds to undergo cell death or apoptosis. This coordinated cell fate specifies the individual digits that will eventually become adult fingers. In developing tissues, cells can correspondingly compensate for this loss of large numbers of apoptotic cells by inducing proliferation of neighboring cells. Drosophila apoptosing imaginal disc cells are known to contain morphogenic proteins (Wnt) that promote proliferation and proper formation of the surrounding architecture (Ryoo, Gorenc et al. 2004) (Perez-Garijo, Martin et al. 2004). Little is known how a cell decides to differentiate into a different cell type or balance proliferation with opposing cell death mechanisms. However, emerging evidence is pointing to an interconnected regulatory network involving multiple mRNAs and distinct levels of translation control.

## *Translational Control and Cellular Homeostasis*

### *The living cell (Proliferation)*

The cell division cycle is a necessary step in the proliferation and differentiation of a fertilized egg into a multicellular organism. The cell cycle consists of four unidirectional phases, G1, S, and G2 which comprise intephase and a mitotic (M) phase (Schafer 1998). The G1 or first gap phase is defined by cell growth (increased protein, lipids, and organelles) prior to DNA

gap phase (G2) allows for additional growth and ensures that cellular DNA is properly duplicated in preparation for cell division during the M phase. Finally the mitotic phase is marked by reduced growth while the cell prepares to undergo the karyokinetic (nuclear division) and cytokinetic (cellular division) steps required for the viability of progenitor (Schafer 1998).

The organization of these phases is partly regulated by checkpoint controls which ensure that each phase is properly completed before progressing to the next. The progression is driven by the direct association of cyclins and serine/threonine cyclin-depedendent kinases (CDKs) (Boonstra 2003). Both binding partners synergistically coordinate entry into successive phases through the heterodimerization of the regulatory cyclins and catalytic CDKs. Together these complexes phosphorylate downstream targets such as the CDK-dependent retinoblastoma protein (pRB). In this instance, hyperphosphorylation promotes the dissociation of pRB from the E2F transcription factor which is a checkpoint that allows for progression into S phase (Stevaux and Dyson 2002). Formation of cyclin/CDK complexes is also stage-dependent because cyclins are only synthesized during a particular phase (G1/G2) in the presence of environmental cues (Boonstra 2003). Thus it is critical that appropriate checkpoint controls are maintained to ensure proper division and proliferation of daughter cells.

The regulation of cell cycle in many eukaryotic organisms is tightly controlled at the level of mRNA translation. Recent evidence has shown that specific proteins can affect the progression of the cell cycle during development. One of the most conserved regulatory events in animal gametogenesis is cell cycle arrest during prophase I of meiosis I (Sugimura and Lilly 2006). During this period huge stockpiles of mRNAs and proteins must be generated, while maintaining the genomic integrity of the cell, to drive subsequent meiotic divisions in oocytes

and mitotic divisions in the early embryo. Cells initiate arrest during the onset of meiosis through inhibition of the mitotic kinase Cdk1. For example, the *Drosophila* translational inhibitor, Bruno, inhibits the expression of cyclins after meiotic entry preventing re-entry into mitosis. Bruno facilitates inhibition through 3' untranslated region (UTR)-mediated repression of cyclin A mRNA translation which may be required to positively regulate Cdk1 (Sugimura and Lilly 2006). Amphibians employ a mechanism whereby meiotic arrest is mediated by decreased cyclin B translation resulting in low Cdk1 activity (Yamashita 1998). Likewise, dissociation of mRNA cap-binding complexes and derepression also correlate with cyclin B translation upon fertilization of sea urchin eggs (Cormier, Pyronnet et al. 2003). Therefore it is clear that multiple translation control mechanisms involved in cell cycle progression are necessary to maintain stable proliferative processes during mitosis, or the alternative of meiotic differentiation.

*Aberrant proliferation (Cancer)*

Dysregulated proliferation found in many transformed cells is marked by elevated levels of protein synthesis following mitogen stimulation (Heys, Park et al. 1991; Vary, Jefferson et al. 2000). Numerous studies have reported translational upregulation of several cell growth and proliferation proteins upon overexpression of translation factors. Overexpression of the mRNA cap-binding protein eIF4E was shown to induce the transformation of immortalized NIH-3T3 cells as well as increased activation of the oncogene, *ras* (Lazaris-Karatzas, Smith et al. 1992). High levels of eIF4E in c-Myc expressing rat embryo fibroblasts also rescued cells from DNA damaging (genotoxic) drugs thereby suppressing apoptosis and contributing to cell survival (Tan, Bitterman et al. 2000). Additional evidence showed that tumors from breast cancer, head and neck squamous cell carcinoma and Non-Hodgkin's lymphoma also contained increased eIF4E

(De Benedetti 2004). These results along with reports of additional aberrantly regulated translation factors (eIF3, eIF2, eIF4B, and eIF4G) indicate that protein synthesis contributes significantly to malignancy (Silvera, Formenti et al.).

The cause of malignant proliferation, as result of high levels of translation factors like eIF4E, is the increased synthesis of growth- and cell cycle-related proteins (De Benedetti and Graff). Many of these proliferative-promoting "weak" mRNAs (long and highly structured 5'UTRs, c-Myc) are outcompeted by "strong" mRNAs (short, unstructured 5'UTRs, actin) under normal conditions where eIF4E protein levels are limiting (Lodish 1974; Rhoads 1993). However during oncogenesis, elevated eIF4E levels promote enhanced synthesis of "weak" proteins underlying dysregulated cell growth and proliferation. Ornithine decarboxylase (ODC) which is involved in the synthesis of polyamines for S phase is more efficiently (30-fold over normal) translated in cells overexpressing eIF4E (Shantz and Pegg 1994). It was determined that stimulation during G1/S transition is strongly dependent on the expression of eIF4E as ODC synthesis is inhibited by rapamycin, an eIF4E kinase inhibitor. Again the reason for the upregulation of ODC like other growth-regulated mRNAs is attributed to its long and highly structured 5' UTR which is more sensitive to eIF4F helicase activity (Svitkin, Pause et al. 2001). Other mRNAs affected by increased eIF4E include cyclin D1 (cell cycle), c-Myc (oncogenesis), human fibroblast growth factor (FGF-2) and vascular endothelial growth factor (VEGF) which are also involved in cell proliferation and angiogenesis (De Benedetti and Graff 2004). Thus, changes in the expression of translation factors are critical to the controlled synthesis of proteins mediating aberrant proliferation and oncogenesis.

*The death of the cell (Apoptosis)*

Apoptosis is equally as important as proliferation in maintaining cellular homeostasis. Multicellular organisms require programmed cell death for the removal of damaged and unnecessary cells, morphogenetic patterning, and protection from autoimmunity. The morphology of cells attributed to apoptosis distinguishes it from other types of cell death including autophagy (lysosome-mediated) and necrosis (uncontrolled). Many of the characteristics that arise from apoptosis (chromatin condensation, nuclear fragmentation, membrane blebbing, and cell shrinkage) are the result of a signaling cascade that employs a hierarchical assembly of initiator and effector proteases called caspases that help break down the cellular infrastructure. Without this caspase cascade, many of the inflammatory contents within cells would affect the viability of neighboring cells.

Caspase activation is mediated by two apoptotic pathways, intrinsic and extrinsic (Fulda and Debatin 2006). The extrinsic or death-receptor pathway utilizes extracellular ligands to activate their corresponding receptors. The fibroblast associated antigen (Fas) binds FasR which contains a cytoplasmic death domain. Ligands induce formation of the Death Inducing Signaling Complex (DISC), trimerization, and endosomal internalization of the receptor. The cytoplasmic adapter protein FADD is then recruited and associates with the receptor death domain. The anchored FADD proteins subsequently bind the death effector domain (DED) of the initiator pro-caspase-8. Active caspase-8 then cleaves downstream executioner caspases to proteolytically process several cytoskeletal and nuclear proteins. The intrinsic or mitochondrial-mediated pathway is alternatively activated by extra-and intracellular signals (oxidative stress or irradiation) that cause disruption of mitochondrial membrane integrity. An apoptotic signal results in recruitment of pro-apoptotic Bcl-2 family proteins (Bax/Bak) which are shuttled from the intracellular membrane to the mitochondrion (Hengartner 2000). Oligomerization of these

proteins through pore formation or fragmentation of the membrane promotes the release of the mitochondrial oxidative phosphorylation protein, cytochrome c, from the intermembrane space. The release of cytochrome c along with dATP, procaspase-9, and the apoptotic protease activating factor 1 (Apaf-1) form a large multimeric holoenzyme called the apoptosome (Hickman and Helin 2002; Shi 2008). The apoptosome activates caspase-9 thereby facilitating proteolysis of downstream executioner caspases.

Cysteine aspartate specific proteases (caspases), first characterized in *C. elegans*, are critical in the disassembly of cellular proteins during apoptosis (Yuan and Horvitz 1992; Xue, Shaham et al. 1996). As the name suggests, caspases recognize apoptotic substrates by a tetrapeptide motif where cleavage occurs at a carboxyl terminal to an aspartic acid residue (Thornberry 1998). All caspases are generated as inactive zymogens that, upon activation by upstream caspases, form heterotetramer complexes consisting of large and small catalytic subunits. Cleavage of numerous intracellular proteins are mediated by these effector caspases including cytoskeletal (actin and adherens), nuclear (lamins), DNA metabolism (PARP), cell cycle (p21), and protein synthesis (PABP and eIF4G) (Chowdhury, Tharakan et al. 2008). While the list of substrates is growing, it is becoming clear that their processing is dependent on not only the type of stress but the transcriptional and translational regulation of caspase activation.

Translational control affords apoptotic cells rapid responses to a variety of stimuli. The result is the spatial and temporal expression of many pro-apoptotic factors that affect the architectural character of developmental cells. In *Drosophila* the Nanos (Nos) protein regulates developmental apoptosis by translationally regulating the survival of germ cells that will become mature gametes (Sato, Hayashi et al. 2007). Nanos specifically is required early for production

of germ cells during oogenesis and later for embryonic polarity (Wang and Lehmann 1991; Wang, Dickinson et al. 1994). The binding of Nos represses the translation of the caspase activator, head involution defective (hid) to suppress apoptosis and promote proper germline development (Sato, Hayashi et al. 2007). Likewise, the *C. elegans* gene encoding the p53 tumor suppressor (*cep-1*) is required to maintain genome stability during meiosis (Schumacher, Hofmann et al. 2001). The CEP-1 transcription factor is responsible for the immediate response to DNA damage-induced apoptosis. Anton Gartner and colleagues have shown that *cep-1* mRNA is translationally repressed by the oocyte differentiation RNA-binding protein, GLD-1, to prevent the death of germ cell subsets undergoing meiotic recombination (double stranded DNA breaks). Likewise, microRNAs (miRNAs) have recently joined the foray of apoptotic regulators during development. These small noncoding RNA molecules can incorporate into the regulatory RNA-induced silencing complex (RISC) that participates in the translational repression and degradation of complementary mRNAs. In *Xenopus*, depletion of miR-24a causes dramatic increase in retinal apoptosis as well as defects in eye morphogenesis (Walker and Harland 2009). It was concluded that miR-24a inhibits apoptosome assembly by negatively regulating the translation of caspase-9 and Apaf-1. Protein synthesis regulation is therefore essential to ensuring the genomic integrity and morphogenetic competence of developmental cells through the manipulation of the apoptotic program in addition to forward differentiation.

Dysregulation of apoptosis can contribute to the proliferative potential of oncogenic cells, suggesting that the protein synthesis mechanisms that govern each might also be linked. Increasing evidence is linking aberrant translational control mechanisms to the malignant potential of a cell. For example, cancer cells overexpressing the p53 regulator, murine double minute 2 (Mdm2) have shown drastic increases in the expression of anti-apoptotic X-linked

inhibitor of apoptosis (XIAP) protein (Gu, Zhu et al. 2009). Further evidence revealed that Mdm2 upregulates XIAP at the translational level by directly binding an internal ribosome entry site (IRES) in the 5'UTR. The corresponding increase in XIAP levels also conferred resistance to irradiation induced apoptosis. Upregulation of other anti-apoptotic factors such as B-cell lymphoma 2 (Bcl-2) and Inhibitor of Apoptosis Proteins (IAPs) have also been implicated in cancer cells that are insensitive to chemotherapy (Nomura, Yamasaki et al. 2005; Silvera, Formenti et al. 2010). Additional research into basic mRNA translation mechanisms underlying the modulation of anti-apoptotic signals may provide a glimpse into the adaptive responses employed by both cancer and other highly proliferative (gametes and embryos) cells.

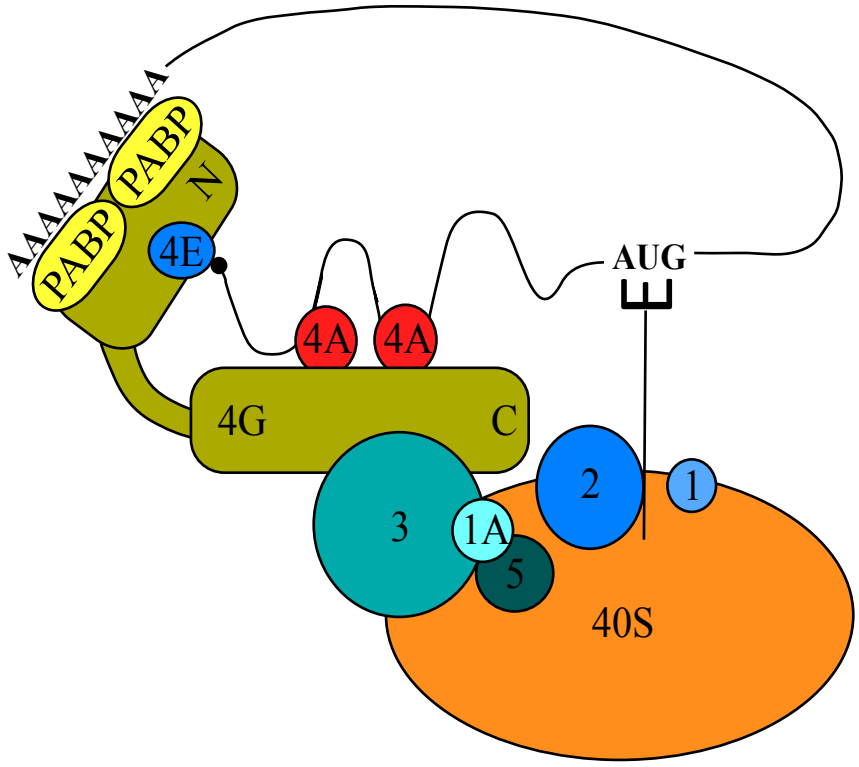
## *Translation Regulation and mRNA Recruitment*

### *Translation initiation*

Control of protein synthesis is most often exerted at the level of initiation where messenger RNAs (mRNAs) are recruited to ribosomes via a group of eukaryotic initiation factors (eIFs). The current model of eukaryotic translation initiation involves recognition of the 5' end of the mRNA followed by 5'-3' scanning to the initiation codon (Figure 1.1) (Kapp and Lorsch 2004). The process begins with generating a pool of ribosomal 40S subunits. A methionine-loaded initiator tRNA binds a GTP-coupled eIF2 to form a ternary complex. The ternary complex binds free 40S subunits along with eIF1, eIF1A, and eIF3 to form the 43S pre-initiation complex. mRNA recruitment involves the ATP-dependent binding of eIF4F comprised of the protein eIF4E, which binds the 7-methyl guanosine cap at the mRNA 5' end, eIF4A, an mRNA

**Figure 1.1 Schematic of the Pre-Initiation Complex.** Complex depicted is involved in the committed step of translation initiation. Eukaryotic initiation factors (eIFs) are involved in the recruitment of mRNAs to the ribosome. Shown are eIF4 factors, eIF4E (color), eIF4G (green), and eIF4A (red), which respectively facilitate mRNA cap-binding, complex assembly, and helicase activity. eIF4G acts as scaffold for other initiation factors in the complex such as eIF3 (3) which directly associates with the 40S ribosomal subunit. Other factors shown are eIF2 (2), Met-tRNA$_i^{Met}$, eIF1 (1), eIF1A (1A), eIF5 (5) and the poly(A)-binding protein (PABP) which binds the mRNA poly(A) tail.

Figure 1.1



helicase, eIF4B, an annealing protein, eIF4G, a scaffold protein all of which catalyze joining of mRNA to the 40S ribosomal subunit via eIF3 (Keiper, Gan et al. 1999). The binding of the mRNA-eIF4F complex with 43S promotes scanning of the mRNA until it encounters the start codon (AUG) in the proper context and forms the 48S initiation complex. The assembly of a stable 48S initiation complex is the committed step for protein synthesis for essentially all mRNAs. Start codon recognition results in GTP hydrolysis by eIF2, dissociation of several initiation factors, binding of 60S subunits, and release of the Met-tRNAi into the P site of the 40S ribosome. As peptide elongation occurs, eIF2-GDP is recycled in the presence of eIF2B for successive rounds of initiation (Merrick and Hershey 1996).

Under normal conditions (unstressed), cells recruit most mRNAs by a 5' cap-mediated scanning mechanism, referred to as cap-dependent initiation. This mode of initiation requires recognition of the m7G cap structure by eIF4F (specifically eIF4E) to commit the complex to protein synthesis. However, when cells experience conditions of acute stress such as viral infection there is a dramatic reduction in cap-dependent translation as a result of disruption of eIF4F. Evidence has shown that viral processing of eIF4G results in decreased translation of host capped mRNAs while maintaining the synthesis, via the C-terminal fragment, of uncapped blot were performed as follows. Samples were boiled for 10 min??at 100oC in 4x SDS sample Keiper and Rhoads 1997). This alternative mode of cap-independent initiation does not require eIF4E association with the methylated cap and allows internal binding of the ribosomal complex to the mRNA. Like viral RNAs, many cellular mRNAs also support this type of translation during periods of early development. For example, many maternally derived mRNAs in premature oocytes of *Xenopus laevis* can support cap-independent initiation despite disruptions to eIF4G integrity (Keiper and Rhoads 1997). Thus a dynamic switch between cap-dependent

and –independent modes of protein synthesis may be necessary to provide rapid responses to a myriad of environmental and internal conditions encountered by the cell.

*Translational control (Global)*

Modifications to the eIF4F (complex containing eIF4E, eIF4A, and eIF4G) and eIF2 factors form the basis of most translational control (Keiper, Gan et al. 1999). This control is due to the differences in translational efficiencies among mRNA populations. For example, studies have indicated that phosphorylation of eIF2 can regulate the translation of global mRNAs with short unstructured 5'UTRs that encode "housekeeping" proteins (β-actin) (Rhoads 1993). In contrast, changes in eIF4F activity can stimulate the translation of a small disproportionate array of poorly translated mRNAs that contain long highly structured 5' UTRs and are typically involved in cell cycle growth and proliferation (c-myc, and cyclin D1) (De Benedetti and Harris 1999). Therefore, it is generally the case that eIF2 controls with some exceptions (GCN4 and ATF4) the overall rate of protein synthesis and the eIF4F factors affect the spectrum of mRNAs translated (Rhoads 1988) (De Benedetti and Harris 1999) (Keiper 2002).

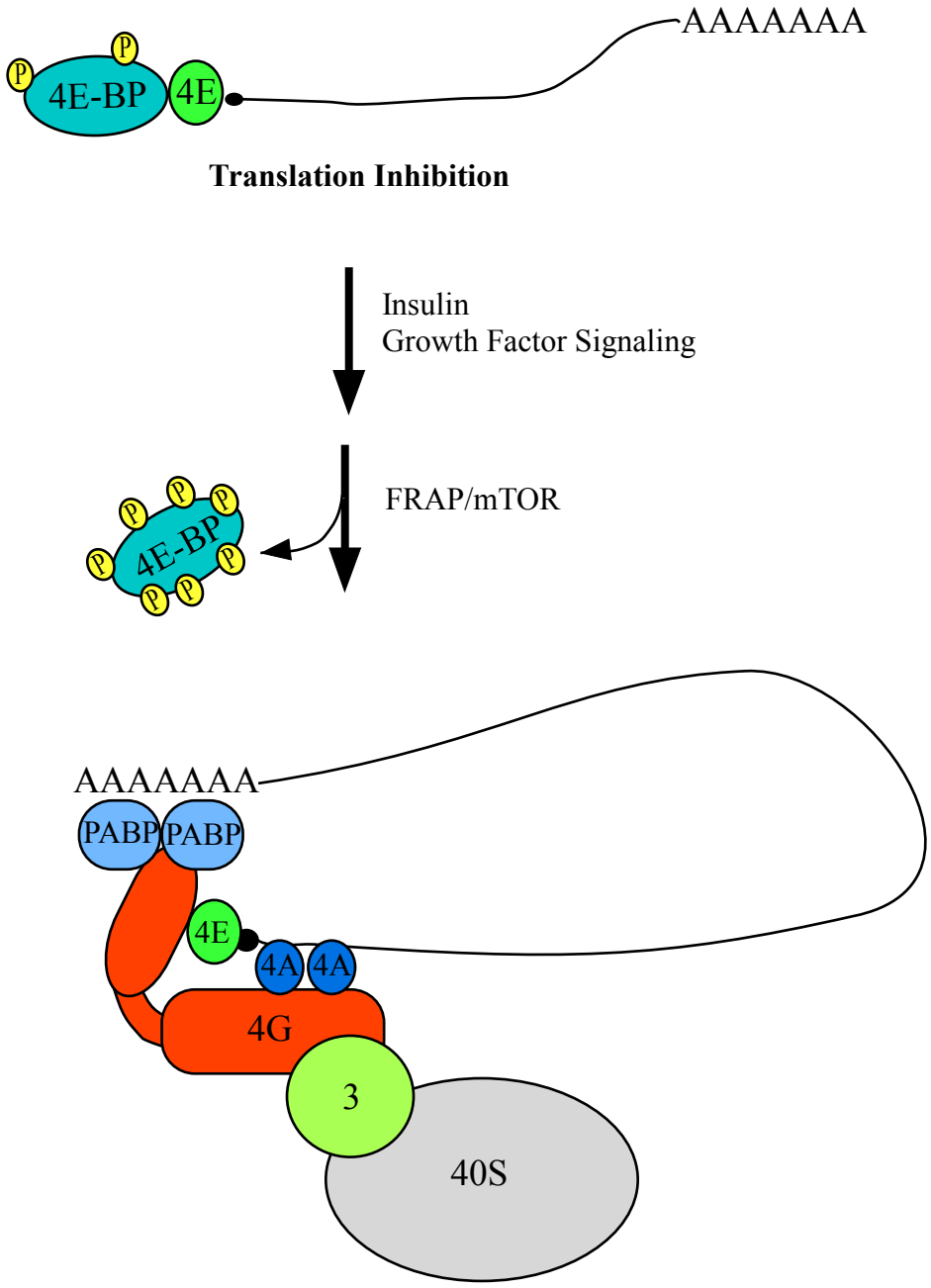
eIF2α phosphorylation at Ser51 can be utilized under several aberrant cellular conditions including nutrient deprivation, iron deficiency, viral infection, and endoplasmic reticulum (ER) stress. Most of these effects involve disrupting the guanine nucleotide exchange between eIF2 and eIF2B. This perturbation to ternary complex formation affects not only synthesis from the majority of mRNAs but increased translation of specific mRNAs (Lackner and Bahler 2008). Amino acid starvation causes the activation of the general control nonderepressible 2 (GCN2) kinase which reduces the level of ternary complex allowing more initiating ribosomes to bypass inhibitory upstream open reading frames (uORFs) in the GCN4 transcript (Wek 1994). The

increased translation of this transcription factor promotes the activation of genes required for amino acid biosynthesis in yeast (Harding, Novoa et al. 2000; Gebauer and Hentze 2004). Likewise, the activating transcription factor 4 (*atf4*) mRNA contains uORFs that are regulated during ER stress and amino acid deprivation. Upregulation of ATF4 causes the increased expression of downstream proteins involved in cellular recovery and apoptosis (Wek, Jiang et al. 2006). While there appear to be stress-specific responses as a result of eIF2α phosphorylation, most conditions of ternary complex depletion predominantly result in global disruption of protein synthesis. For example global translation is affected by haem depletion. Low levels of haem in erythrocytes cause a corresponding increase in phosphorylation of eIF2α by the haem-regulated inhibitor (HRI) kinase. The HRI kinase ensures that no globin chains required for hemoglobin are synthesized until more haem becomes available. Furthermore, the infection of the host by many viruses causes an increase in foreign RNAs. As a result the cell generates an interferon-induced activation of the protein kinase activated by dsRNA (PKR) that reduces translation to prevent synthesis of new viral proteins. Lastly, as a result of hypoxia unfolded proteins can accumulate in the ER causing the cell to activate the PKR-like ER eIF2a kinase (PERK). The resulting eIF2a phosphorylation which disrupts the overall rate of translation allows the cell time to degrade and remove improperly folded proteins. These examples show that translational control is required to direct global responses that allow the cell to recover from a variety of physiological stress.

Repression of cap-dependent translation by inhibition of eIF4E-eIF4G interaction can also result in a reduction in protein synthesis. During stress, the eIF4E-binding proteins (4E-BP) can compete with eIF4G and directly prevent its association with the mRNA cap-binding protein (Teleman, Chen et al. 2005) (Figure 1.2). 4E-BPs are regulated by the cell growth regulator,

**Figure 1.2 mTOR prevents 4E-BP repression of cap-dependent translation.** Growth during development is mediated in part by mTOR (mammalian target of rapamycin) and the eIF4E-binding protein (4E-BP) which serves as a regulator, repressing translation by preventing recruitment of capped mRNAs to ribosomes. Under low mTOR conditions, 4E-BP is associated with eIF4E in a hypophosphorylated state. Upon growth factor signaling/insulin, hyperphosphorylation causes dissociation of 4E-BP, allowing eIF4G to bind and translation to resume.

# Figure 1.2


AAAAAAA
P
P
4E-BP
4E
Translation Inhibition
Insulin
Growth Factor Signaling
FRAP/mTOR
P
P
P
P
P
P
P
4E-BP
AAAAAAA
PABP
PABP
4E
4A
4A
4G
3
40S
Translation Stimulation

mammalian target of rapamycin (mTOR). As a result of growth factor signaling (insulin) 4E-BP is hyperphosphorylated disrupting its association with eIF4E thereby allowing eIF4G to bind and translation to proceed. Other repressors such as the small yeast p20 protein also inhibit eIF4E-eIF4G binding with a corresponding reduction in cap-dependent translation (Altmann, Schmitz et al. 1997). In addition disruption of global synthesis by post-translational modifications and cleavage of eIF4G has also been described. The p21-activated protein kinase 2 (Pak2) is activated by nutrient deprivation, irradiation, and apoptosis and has also been shown to compete with eIF4G for eIF4E binding (Orton, Ling et al. 2004; Ling, Morley et al. 2005). Concomitantly eIF4G is also utilized during viral infection to reduce translation rates of cellular mRNAs. Rotoviral RNAs are unlike many viruses are capped but not polyadenylated. These viruses encode non structured proteins (NSP3) that act as PABP substitutes for 3' end assembly of eIF4F complexes (Piron, Vende et al. 1998). Further evidence has shown that influenza virus uses a similar mechanism. However the viral NS1 protein binds to the N-terminus of eIF4G and the 5' UTR of viral mRNAs mimicking the function of the eIF4E protein and host cap-dependent translation. Thus, the cell and viruses have evolved numerous ways of modulating both the total and specific synthetic output of proteins during periods of stress and infection.

*Translational control (mRNA-specific)*

*mRNA 3'UTR repression*. Regulation of protein synthesis plays an important role in genome expression. mRNA translational control is especially essential to temporally regulate the appearance of developmental inducers at times when transcriptional control exerts relatively little control over gene expression (Davidson 1986; Dworkin and Dworkin-Rastl 1990; Goodwin and Evans 1997). During oogenesis huge stockpiles of maternal mRNAs are transcribed but wait

to become translationally active until ooctye maturation, fertilization, or early embryogenesis (Mendez and Richter 2001). Translational control allows for the immediate synthesis of these proteins without the need for new transcription. In *Xenopus laevis*, for example, c-Mos and cyclin B mRNAs are dormant until oocyte maturation and stored in an inhibitory mRNP complex that includes a protein called Maskin (Stebbins-Boaz, Cao et al. 1999). Maskin is an inhibitor that prevents the formation of the translation initiation complex. Maskin and the mRNA cap binding protein, eIF4E, bind the cytoplasmic polyadenylation element binding protein (CPEB) in a repressed complex within the 3'UTR of many dormant mRNAs. Upon stimulation of oocyte meiotic maturation by progesterone, CPEB is phosophorylated leading to the dissociation of Maskin from eIF4E and the active translation of several maternal messages. A similar mRNP particle known as a P granule stores maternal mRNAs in germ cells of the nematode *C. elegans* and sequesters an isoform of eIF4E bound to the RNA-binding protein, PGL-1 (Amiri, Keiper et al. 2001). However the mechanism of this analogous recruitment to P granules is not well understood. Thus changes in gene expression are exerted not only by an increase in the overall (quantitative) rate of protein synthesis but also the preferential (qualitative) translational activation of certain mRNAs involved in cell growth and proliferation, and as will be discussed later, apoptosis.

*IRESs.* Internal ribosome entry sites add an alternative mode of translational control for specific mRNAs during periods or viral infection and cellular apoptosis. The first report of IRESs stemmed from the discovery that polysome-associated uncapped picornaviral RNAs had a 5' pUp terminus instead of the normal cellular methylated cap (Nomoto, Detjen et al. 1977; Hellen and Sarnow 2001). It wasn't until much later that infected cells, containing uncapped polio or encephalomyocarditis virus (EMCV) RNAs, were found with ribosomes bound to the

internal portions of 5'UTR viral transcripts (Jang, Krausslich et al. 1988; Pyronnet, Dostie et al. 2001). The function of IRESs is the internal translation initiation of mRNAs during conditions where cap-dependent protein synthesis is compromised; thus it does not require eIF4E nor its association with the initiation complex. This cap-independent mode of initiation does, however, (with one exception) still require eIF4G. In addition to some primary sequences and secondary structure which have been attributed to viral IRESs, there are certain features that characterize a potential IRES. These features include long highly structured polypyrimidine regions, often with multiple AUGs that under normal growth conditions inhibit cap-dependent scanning (Hellen and Sarnow 2001). The recruitment of ribosomal complexes, however, is similar to the cap-mediated scanning complex except it does not require eIF4E and the N-terminus of eIF4G (Lomakin, Hellen et al. 2000). However there have been distinct translation sub-mechanisms of viral IRES-dependent initiation. Studies have shown that viral IRES containing RNAs can use minimally the central RNA/ribosome binding domain of eIF4G, eIF4A to assemble initiation complexes. Alternatively, some viral transcripts like those of HCV can associate with the 40S subunit directly and insert ternary complexes in the P site without any eIF participation. Even more intriguing is that the CrPV-like IRES can assemble 80S ribosomes and bind the P site directly without eIFs or ternary complexes, essentially beginning its translation at an elongation step (Hellen and Sarnow 2001). Additionally, studies have indicated that IRES-specific trans-acting factors (ITAFs) are also necessary to stabilize 5'UTR secondary structure required to bind the translational apparatus (Kaminski and Jackson 1998). The La autoantigen is one of many that stimulates the activity of the poliovirus IRES. Despite the variability of assembly, one fundamental connection between all of these mechanisms is their lack of requirement for eIF4E.

The first cellular IRES described was the immunoglobulin heavy chain binding protein (BiP) which was found actively translating on polyribosomes in virally infected cells despite global repression of cap-dependent synthesis (Sarnow 1989; Macejak and Sarnow 1990). Soon after several others were identified, many involved in regulating the expression of genes during development, cell proliferation, cell cycle, and apoptosis (Komar and Hatzoglou 2005). It is believed that similar ITAF stabilization of cellular IRES structure mediate recruitment of translation complexes and enhance activity numerous transcripts (Sella, Gerlitz et al. 1999; Mitchell, Brown et al. 2001; Holcik and Sonenberg 2005). Interestingly, some evidence has even suggested that Shine-Delgarno-like sequences complementary to 18S ribosomal RNAs may be involved (Hellen and Sarnow 2001).

Translational regulation of cellular IRESs provides a rapid response to changes in cell cycle, stress, and apoptosis. The global synthesis of proteins during the G2/M phase of the cell cycle is significantly reduced partly as a result of eIF4E dephosphorylation (Bonneau and Sonenberg 1987; Huang and Schneider 1991). Yet several IRES containing mRNAs including ornithine decarboxylase, the G2/M cell cycle kinase $p58^{PITSLRE}$, and c-Myc remain active (Pyronnet, Pradayrol et al. 2000). Because not all IRES-containing transcripts are activated during G2/M, these results indicated that cells have adapted mechanisms to synthesize stage-specific cell cycle proteins. Both anti- and pro-apoptotic IRES-containing mRNAs are regulated during stress and cell death. During hypoxia and genotoxic stress, VEGF and the cellular inhibitor of apoptosis c-IAP1 IRES activity are increased which gives the cell time to recover from these transient stressors (Ekedahl, Joseph et al. 2002; Van Eden, Byrd et al. 2004). However, prolonged exposure to chronic stress such as UV irradiation can cause the upregulation of apoptogenic IRESs like Apaf-1 that protect neighboring cells by killing the damaged ones

(Ungureanu, Cloutier et al. 2006). Apaf-1 IRES regulation during stress and apoptosis will be addressed in Chapter 3. Therefore IRES-mediated synthesis allows the cell to direct spatial and temporal responses depending on the severity of the insult.

*Alternative eIF4F assembly*. Of the eIF4 factors, eIF4E and eIF4G are specifically involved in mRNA recruitment to ribosomes and have been characterized in a variety of species. At least three eIF4E isoforms exist in human, termed eIF4E-1, 4EHP and eIF4E-3; three in *Arabidopsis thalania*; eight in *Drosophila melanogaster* and five in the nematode worm, *Caenorhabditis elegans* (Hernandez and Vazquez-Pianzola 2005). In addition, nematode eIF4Es (IFEs) have specialized roles in certain cell types and in certain stages of development. For example, IFE-1 and IFE-3 are expressed in the *C. elegans* gonad, but only IFE-3 is essential for embryonic viability (Keiper, Lamphear et al. 2000). IFE-1, on the other hand, is sequestered in germ (P) granules that contain maternal mRNA and plays a vital role in spermatogenesis (Keiper, Lamphear et al. 2000; Amiri, Keiper et al. 2001; Henderson, Cronland et al. 2009). eIF4E isoforms with unique individual cellular and subcellular distributions may therefore confer selective recruitment of mRNAs in order to affect the translational control of gene expression. Interestingly, all of these isoforms must presumably exert their translational functions through one eIF4G in worms, IFG-1 (see below). These studies suggest that the regulation of translational control by many eIF4F members may enable alternative initiation complexes to regulate gene expression in specific tissues and/or at different stages of development.

In human cells, there are three isoforms of eIF4G that likewise involve multiple levels of translational control during growth (cell size), proliferation (cell number), and apoptosis (cell death). eIF4GI and eIF4GII are eIF4E binding isoforms that are encoded by separate genes and

are broadly co-expressed (Figure 1.3)(Imataka and Sonenberg 1997). A third shorter eIF4G isoform, p97/NAT1/DAP5, contains domains which interact with both eIF3 and eIF4A, but not the N-terminal eIF4G domain which binds eIF4E. p97 was originally described as a general repressor of translation, but has recently been shown to promote translation of cell cycle and apoptotic mRNAs containing an IRES (Hundsdoerfer, Thoma et al. 2005; Lee and McCormick 2006). The eIF4G protein can be divided into three modular domains based on characterized structure: N-terminal, central, and C-terminal. The N-terminus binds to PABP and eIF4E and thus coordinates the 5' cap and 3' poly (A) tail of an mRNA to circularize it, causing a synergistic interaction in translation initiation. A hinge region links the N-terminal domain to the central domain, the region most highly conserved among all eIF4G-like proteins (MIF4G), which contains binding sites for eIF3, eIF4A, and mRNA (Lamphear, Kirchweger et al. 1995). The C-terminal domain contains another binding domain for eIF4A and Mnk-1 kinase.

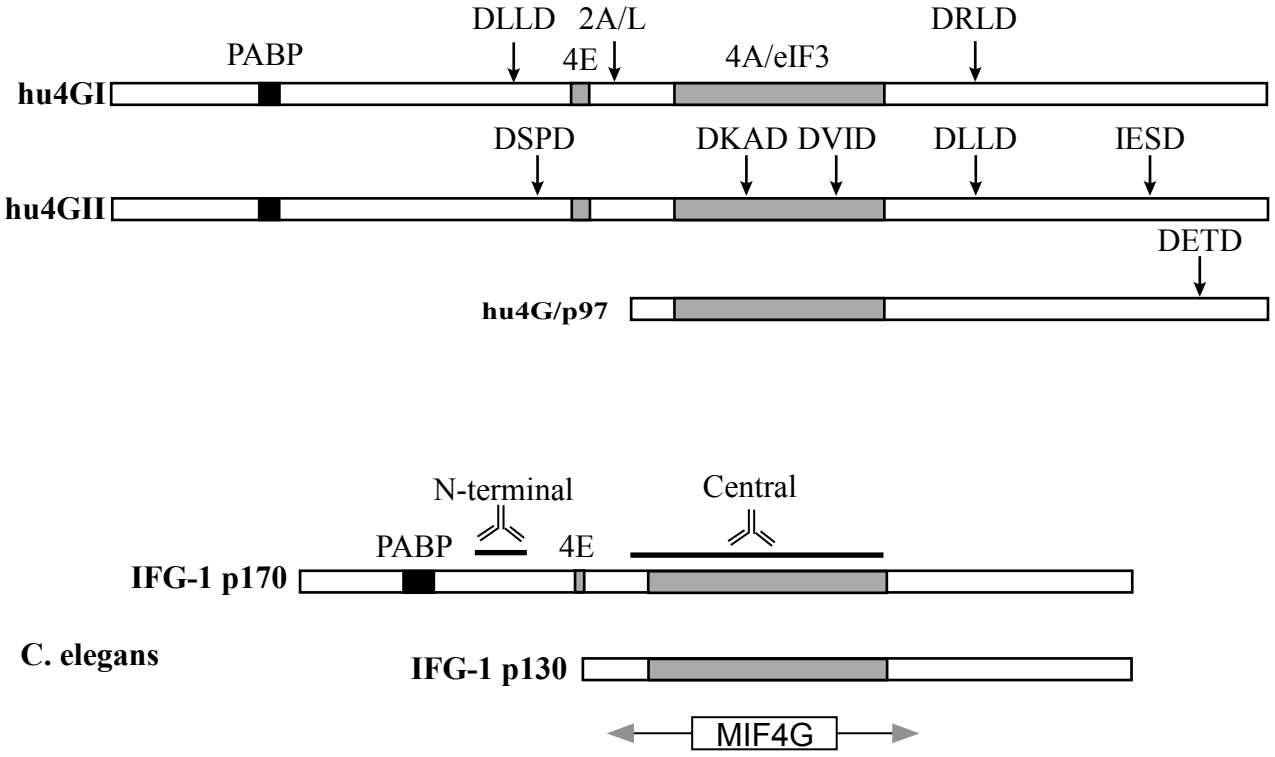
The recent discovery of minor variations in eIF4G structure suggests additional cellular mechanisms which may alter translation initiation activity. For example, human eIF4GI is encoded by at least seven distinct mRNAs that arise from three alternate promoters and numerous alternative splicing events, all of which contribute to the differences in the N-terminal domain (Antonelli, Farwell and Keiper; unpublished data (Bradley, Padovan et al. 2002; Han and Zhang 2002; Byrd, Zamora et al. 2005). There are three promoters (α, β, y) that generate seven transcripts from alternative splicing of the first eight exons (Coldwell and Morley 2006). Most of these isoforms originate from the α and β promoters. Interestingly depletion of the α/β transcripts increases translation of y mRNAs which lack the sequences encoding the PABP binding site. Concomitantly, post-translational modifications including N-glycosylation, phosphorylation, and ubiquitylation also add to heterogeneity in functional potential between

isoforms. For example, eIF4GII but not eIF4GI phosphorylation is increased during serum starvation leading to a decrease in translation during G2/M of the cell cycle by an unknown mechanism. While the complexity of eIF4G isoform representation is growing, it remains unclear how these alternative structures affect initiation complex formation or what biological requirement necessitates them.

*eIF4G and cancer***.** Increased eIF4G activity has been shown to enhance cell cycle and proliferation. This stimulation is just one of several characteristics attributed to cancer. The onset of increased protein synthesis in affect allows oncogenic cells to support their rapid proliferation (Caraglia, Budillon et al. 2000). The modulation of eIF4 factor activity can affect the regulation of growth-related mRNAs such as c-Mos, cyclin B1, and cdk2 that are essential for cell cycle progression (Keiper and Rhoads 1999). Overexpression studies have shown that human eIF4GI is involved in tumorigenesis (Fukuchi-Shimogori, Ishii et al. 1997; Bauer, Brass et al. 2002). The pathology of aggressive inflammatory breast cancer (IBC) has also been attributed to overexpression of eIF4GI. The transformation of breast cancer cells resulted in the IRES-dependent translation of p120 (E-cadherin interacting protein) which promoted increases in tumor growth (Silvera and Schneider 2009). Squamous cell lung carcinoma (SCC) accounts for 75-80% of all malignant lung tumors in the United States (Bauer, Brass et al. 2002). Characterization of a high percentage of SCC transformed cell lines show that increased eIF4F complex formation enhances expression of growth related mRNAs. These mRNAs, which include numerous cyclins, c-Myc, FGF, ODC and VEGF show a high degree of secondary structure in their 5' UTR's. This extensive secondary structure causing limited progression of the complex along the mRNA and requires increased helicase activity to be unwound during AUG scanning and initiation. It is therefore clear, from these findings, that limiting or increasing

**Figure 1.3 Diagram of human and *C. elegans* eIF4G proteins.** Three human eIF4G genes (hu4GI, hu4GII, hu4G/p97) exist in human encoding the three protein isoforms depicted. The *C. elegans* eIF4G protein isoforms (IFG-1 p170 and p130) which are derived from a single gene are also shown along with sites of recognition by IFG-1 N-terminal and central domain antibodies. Binding sites for eIF4E, PABP, and the conserved eIF3/eIF4A domain are also indicated (MIF4G). eIF4G is susceptible to both apoptotic and viral protease digestion to alter the mechanism of protein synthesis. Cleavage sites for both caspase-3 and picornaviral 2A/L protease are shown.

# Figure 1.3


DLLD
2A/L
DRLD
PABP
4E
4A/eIF3
hu4GI
DSPD
DKAD DVID
DLLD
IESD
hu4GII
DETD
hu4G/p97
N-terminal
Central
PABP
4E
IFG-1 p170
C. elegans
IFG-1 p130
MIF4G

the number of interactions between eIF4G and other factors, can alter dramatically both the rate of protein synthesis and the types of proteins made.

*eIF4G and viral infection***.** Several modifications to eIF4G and its activity within the cell can affect the types and amounts of mRNAs that are recruited. For instance, viruses have adapted mechanisms to hijack the host translational machinery during replication of its genome. This is designed to maximize efficiency and promote the selective translation of viral mRNAs over intracellular host transcripts (Alvarez, Menendez-Arias et al. 2003).

The picornaviruses (rhinovirus, coxsackievirus) encode 2A proteases that cleave both human eIF4GI and eIF4GII separating the proteins into an N-terminal domain that binds eIF4E from the C-terminal domain that binds eIF4A and eIF3 (Lamphear, Kirchweger et al. 1995). This results in the loss of cap binding activity, leaving only the RNA helicase and ribosomal binding activities (Ohlmann, Rau et al. 1996; Keiper and Rhoads 1997; Hellen and Sarnow 2001). Loss of cap-binding activity correlates with a dramatic shutdown of host cellular protein synthesis (Etchison, Milburn et al. 1982; Krausslich, Nicklin et al. 1987). The resulting eIF4G central domain and carboxy terminus is still capable of supporting translation in the absence of the N-terminal domain (Leibowitz and Penman ; Perez and Carrasco 1992; Aldabe, Feduchi et al. 1995; Irurzun, Sanchez-Palomino et al. 1995; Keiper and Rhoads 1997; De Gregorio, Preiss et al. 1999). Translation of viral mRNAs, which contain internal ribosome entry sites (IRESs) is therefore sustained via cap-independent initiation. Interestingly, eIF4G cleavage does not completely account for translation shutdown. Studies have shown that cellular translation is only partially inhibited in the presence of viral replication inhibitors despite significant processing of eIF4GI (Perez and Carrasco 1992; Irurzun, Sanchez-Palomino et al. 1995; Prevot, Darlix et al.

2003). Concomitantly, 2A protease digestion of eIF4GI in *Xenopus oocytes* also results in modest decreases in protein synthesis (Keiper and Rhoads 1997). This discrepancy may partly be attributed to the slower cleavage of eIF4GII and PABP which could temporarily compensate for host synthesis for longer periods (Prevot, Darlix et al. 2003). Thus, viral stress initiates a multi-pronged attack of cellular translation apparatus through eIF4G in order to promote a modal switch from cap-dependent to cap-independent initiation.

*eIF4G and apoptosis*. Likewise, eIF4G undergoes similar biochemical alterations during apoptosis. During apoptosis, translation is dramatically reduced (~70%) following eIF4G cleavage (Morley, McKendrick et al. 1998). All three eIF4G proteins (I, II, and p97) are cleaved by apoptotic caspases (Figure 1.3) (Marissen and Lloyd 1998; Henis-Korenblit, Strumpf et al. 2000; Marissen, Gradi et al. 2000). Like picornaviral 2A proteases, caspase cleavage occurs in close proximity to the eIF4E binding site of eIF4GI/-II (Figure 1.3). However, the processed fragments generated by caspases do not cause bifurcation of the protein. The aspartate-specific protease, caspase-3 was shown responsible for eIF4G processing into several distinct fragments (Morley, McKendrick et al. 1998; Bushell, McKendrick et al. 1999; Marissen, Gradi et al. 2000). Caspase-3 proteolytically digests eIF4GI at two sites ($DLLD^{532}$ and $DRLD^{1176}$) resulting in three fragments (Figure 1.3) (Bushell, Poncet et al. 2000). Of the three fragments, the 76 kDa middle fragment of eIF4G (M-FAG) has been implicated in stimulation of IRES-mediated translation during cell death (Nevins, Harder et al. 2003; Hundsdoerfer, Thoma et al. 2005; Marash and Kimchi 2005). eIF4GII cleavage is believed to completely disrupt function (Marissen 2000). Cleavage of the he eIF4G isoform p97/NAT1/DAP-5 ($DETD^{790}$) generates a 86 kDa fragment that has also been shown to promote the translation of IRES-containing apoptotic mRNAs such as p97, Apaf-1, and XIAP (Henis-Korenblit, Shani et al. 2002). While both M-FAG and p86

contain the core ribosome recruitment domain, they differ in the capacity to associate with eIF4E (Marash 2005). It is therefore not fully understood how M-FAG contributes to the induction of IRES-mediated translation during apoptosis. IRES elements can be found in many survival factors, oncogenes, and other proteins involved in the critical control of apoptosis such as c-myc, VEGF, Bcl-2, p97, XIAP and Apaf-1 (Holcik, Sonenberg et al. 2000). However, the apoptotic cascade likely requires a stage-dependent influence on specific IRESs depending on the integrity of 4G and the severity of insult (Marash and Kimchi 2005; Graber and Holcik 2007). The relationship between eIF4G cleavage and the cell death pathway will be further addressed in Chapter 4. Despite extensive *in vitro* characterization of eIF4G cleavage products and their effects on cap-independent initiation, much is still unknown about the signals that potentiate stress-specific IRES mRNA translation during apoptosis. Furthermore the translational switch during apoptosis and its genetic influence on the caspase cascade have not been studied due the complexity of human eIF4G isoforms and corresponding limitation of cell lines. The studies of this dissertation revolve around a whole animal approach using the nematode *Caenorhabditis elegans* to address the relationship between eIF4G isoforms and the decision to adopt an apoptogenic fate.

### *C. elegans as a Model Organism for Gene Regulation in Apoptosis*

*C. elegans* is a simple eukaryote that is used to study complex multicellular eukaryotic processes during development (Riddle, Blumenthal et al. 1997). Although anatomically simple, the worms are directly comparable to higher vertebrate systems in that they transition through various phases including ovulation, fertilization, gastrulation, growth of juvenile forms, and sexual maturation of adults. The nematode can exist as a self-fertilizing hermaphrodite or sperm producing male (Figure 1.4). Both forms go through rapid life cycles, maturing through four

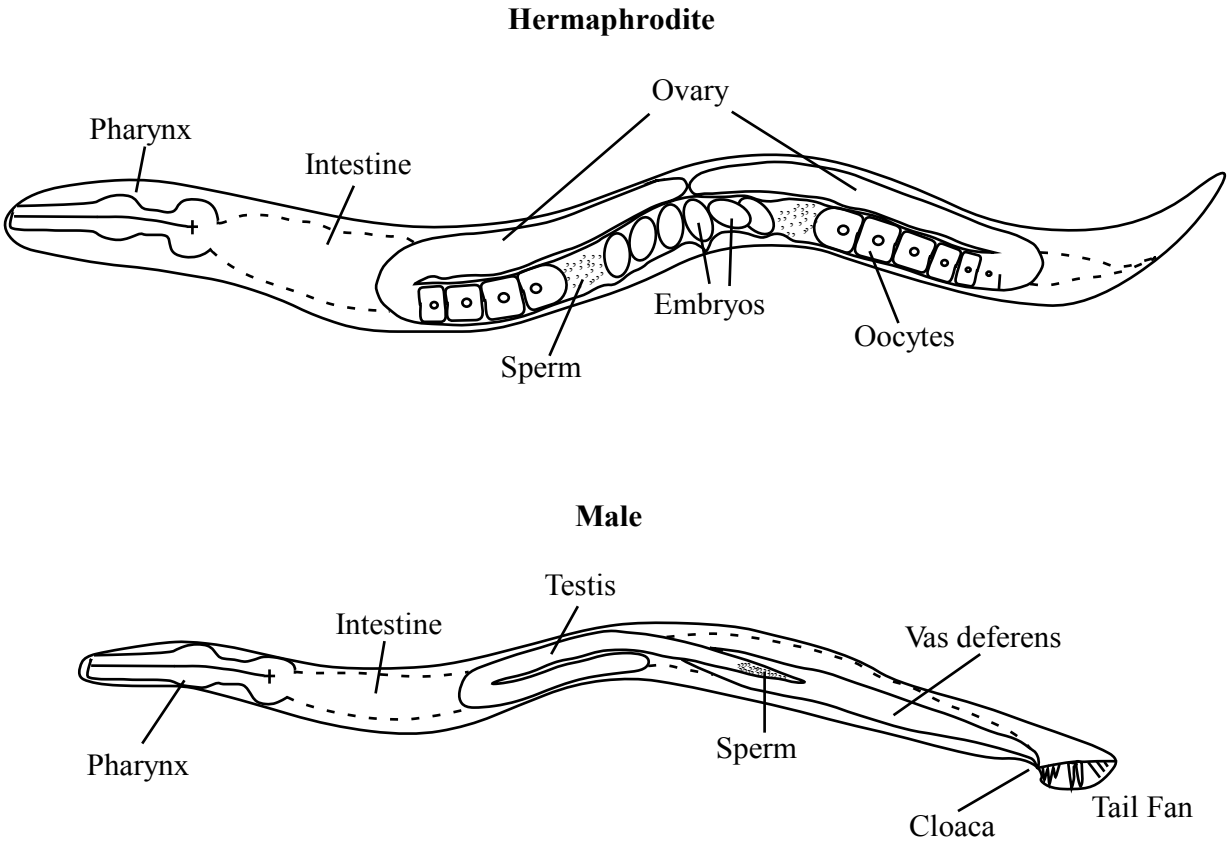
larval stages (L1-L4) to adulthood in two days at 20$^{o}$C (Riddle, Blumenthal et al. 1997). Perhaps one of the most interesting characteristics of these worms is their relative optical transparency, which allows for easy microscopic observation of nearly all organ systems. In the case of adult hermaphrodites, this is especially beneficial to observe sexual maturation and gamete formation.

The hermaphrodite reproductive system consists of two functionally independent anterior and posterior U-shaped arms. Each reflexed arm contains a distal portion containing immature oocytes, followed by a more proximal portion containing maturing oocytes, and a spermatheca (organ containing sperm) connected to a common uterus containing fertilized eggs (Figure 1.4). Gametogenesis begins by producing sperm from the original germ cells during late larval stages and then switches to oocyte production as an adult (Schedl 1997). This is important for the purposes of this study as oogenesis is a process requiring massive cell growth and deposition of maternal organelles, mRNAs and proteins, thus necessitating increased protein synthesis. This allows for observations of oocytes that are growing and stockpiling cytoplasmic contents and transcripts for later developmental stages. The cellular morphology during oogenesis is marked by the linear progression of immature germ cells in the distal gonad to maturing oocytes in the proximal gonad (Schedl 1997). Germ cells in the distal gonad are defined as a nucleus, its cytoplasm, and semi-enclosed membrane (Hansen, Hubbard et al. 2004). In adult hermaphrodites, the distal most germ cells serve as a stem cell population led by a distal tip cell (DTC). As distal germline nuclei progress proximally during meiotic prophase I, they become fully enclosed by membrane, cell and nuclear volumes increase, and chromosomes condense. Meiosis pauses and cells/nuclei do not complete divisions until meiotic maturation has occurred. This delay mechanism during prophase is a common feature of oocyte meiosis in animals (Navarro, Lehmann et al. 2001). Wild type oocytes at this stage contain six bivalent or

**Figure 1.4 Schematic of the *Caenorhabditis elegans* model system.** The nematode**,** *C. elegans* exists as both hermaphrodites and males. Worms contain a pharynx that contains a simple nervous system, a rudimentary gut, and egg and sperm producing bilobed gonad (hermaphrodites) or sperm producing unilobed testes (males). The worm has proven beneficial to the study of cellular differentiation and apoptosis due to their transparency.

# Figure 1.4

## *Caenorhabditis elegans*



homologous pairs of chromosomes in the proximal gonad (Albertson, Rose et al. 1997). Meiotic maturation involving nuclear envelope breakdown and ovulation is marked by a transition from diakinesis to metaphase of meiosis I (Schedl 1997). Upon fertilization, subsequent divisions of meiosis I and II occur in the uterus followed by the onset of a rapid mitotic cell cycle.

The germline is also useful in studying programmed cell death. Apoptosis is a highly conserved process that plays a crucial role in cellular homeostasis during both oogenesis and embryonic development (Baum, St George et al. 2005). Cells undergoing apoptosis have been shown to proceed through three distinct stages: the specification of the dying fate, execution of cell death, followed by recognition and engulfment of the dying cell (Fraser 1999). Apoptosis has been extensively characterized in the worm. The *ced* (cell death abnormal) genes are responsible for the execution and engulfment of cells fated to die in the worm (Hengartner 1997). They consist of the survival factor CED-9, a Bcl-2 homolog, the pro-apoptotic protein Apaf-1 homolog, CED-4, that assembles the apoptosome, and the caspase-3 homolog, CED-3 (Twomey and McCarthy 2005) (Liu and Hengartner 1999); (Xue, Shaham et al. 1996). Apoptotic cells are then phagocytized or engulfed in the presence of CED-1 (Zhou, Hartwieg et al. 2001). Over half of all cells that begin oogenesis are eliminated by programmed cell death during meiotic growth in the gonad (Gumienny, Lambie et al. 1999). These apoptotic cells termed "corpses" appear as highly refractile button-like objects. (Hengartner 1997). In the germline, corpses are phagocytosed and degraded by their undifferentiated sister cells. It has been suggested that this death is a mechanism used to eliminate excess germ cell nuclei. These eliminated germ cells may provide the same function as that of "nurse cells" in vertebrate ovaries by synthesizing cytoplasmic components required by those oocytes that will survive to be fertilized. Studies show high levels of transcriptional activity occurring in these cells and that the resulting

transcripts are eventually exported to the common cytoplasmic core (Gumienny, Lambie et al. 1999). Much evidence indicates that apoptosis is critical for normal homeostasis and establishment of tissue architecture. Overall the relative ease of assaying the many developmental pathways characterized in this model organism provided a versatile means to study eIF4G and its role in translational control during cell differentiation, tumorigenesis, and apoptosis.

# CHAPTER 2: TWO STRUCTURALLY DISTINCT eIF4G (IFG-1) ISOFORMS IN *C. ELEGANS*

## *Introduction*

Selective recruitment of mRNAs to ribosomes can affect the regulation of protein synthesis levels in specific cell types (Morley and Coldwell 2008). The eIF4F complex (eIF4A, eIF4E, and eIF4G) catalyze the joining of mRNAs to the 40S small ribosomal subunit. Therefore the modulation of eIF4F factors and the dynamic interactions facilitating its assembly can be important for exerting control mechanisms that require rapid stimulation of gene expression from mRNA pools (Coldwell and Morley 2006). The eIF4F scaffold protein, eIF4G, is a central hub in the recruitment phase during translation initiation that acts as a molecular bridge between the mRNA and the ribosome. Dysregulation of eIF4G either through overamplification of the gene or ectopic overexpression has been shown to induce aberrant proliferation of cells (Hayashi, Nishimura et al. 2000; Comtesse, Keller et al. 2007).

Configurations of the eIF4F complex that promote the cap-dependent mode of translation also leads to mRNA selection of a subset that lead to cell growth, proliferation, and often oncogenesis. Studies in tumors and tumor cell lines have shown that the types of mRNAs selected for translation are directly affected by the levels of eIF4E and eIF4G, alone or in concert with one another (Schneider 2007). Increased activity or expression level of translation factors stimulate the synthesis of several growth and pro-survival proteins including vascular endothelial growth factor (VEGF), ornithine decarboxylase (ODC), c-Myc, and fibroblast growth factor 2 (FGF-2) (Kevil, Carter et al. 1995; Kevil, De Benedetti et al. 1996; Rousseau, Kaspar et al. 1996; Schmidt 2004). *De novo* synthesis of such proteins modifies the metabolism of the cancer cell and concomitantly increases angiogenesis, metastasis, and insensitivity to apoptosis. In a recent

study in inflammatory breast cancer cells, depletion of human eIF4GI was shown to reduce the preferential translation of metastatic proteins like E-cadherin/p120, without affecting global protein synthesis (Silvera and Schneider 2009). These results indicate that increased levels of eIF4GI contribute significantly to the hyper-malignant phenotype and synthetic competence of oncogenic cells. Evidence from these studies also indicates that depletion of the overexpressed eIF4GI causes a reversion to the normal growth phenotype and normal protein synthetic patterns. Therefore understanding how modular disruptions of eIF4G activity(ies) affect the translation of certain mRNAs could aid in the design of novel adjuvant therapies that selectively sensitize malignant cells to apoptosis while minimizing side effects in normal cells.

Physiological modulation of eIF4G activity appears to be complicated by the fact that it exists as multiple isoforms in several species (Prevot, Darlix et al. 2003). However, little is known of how structural differences between these isoforms contribute functionally to translation initiation (Coldwell and Morley 2006). Studies have shown that these different isoforms can participate in overlapping but distinct pathways under conditions where translation is compromised. For example, silencing of eIF4GI produces impaired mitochondrial activity and AMPK activation (Kundu and Thompson 2008; Ramirez-Valle, Braunstein et al. 2008). However, the depletion of eIF4GII and p97 show no effects on cellular bioenergetics. In addition, eIF4GII is specifically recruited to translation complexes upon megakaryocyte differentiation and is subject to selective phosphorylation events during G2/M phase of the cell cycle that are not observed for eIF4GI (Pyronnet, Dostie et al. 2001; Qin, Raught et al. 2003; Caron, Charon et al. 2004). While providing little description of their unique activities in protein synthesis, these and numerous other observations suggest that eIF4G isoforms are not interchangeable.

It is becoming clear that cells require acute protein synthetic responses to cell stress and apoptosis (Graber and Holcik 2007). Many of the initial responses following a cellular insult involve modifications of the eIF4 factors that mediate mRNA recruitment during periods of crisis. Cleavage of human eIF4GI, eIF4GII and p97 during viral infection and apoptosis alter the recruitment of mRNAs to meet the synthetic needs required to promote spatial and temporal changes in the cell. For example, picornaviral genomic RNAs also serve as mRNAs but lack mRNA 5'-caps and have adapted mechanisms to usurp the host translational complex in order to synthesize new viral proteins (Alvarez, Menendez-Arias et al. 2003). The viral 2A protease processes eIF4GI eliminating the cap-binding N-terminus and using the ribosome binding central domain to recruit 40S subunits for translation (Lamphear, Kirchweger et al. 1995; Ohlmann, Rau et al. 1996). Likewise, during somatic cellular injury, eIF4GI and p97 are cleaved to generate fragments that only support the synthesis of apoptotic proteins that do not require cap-mediated translation. Perhaps, most surprising is that selective recruitment of mRNAs particularly during apoptosis is not an all or none response but rather a hierarchical network designed to adapt to various stressors as well as the amount of time under duress (transient vs. chronic signals). For example, during etoposide (DNA topoisomerase II inhibitor) treatment, anti-apoptotic HIAP2 IRES-containing mRNAs are not activated (Holcik and Sonenberg 2005). However, during ER stress-induced apoptosis, caspase cleavage of p97 enhances the translation of the HIAP2 IRES presumbably to allow cellular recovery (Warnakulasuriyarachchi, Cerquozzi et al. 2004). Thus, there appear to be multiple levels of translation regulation that depend upon the integrity of eIF4G isoforms and their representation in a cell under a specific stress.

The characterization of human eIF4GI has shown that it can be regulated at the transcriptional, translational, and post-translational levels. Human eIF4GI exists as a modular

protein encoded by three genes and multiple alternative mRNA splice forms. The gene also has three promoters and an IRES which provide additional regulation. Recently, we discovered the existence of multiple isoforms of the *C. elegans* eIF4G (IFG-1). The two major isoforms (p170 and p130) differ in their N-termini and presumably in the capacity to assemble in cap-binding translation complexes (Figure 1.3). The worm genome encodes just a single gene (*ifg-1*) for eIF4G, in contrast to mammals, plants, flies, frogs, fish and even yeasts (Prevot, Darlix et al. 2003; Contreras, Richardson et al. 2008). This chapter will address the sequence and structural interactions which distinguish individual isoforms of eIF4G and their physiological consequences in the context of initiation complex assembly and germ cell development versus apoptosis.

## *Results*

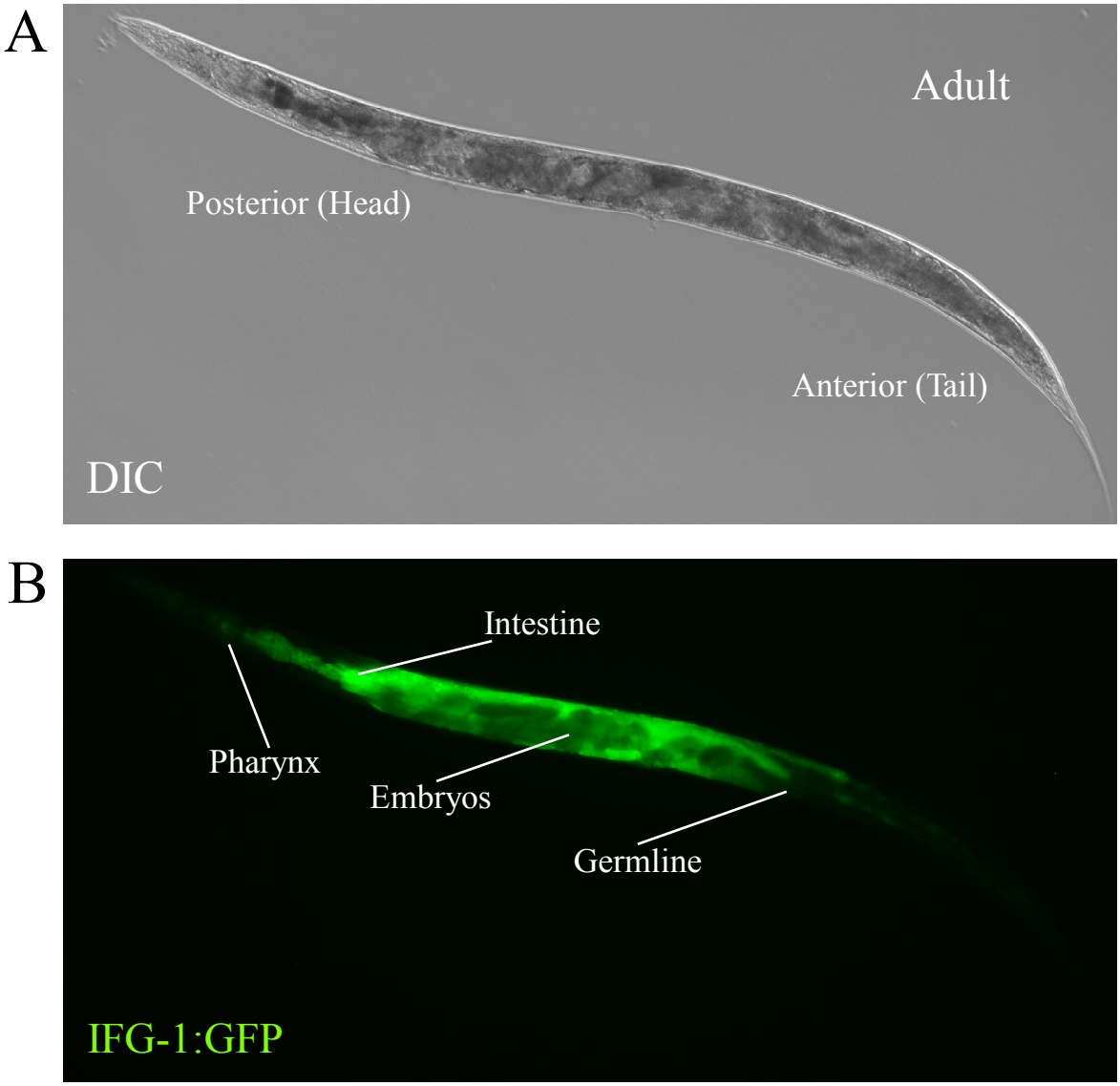
### *Developmental expression of IFG-1*

The expression pattern of proteins can provide valuable information on how its movement and localization influences physiological processes in the cell. In order to understand how IFG-1 is involved in the biological assembly of *C. elegans*, it was necessary to determine its expression and localization, both temporally and spatially. We expressed an in-frame translational fusion to green fluorescent protein (GFP) containing the full length (nine exons) *ifg-1* gene under the control of its endogenous promoter. Introduction of transgenic DNA was performed by microinjection of extrachromosomal arrays directly into the common cytoplasm of the worm gonads (Mello and Fire 1995). This method has the advantage of delivering multiple copies of the transgene that will be shared by many progeny. The recipient *unc-119* strain carries a mutation that creates uncoordinated movements but normal development and fertility.

When the transgene is injected into these worms along with a selectable marker, which in this case was an *unc-119* rescue plasmid (wild type gene), transgenic progeny are easily identified by the return of natural sinusoidal mobility. Positive transgenic F1 daughters of injected hermaphrodites were selected for microscopy based on mobility. These hermaphrodite worms appeared to exhibit low fluorescence but have ubiquitous expression of IFG-1 (Figure 2.1). This observation was expected considering the transgene encodes both IFG-1 isoforms. However there were clearly areas of enriched IFG-1 expression that included the pharynx and gut. The germline and uterus displayed extremely low or no GFP expression. This is consistent with previous reports of germline silencing of extrachromosomal arrays which mimics epigenetic regulation during X chromosome assembly (Schaner and Kelly 2006). Finally, many progeny had no detectable expression which likely indicates that these worms do not carry the heritable array. The heritability and low expression due to genetic instability thus ensures that observations of the IFG-1 transgene could not be properly addressed. In order to stabilize heritability of *ifg-1:gfp* and improve expression among individual worms, the transgene was integrated into the *C. elegans* genome. Ultraviolet radiation was used to induce chromosomal breaks which facilitate ligation of transgenic copies during the repair process. As a result of radiation-induced non-specific mutations, worm chromosomal integrity had to be subsequently restored by backcrossing at least six times with wild type males. Gross observations indicated that the integrated transgene was also broadly expressed in all worms, yet also showed increased expression in the tissues previously described. Upon closer examination of developmental stages, we observed that expression of IFG-1 appeared during gastrulation stages (Figure 2.2A). However there was no expression in early embryos (1-2 cell) suggesting that repression mechanisms during the first few cleavage events may account for this delayed expression (see

**Figure 2.1 IFG-1:GFP is broadly expressed.** DIC and fluorescence images of F1 progeny immediately observed following injection of the transgenic array. Worms had low but ubiquitous expression of IFG-1:GFP throughout somatic tissue (gut, pharynx, cuticle). There was no or very low expression in the germline presumably due to mitotic instability of the transgene. Despite the low expression, IFG-1 appeared enriched in the intestine and pharynx which are known sites of protein trafficking and neuronal function.

# Figure 2.1


A
Adult
Posterior (Head)
Anterior (Tail)
DIC
B
Intestine
Pharynx
Embryos
Germline
IFG-1:GFP

uterus of hermaphrodite, Figure 2.3). Upon hatching, the first larval staged (L1) worms exhibit IFG-1:GFP expression throughout the body with an enrichment in pharynx which comprises almost half of the body plan and the tissues encompassing the intestine (Figure 2.2A). This is expected as the majority of ventral and motor neurons which surround the pharynx and throughout the body plan develop during this hatchling phase (Sulston and Horvitz 1977). The germline, marked by the precursor cells Z1-Z4, were difficult to discern at this stage. The second stage (L2) showed increased growth of the body with an enrichment of expression in the pharynx and gut relative to the outer cuticle (Figure 2.2B). Interestingly, the germline was observed and appeared to be devoid of expression indicating that germline silencing may still be present. There were no dramatic changes in expression among somatic tissues during late larval stages (L3/L4) and the germline still displayed no expression (Figure 2.2C). During adult stages, expression was enriched in the pharynx, gut, vulva, and the spermatheca (Figure 2.3). The increased expression of IFG-1 in the sperm-containing organ was a surprise considering the expression in oocytes of the germline was non-existent. It is not clear however whether this expression is attributed to the sperm or somatic organ containing them. Interestingly, adult males also exhibited enriched expression in the pharynx and intestine as well as a sensory ray located within the male tail fan (data not shown). These results may indicate a role for IFG-1 in sperm activity and copulatory sensation. Nevertheless, it would appear that enrichment of IFG-1 could contribute to specialized roles in certain organs, i.e. pharynx and intestine, hermaphrodite spermatheca, and male tail relative to the body hypodermis. The GFP expression of IFG-1 arises presumably from the synthesis of both p170 and p130. As a result, it is not possible to determine whether isoform protein levels are differentially enriched during each developmental stage. *C. elegans* adults develop over a 72 h period at 20$^{o}$C from four larval stages following

**Figure 2.2 Expression of IFG-1:GFP throughout development.** Following integration of the *ifg-1:gfp* transgene into the worm genome, several developmental stages (embryo, L1, L2, L3) were analyzed for any changes in expression. Expression appeared higher than non-integrated worms but still relatively weak. Uniform expression was observed for all stages (A) eggs, (B) L1, (C) L2, and (D) L3. The germline (shown outlined as white dashes) showed no GFP expression indicating that the integrated transgene still experiences silencing.

# Figure 2.2

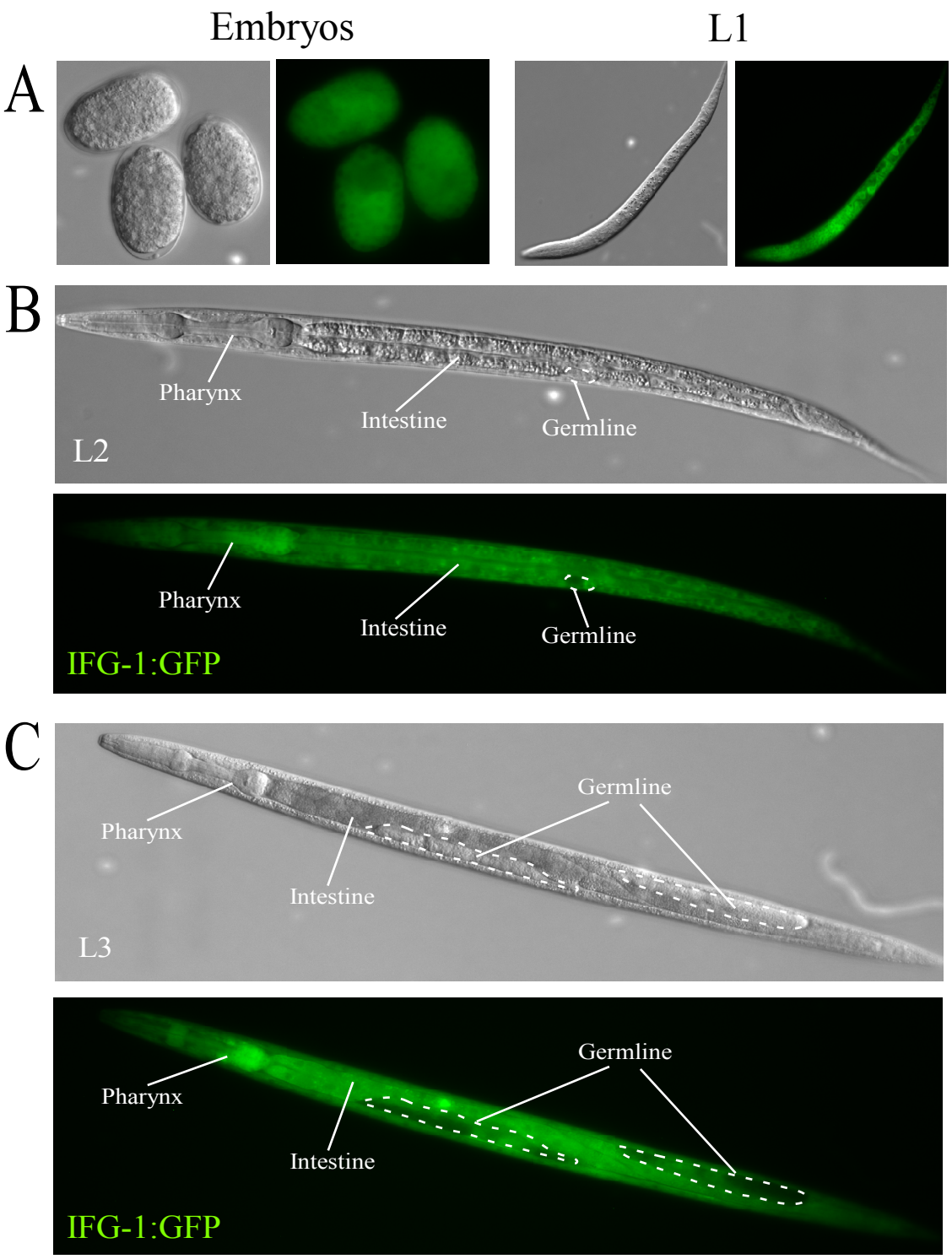
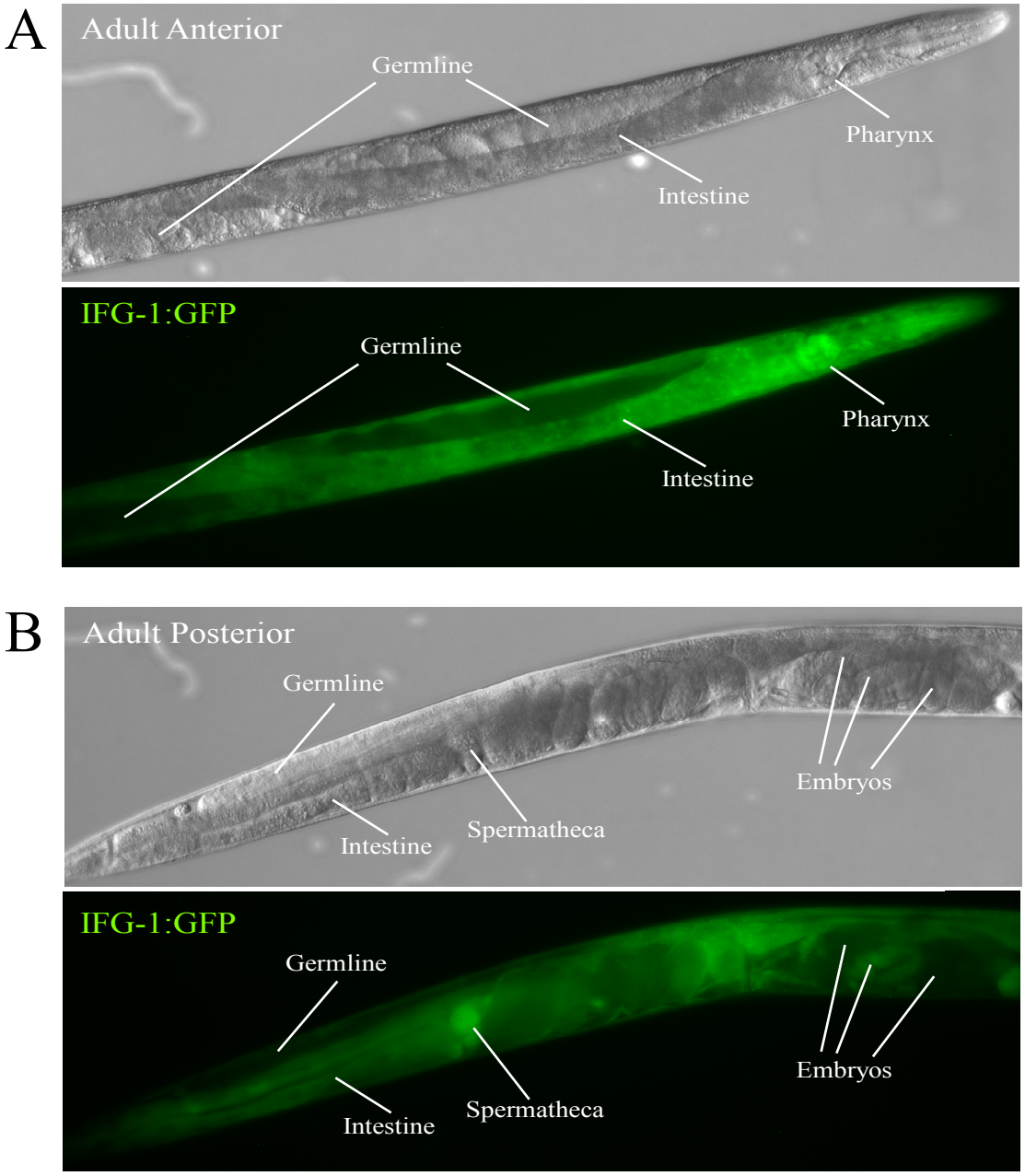

Embryos
L1
A
B
Pharynx
Intestine
Germline
L2
Pharynx
Intestine
Germline
IFG-1:GFP
C
Germline
Pharynx
Intestine
L3
Germline
Pharynx
Intestine
IFG-1:GFP

**Figure 2.3 IFG-1:GFP enrichment in pharynx, intestine, and sperm organs.** Adult expressing IFG-1:GFP shows expression in all cells except the germline. There appears to be higher relative to somatic body tissue, expression in the pharynx, intestine, and spermatheca. A change in IFG-1 expression from none in early embryos to moderate in later embryos was observed in the uterus (arrows).

# Figure 2.3

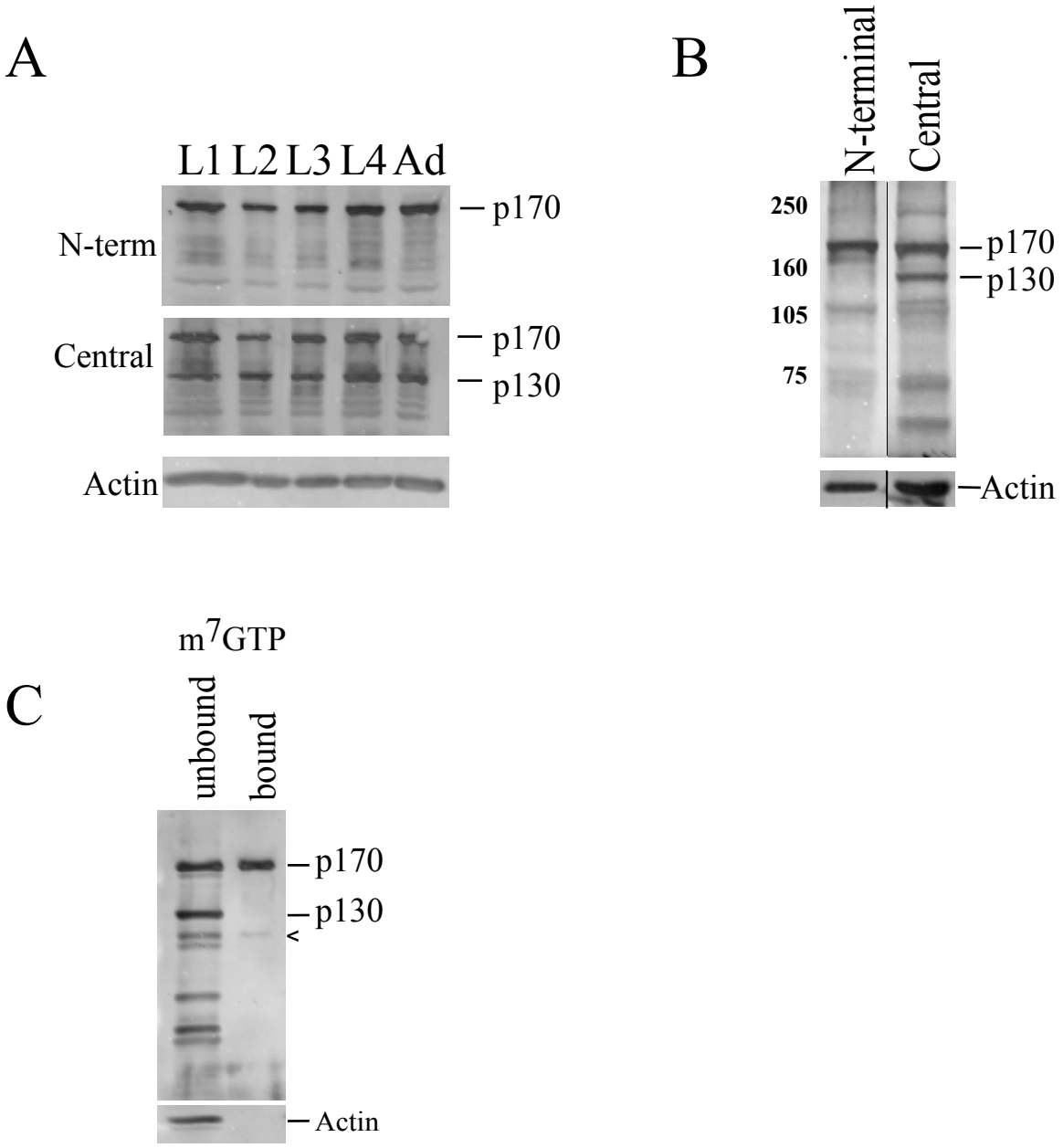

A
Adult Anterior
Germline
Pharynx
Intestine
IFG-1:GFP
Germline
Pharynx
Intestine
B
Adult Posterior
Germline
Embryos
Intestine
Spermatheca
IFG-1:GFP
Germline
Embryos
Intestine
Spermatheca

embryogenesis (L1-L4), much like other nematodes. To determine whether IFG-1 p170 and p130 are differentially expressed throughout development, western blotting, using either an IFG-1 N-terminal or central domain antibody, was performed using wild type extracts from each larval stage. Differences in protein level among larvae were compared to ascertain whether expression is consistent throughout development or changes during one of the four stages (Figure 2.4). IFG-1 p170 was detected at all developmental stages using antisera against the N-terminal (p170) domain. However levels appeared slightly less in L2/L3 staged worms that are consistent with previously published reports (Pan, Palter et al. 2007). Worm extract probed with the central IFG-1 antibody which overlaps the highly conserved MIF4G domain (p130), also showed no difference in isoform distribution (except L2) between larvae (Figure 2.4A). Interestingly there is a slightly lower level of the p130 isoform in early larval stages when compared to late larval and adult stages. This may indicate an acute requirement for the shorter isoform during tissue expansion and germ cell proliferation during these later stages (L4/Adult). Coincidentally, Brett Keiper showed that when the distribution of isoforms from worms devoid of germline was compared with wild type worms (containing both somatic and germline), there was no observed enrichment in either IFG-1 p170 or p130 (Contreras 2008). This indicated that the IFG-1 isoform protein levels do not change during larval development and are not enriched in differentiated (somatic) or non-differentiated (germline) cell types. The western blot results correlate with the broad protein levels observed in IFG-1:GFP worms indicating that there is ubiquitous expression of both isoforms during larval and adult stages.

Unlike human eIF4G, there is a single *ifg-1* gene in *C. elegans* encoding two major isoforms p170 and p130 (Figure 2.4B). Our evidence showed that these two major isoforms differ in their N-termini. The mammalian N-termini of eIF4G and eIF4GII possess binding sites

**Figure 2.4 Two structurally distinct isoforms of IFG-1.** (A) Western blot of whole worms for each developmental stage show no enrichment of IFG-1 for either isoform. All stages also show similar proportions of p170 and p130. (B) N-terminal and central domain IFG-1 antibodies detect two major protein isoforms. (C) mRNA cap complexes were purified by subjecting worm lysates to $m^7$GTP affinity chromatography. Immunoblotting of elution fractions were performed using the central domain antibody to detect both isoforms. Only p170 was observed in both elution fractions (bound and unbound). IFG-1 was only present in the unbound fraction. Non-specific protein shown in bound lane is likely a breakdown product (carrot).

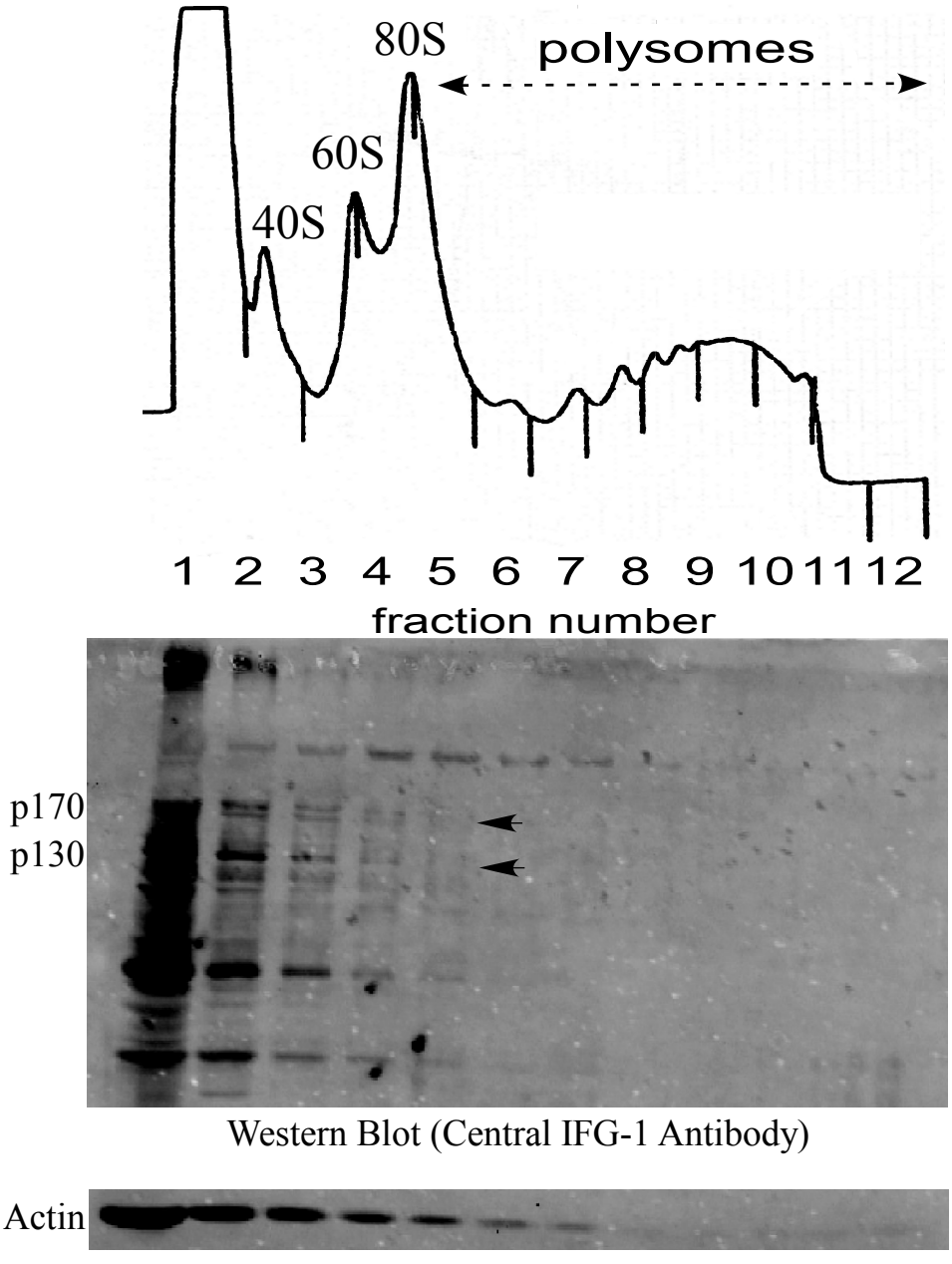
Figure 2.4


A
L1 L2 L3 L4 Ad
N-term
Central
Actin
p170
p170
p130
B
N-terminal
Central
250
160
105
75
p170
p130
Actin
C
m7GTP
unbound
bound
p170
p130
Actin

for the PABP and eFI4E required for poly (A) and cap-dependent translation. p97 lacks these sequences and alternatively participates in cap-independent protein synthesis. To determine if IFG-1 p170 and p130 both participate in in mRNA cap-mediated complex assembly, m7GTP-Sepharose affinity chromatography was performed whole worm extracts to separate mRNA cap-bound complexes. Western blotting of bound and unbound fractions using the central domain antibody showed that only IFG-1 p170 was associated with the mRNA cap-binding complex (Figure 2.4C). In contrast, all of the IFG-1 p130 remained in the unbound fraction indicating that it does not associate with any of the five eIF4E isoforms (IFEs) in *C. elegans* (all of which bind to m7GTP). These results demonstrate that the IFG-1 short and long isoforms likely assemble into different mRNA recruitment complexes. Additionally, IFG-1 p130 may be a paralog of human p97, which also lacks the eIF4E binding region and participates in cap-independent translation. IFG-1 p170 forms canonical mRNA cap-binding complexes and like human eIF4GI and eIF4GII likely associates with polyribosomal complexes. Ribosomal complexes are active centers of protein synthesis that at times require initiation factors for translation. It was therefore important to ascertain whether cap-independent IFG-1p130 associates with ribosomes. Recent evidence has shown that human p97 associates with ribosomal complexes via the eIF3/eIF4A ribosomal recruitment domain (Lee and McCormick 2006). To determine if IFG-1 proteins sediment with ribosomes, wild type worm extracts were resolved by sucrose density gradient velocity sedimentation, fractionated and then analyzed for the presence of p170 and p130 proteins by immunoblot analysis (Figure 2.5). The absorbance at 260 nm was recorded during fractionation and recorded to show the profile of non-translating (fractions 1-4), one ribosome (fraction 5), and more than one ribosome (fractions 6-12) bound. The majority of IFG-1 p170 and p130 were detected in lighter fractions (1-3). However, both were detected in

**Figure 2.5 IFG-1 p170 and p130 association with ribosomes.** Western blotting was perfomed on sucrose gradient fractions from a polysome profile of wild type worms that was kindly provided by Melissa Henderson. The distribution of IFG-1 protein in non-translating, monosome, and polysomes was detected using the central domain antibody. Chart recording containing the fraction number and ribosomal peaks for (40S, 60S, and 80S) were included to correlate detected proteins with sample fraction collected. IFG-1 p170 and p130 are shown (arrows) in subpolysomal fractions (1-5).

Figure 2.5


80S
polysomes
60S
40S
1 2 3 4 5 6 7 8 9 10 11 12
fraction number
p170
p130
Western Blot (Central IFG-1 Antibody)
Actin

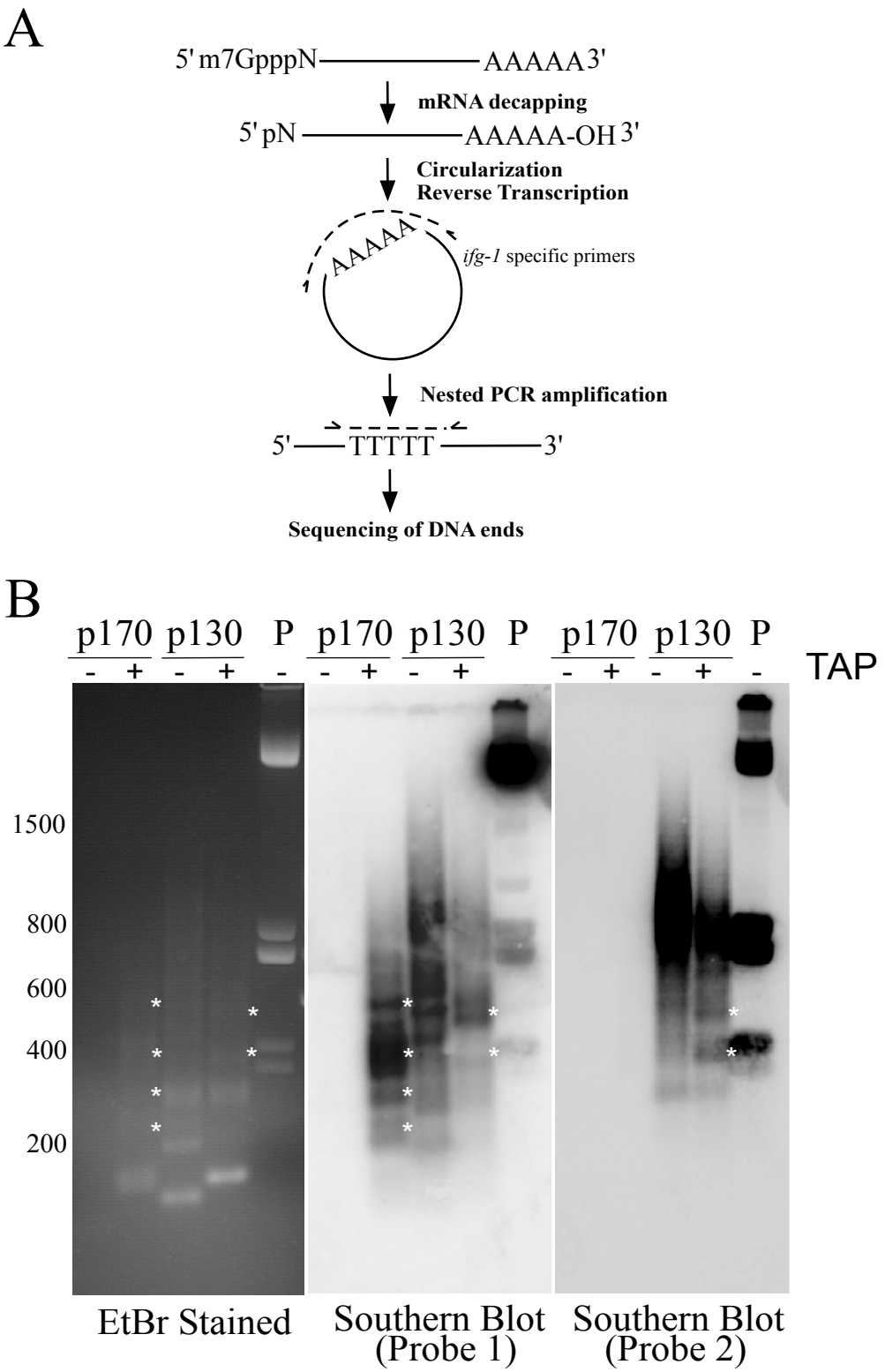
fractions containing 40S and 60S complexes but not in later heavier polysomes suggesting that IFG-1 may associate with initiating ribosomal complexes. Actin antiserum was used to account for non-specific protein aggregation and cytoskeletal association. Actin was observed in most fractions including heavier fraction which may indicate a natural association of polysomal complexes with cytoskeletal proteins. Interestingly, multiple lines of evidence have shown that polysomes and the translation machinery associate with cytoskeletal actin filaments (You, Abe et al. 1992; Hesketh 1994). It is believed that this association contributes to not only the association of polysomes with membranes but protein synthesis compartmentalization (Hesketh 1994). The fact that IFG-1 isoforms are enriched in lighter ribosomal fractions as compared to actin which is found throughout suggests that there is a more specific interaction between these translation factors and ribosomal complexes in the lighter fractions. Thus IFG-1 p170 and p130 are both likely ribosome-associated factors. However further information will have to be collected to address whether these proteins are canonical initiation factors.

Characterization of the primary sequences encoding the human eIF4G isoforms have shown heterogeneity at the 5' end of eIF4GI but little is known how this contributes to their individual function. IFG-1 p170 and p130 likewise differ in their N-terminal ends that likely define not only the cap-dependent versus –independent functions but the types of protein complexes assembled during translation. In order to better characterize the IFG-1 isoforms, we have analyzed the primary coding sequences for both mRNAs. Over thirty expressed sequence tags (ESTs) have been described encoding *ifg-1* mRNAs (Contreras 2008). This evidence indicated that there were *ifg-1* coding mRNAs differing in their 5' start sites that encodes the N-terminal third of eIF4G. Northern blot analysis confirmed two major transcripts at approximately 4500 nt and 2500 nt that correspond with the EST predictions. In addition RNase

protection was attempted to show more defined 5' ends. Using an antisense probe complementary to exons 1-6, it was discovered that long protected fragments representing alternatively spliced *ifg-1* p170 mRNAs contained end points near the 5' ends of exons 1-4 (Contreras 2008). The shorter mRNA (2500 nt) yielded a protected fragment with a mapped start site at the end of exon 4. To more precisely map the 5' and 3' ends of the *ifg-1* p170 and p130 transcripts, we performed mRNA circularized reverse transcription polymerase reaction (cRT-PCR). This method specifically amplifies the ligated ends of an mRNA and shows both the transcriptional start and 3'UTR sequences. Circularization of *C. elegans* wildtype mRNA by T4 RNA ligase was performed following removal of the 5' cap using tobacco acid pyrophosphatase (TAP) and cDNA synthesis across the ligated junction by PCR (Figure 2.6A) (Fromont-Racine, Bertrand et al. 1993; Couttet, Fromont-Racine et al. 1997; Mullen and Marzluff 2008). cRT-PCR using total worm RNA and gene-specific primers that flank the *ifg-1* 3' UTR and exon 1 were used to initially identify IFG-1 p170 start site. Gel electrophoresis followed by ethidium staining showed that in the absence of TAP, no p170-derived PCR products were detected. This suggested that all p170 mRNAs were capped since ligation and circularization did not occur (Figure 2.6B, EtBr Stained, Lane 1). Upon addition of TAP, however, several stained bands are observed at approximately 125 bp, 210 bp, 275 bp, 350 bp, and 450 bp, respectively (Fig. 2.6B EtBr Stained, Lane 2). To further characterize the observed sizes and determine if these cDNA fragments corresponded to *ifg-1* sequences, we transferred the gel products to a nylon membrane and performed southern blotting using $^{32}$P-labeled probes that were complementary to *ifg-1* exons 7-9. Hybridization of the *ifg-1* 3' probe with TAP-treated p170 products yielded discrete bands at 210 bp, 275 bp, and 500 bp as well as heterogeneous sizes between 325 bp and 450 bp (Figure 2.6B Southern, Probe 1). The most abundant TAP-

**Figure 2.6 Mapping *ifg-1* p170 and p130 5' ends by cRT-PCR.** (A) 5' and 3' ends of mRNAs can be analyzed by isolating total RNA, decapping mRNAs in the presence of tobacco acid pyrophosphatase (TAP), and ligating the ends with T4 RNA Ligase to circularize the transcript. Primers flanking the junction can then amplify both ends of the mRNA to generate cDNA that is subcloned and sequenced. (B) PCR products are shown by both ethidium bromide staining and southern hybridization with two *ifg-1* specific antisense probes. Unique products in TAP-treated lanes are indicated (*). An *ifg-1* plasmid was used as positive control for hybridization (P).

# Figure 2.6


A
5' m7GpppN
AAAAA 3'
mRNA decapping
5' pN
AAAAA-OH 3'
Circularization
Reverse Transcription
AAAAA
ifg-1 specific primers
Nested PCR amplification
5'
TTTTT
3'
Sequencing of DNA ends
B
p170 p130 P p170 p130 P p170 p130 P
- + - + - - + - + - + - + -
TAP
1500
800
600
400
200
EtBr Stained
Southern Blot (Probe 1)
Southern Blot (Probe 2)

derived p170 products were subsequently subcloned and sequenced. An alignment of sequenced clones identified a shared start site (AUG) at nucleotide positions 24-26 preceded by a *C. elegans* SL1 trans-splice leader added just 23nt upstream (Figure 2.7A). Interestingly, the sequenced 3' ends of several p170 transcripts confirmed the use of a polyadenylation signal located 252 bp from the termination codon as well as an extended 3' UTR indicating that a second polyadenylation signal located 550 bp from the termination codon (298 bp downstream of the first) (Figure 2.7B). This heterogeneity of the 3' end is consistent with the observed broad spectrum of PCR product sizes observed by ethidium staining and southern blot. These results confirm that the PCR products were derived from *ifg-1* capped p170 mRNAs and were all SL1 trans-spliced.

Initial characterization of p130 mRNA showed that the transcriptional start site was located at the 3' end of exon 4. This missing 5' sequence is presumably be responsible for encoding the eIF4E and PABP binding sites, while the downstream sequences, which are identical to *ifg-1* p170, encode the MIF4G ribosome recruitment domain. To determine the exact position in exon 4, cRT-PCR was again performed using a set of primers located immediately downstream of the predicted p130 start site in exon 5, based on several previously characterized ESTs (Contreras, Richardson et al. 2008), and in the *ifg-1* 3' UTR. Unlike p170 mRNA cRT-PCR, there were p130 mRNA-derived PCR products amplified from TAP-untreated total RNA and observed when ethidium stained (Figure 2.6B). These fragment sizes were approximately 100bp, 175bp, 210bp, and 275bp. The presence of products in non-decapped samples may indicate either non-specific PCR artifacts or the presence of a sub-population of p130 mRNAs that exist naturally uncapped. cDNA synthesis of decapped p130 mRNAs, however, showed a slightly modified size distribution of 125 bp, 275 bp, 375 bp, and 475 bp (Figure 2.6B, EtBr,

Lane 4). Of these TAP-treated fragments, only the 275 bp band overlapped with non-decapped products (Figure 2.6B, EtBr, lane 4). The presence of identical amplified products from TAP-treated and untreated RNAs suggest that there are indeed capped p130 mRNAs. Likewise, the unique products amplified without decapping also point to likely uncapped p130 mRNAs. Uncapped forms are unusual for normal RNA Polymerase II transcripts, except as products of the decapping enzymes, DCP-1 and -2, but are common among viral RNAs (discussed further below). To further characterize *ifg-1* p130 mRNA derived products, southern blot analysis was once again conducted using the same *ifg-1* 3' UTR probe. Hybridization to p130 products derived from the TAP-untreated reaction showed major sizes detected at 175 bp, 275 bp, 400 bp, 500 bp, and a heterogeneous population of sizes extending from 550 bp-900 bp. However, the TAP-derived p130 cDNAs probed with the 3' *ifg-1* probe revealed nonoverlapping sizes of 375 bp, 475 bp, and 550 bp. A second *ifg-1* 5' derived probe (nt 24-2083bp), which overlaps the p130 start region, confirmed the 375 bp and 475 bp sized fragments (Figure 2.6B, probe 2). Sequencing of cloned TAP-treated p130 cDNAs showed 5' ends that were all 9-84 bp upstream of a single in-frame AUG (nt 1194-1196) near the 3' end of exon 4 (Figure 2.7). The predicted size of the mRNA using this transcription start is approximately 2300 bp, very similar to our previous Northern and RNase protection characterization of the p130 transcript (Contreras, 2008). In addition, the majority of the sequenced 3' ends of p130, like p170 clones, contained variable lengths of the 3' UTR (Figure 2.7B). These data confirmed that there is also heterogeneity within the 3' end of *ifg-1* p130 mRNA. Unlike p170 mRNA, a splice leader (SL) was never observed in any of the p130 clones, indicating that p130 mRNAs are not produced by trans-splicing of a longer transcript. The diversity of 5' ends therefore indicates that p130

**Figure 2.7 Comparison of *ifg-1* p170 and p130 sequenced endpoints.** (A) cDNA clones for *ifg-1* p170 and p130 were sequenced to identify 5' start sites. The end points are depicted as primary sequence with dots representing mRNA caps to indicate that clones were subcloned from TAP-treated samples only. The SL1 splice leader is also indicated on p170 mRNAs. The first four amino acids including the initiating methionine (underlined) for p170 and p130 are also shown above the nucleotide sequence. (B) Graphical summary of information obtained from sequencing for p170 and p130 mRNAs. Exons (numbered boxes), methylated cap (dots), and *ifg-1* 3'UTR containing the two predicted poly(A) signals (PAS1, PAS2) are also shown as a frame of reference. Primer sets (carrots) and radiolabeled probes (double arrows) are included. The amino acids shown in (A) are also included along with the representative 3' end information (thick bars). One clone contained poly(A) tail sequences and is also indicated.

# Figure 2.7

A

p170 5' Ends

*Splice Leader*

M S N A

*GGTTTAATTACCCAAGTTTGAG*TGACTTCCTCAAACTGAAAGTT ATG TCA AAC GCT

*GGTTTAATTACCCAAGTTTGAG*TGACTTCCTCAAACTGAAAGTT ATG TCA AAC GCT

p130 5' Ends

AGATTGAAAAGAAAAAACTTCGCGAGGAAGCTTTAGAGAAAAGAATCGAGGAGCTTATCCTGGCCGGAAGTGCTGTCATCTCGGATGGAA...

ACTTCGCGAGGAAGCTTTA----------TCCTGGCCGGAAGTGCTGTCATCTCGGATGGAA...

TCATCTCGGATGGAA...

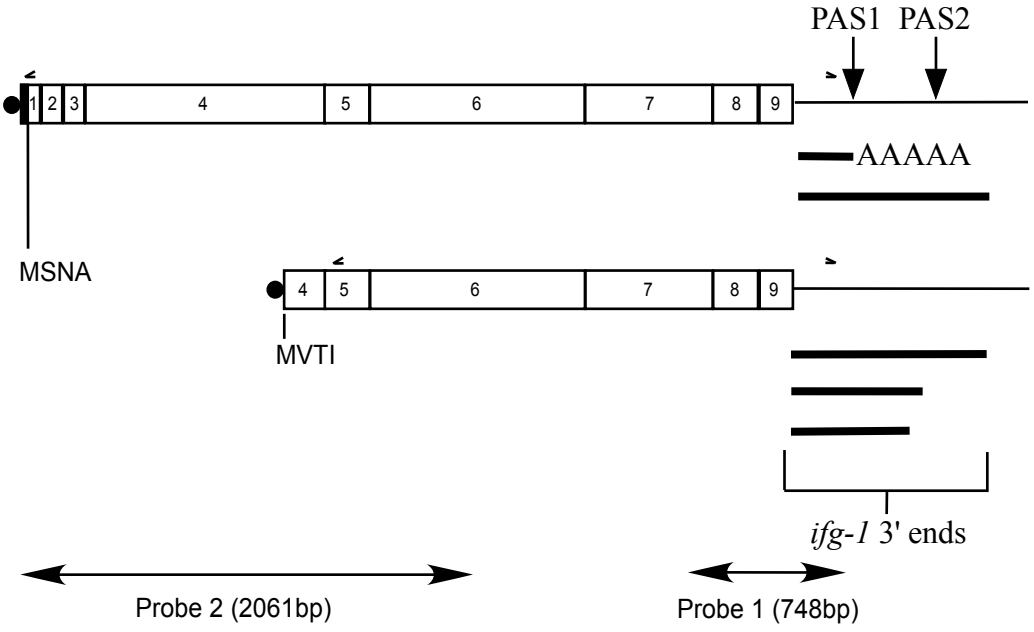
M V T I

...CTTTTGGAAGAGATTTC ATG GTT ACT ATT

...CTTTTGGAAGAGATTTC ATG GTT ACT ATT

...CTTTTGGAAGAGATTTC ATG GTT ACT ATT

B



transcripts are derived from transcription initiation by RNA polymerase via an alternative promoter within *ifg-1* exon 4.

Curiously, one of the three p130 cRT-PCR clones appeared to splice out a small alternative intron within exon 4 and upstream of the relevant AUG initiation codon, indicating even further complexity to *ifg-1* p130 transcription (Figure 2.7A). Collectively, the extensive mRNA mapping and sequencing shows that *ifg-1* p170 mRNAs are SL1 trans-spliced producing a homogeneous 5' start just upstream of the unique start codon (Figure 2.7B, MSNA). By contrast *ifg-1* p130 transcripts are heterogeneous at the 5' end and are not trans-spliced, but nevertheless precede a unique start codon (Figure 2.7B, MVTI). Interestingly, the encoded initiating methionine is immediately downstream of the predicted eIF4E-binding site. In the course of cRT-PCR sequence analysis, some heterogeneity of *ifg-1* p170 and p130 3' ends were also noted. Two predicted poly (A) sites (PAS) for *ifg-1* were mapped 298nt and 550nt downstream of the termination codon. These results indicated that multiple p170 and p130 transcripts were generated by alternative 3' end processing. These calculated sizes could account for the transcripts including the extended 3' end that correlated with sizes estimated by Northern analysis. Unfortunately of the six clones sequenced only one capped p170 mRNA contained some portion of the actual poly (A) tail (~5 nt), though most did encode a mature SL1 spliced leader, confirming that they represent mature processed mRNAs. The addition of a splice leader is evidence of a fully post-transcriptional processed mRNA. The lack of products observed in non-decapped p170 amplified mRNA indicated that degradation intermediates were not being amplified (Figure 2.6B, probe 2, lane 1). These results indicate that *ifg-1* may contain 3'UTR regulatory elements that may contribute to more complex spatial and temporal expression patterns in the transcriptionally quiescent germline. .

*Conclusion*

Aberrant expression (depletion/overexpression) of multiple eIF4G isoforms (eIF4GI, eIF4GII, and p97) has shed new light on the dynamics of mRNA recruitment and translation complex formation. eIF4G proteins are ubiquitously expressed in mammalian tissues. (Coldwell and Morley 2006). Yet, with regard to individual isoforms, transcript levels of eIF4GI are higher in skeletal muscle while eIF4GII is higher in testes (Gradi, Imataka et al. 1998). In addition, p97 protein has been shown to be highly expressed in heart, brain, lung, kidney, and the reproductive system (Gradi, Imataka et al. 1998). Our results presented in this dissertation are consistent with observed expression patterns in mammals. We observed broad expression of *ifg-1:gfp* in all tissues. However there were slightly increased levels in the pharynx, gut, vulva, spermatheca, and male tail fan testes which could be considered analogous to previously observed human organs. Interestingly, the pharyngeal head contains over a third of the 959 cells in the body (White, Southgate et al. 1976). Most of these cells are neurons that form the complex and highly organized ganglia surrounding the pharynx. Likewise, the testes and intestine compose a significant portion of somatic mass in the worm and are also sites of protein trafficking and translational control. Thus increased expression of IFG-1 may be required to sustain the synthetic output of these tissue types over less demanding organs such as the cuticle or body wall, which contained relatively lower GFP expression.

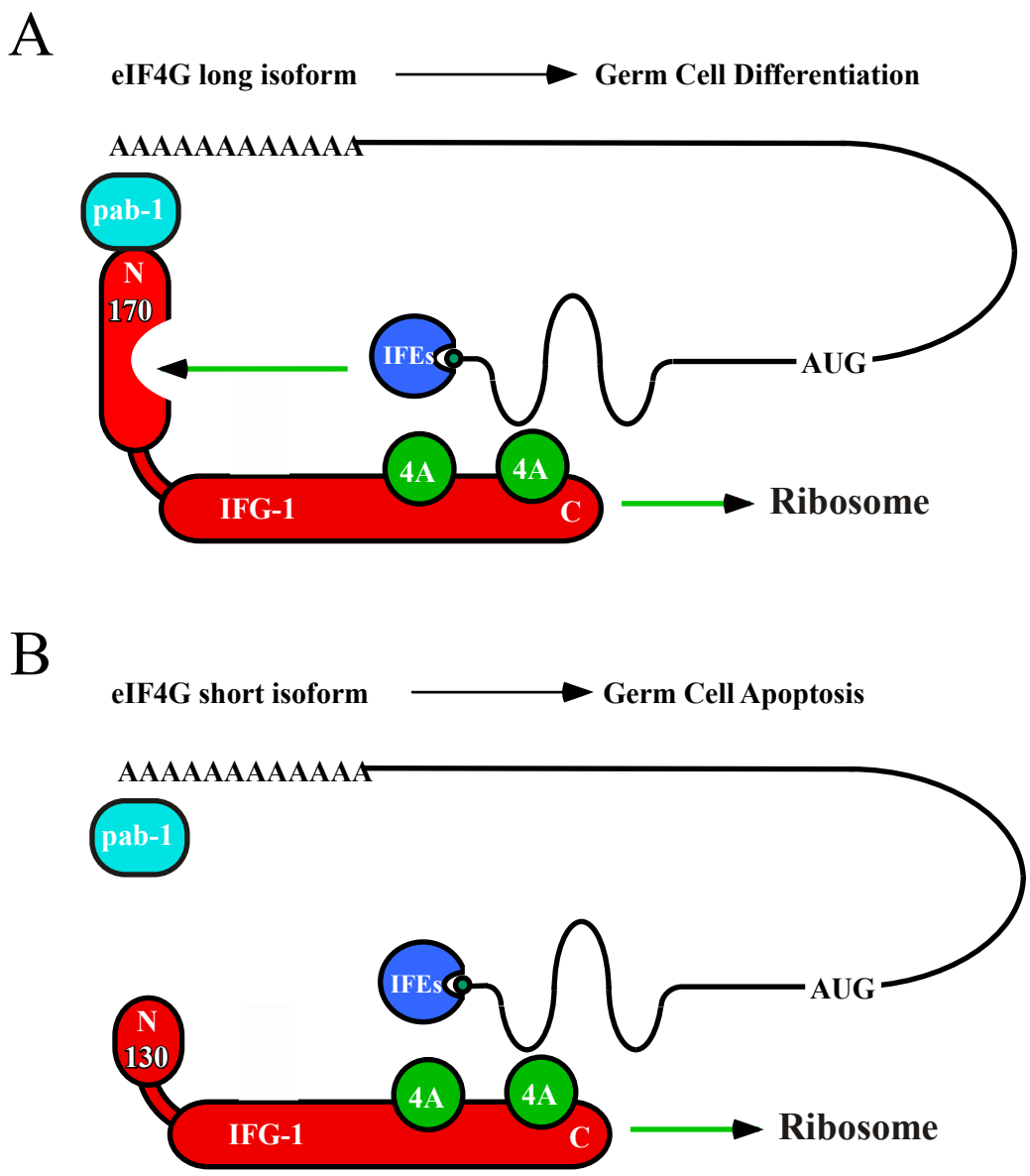
Previous studies have shown that both human eIF4GI and eIF4GII form complexes with eIF4E, while p97 does not interact with cap-binding structures (Gradi, Imataka et al. 1998). Data presented here indicate that there is a difference between IFG-1 p170 and p130 in their capacity to associate with mRNA cap complexes Like mammalian p97, *C. elegans* IFG-1 p130 lacks the capacity to bind eIF4E and is therefore the paralogous cap-independent eIF4G isoform. On the

other hand, *C. elegans* IFG-1 p170, like eIF4GI and eIF4GII, does mediate cap-dependent protein synthesis through association with one or more of the five eIF4E (IFE) isoforms. Such alternatives would necessarily give rise to assembly of heterogeneous mRNA recruitment complexes by the eIF4 initiation factors. In other systems, the potential for alternative translation complex assembly has been shown to promote the selective synthesis of proteins during mitosis, differentiation, and periods of stress and apoptosis (Nousch, Reed et al. 2007; Lewis, Cerquozzi et al. 2008). Curiously, p97 has recently been shown to promote the synthesis of cell cycle proteins during mitosis (Lewis, Cerquozzi et al. 2008). Reports confirm that the highly conserved central domain found in human p97 and IFG-1 p130 can support translation initiation by internal ribosome entry (De Gregorio, Preiss et al. 1999). However during apoptosis, cleavage of p97 activates the IRES-dependent synthesis of Apaf-1 as well as the translation of its own mRNA to positively promote cell suicide (Henis-Korenblit, Shani et al. 2002). We have shown that depletion of IFG-1 p170 causes increased apoptosis among developing gametes in *C. elegans* (Contreras 2005; Contreras, Richardson et al. 2008). It is therefore likely that disruption of cap-dependent translation and continued presence of IFG-1 p130 initiate a corresponding cap-independent mediated cascade during gametogenesis (Figure 2.8). Other labs have found p97 to be recruited to ribosomes following growth factor stimulation and can associate with polysomes in proliferating cells (Lee and McCormick 2006). We found IFG-1 p170 and p130 to likewise associate with ribosomes in unstressed wild type worms. It would be interesting to determine whether apoptosis or cell stress alters the distribution of either IFG-1 p170 or p130 on polysomes.

Multiple eIF4G isoforms have been discovered in many species including human, *Drosophila*, sea urchin, yeast, and *C. elegans*. There is some evidence that there are multiple

**Figure 2.8 IFG-1 isoforms assemble different mRNA complexes during gametogenesis.** (A) During the recruitment of eukaryotic mRNAs to ribosomes, the p170 eIF4G isoform is able to bind eIF4E (IFEs). The long eIF4G complex is competent for initiation events required to synthesize proteins during late meiotic development in oocytes. (B) IFG-1 p130 which lacks the N-terminal eIF4E binding domain, is unable to assemble cap-dependent translation complexes. As a result recruitment of mRNAs is limited to mitotic and early meiotic (mostly nuclear) germ cells and not late stage oocytes which require bursts of protein synthesis prior to fertilization. A balance of IFG-1 complexes must therefore be maintained to prevent germ cells from entering apoptosis rather than finishing oogenesis.

Figure 2.8


A
eIF4G long isoform
Germ Cell Differentiation
AAAAAAAAAAA
pab-1
N
170
IFEs
AUG
4A
4A
IFG-1
C
Ribosome
B
eIF4G short isoform
Germ Cell Apoptosis
AAAAAAAAAAA
pab-1
IFEs
AUG
N
130
4A
4A
IFG-1
C
Ribosome

levels of derivation for these isoforms, several involving post-transcriptional mechanisms. Each mechanism leading to a unique eIF4G isoform adds additional complexity to translational regulation in that cell (Coldwell and Morley 2006). In humans, there are three separate genes encoding three distinct isoforms. Of these genes, eIF4GI encodes seven isoforms through alternative splicing and the use of multiple promoters (Byrd, Zamora et al. 2005). *C. elegans* eIF4G isoforms p170 and p130 are derived from a single gene and differ in their 5' ends. Data presented in this chapter identified the respective mRNA and translation start sites for both isoforms. p170 is generated from a trans-spliced transcript from the canonical, predicted transcriptional start. Recently, our lab discovered that p170 mRNA is also alternatively spliced in exon 2 that adds an additional twelve nucleotides indicating still further heterogeneity in the 5' end (Hao and Keiper, unpublished data).

*ifg-1* p130 mRNAs, however, did not contain a trans-spliced leader and appears to translate from a unique AUG just downstream of the predicted eIF4E-binding site. The presence of heterogeneous transcriptional starts for p130 mRNA clustered within exon 4 further suggests that an alternative internal promoter(s) may be used in *ifg-1*to generate the cap-independent isoform. Human eIF4GI, which contains 3 promoters, generates at least seven transcripts that all encode heterogeneous N-terminal proteins and are capable of binding mRNA cap complexes. The shortest eIF4GI isoform does not however contain the binding site for the PABP, which could affect poly(A) dependent complex assembly. Thus, alternative mRNA regulation of eIF4G may confer a previously unknown regulatory network employing the use of multiple isoforms that assemble specific translational complexes to promote rapid synthetic responses to different environmental stimuli (example shown in Figure 2.8).

Finally, we observed various lengths of the 3'UTR in both p130 and p170 mRNAs, indicating that alternative polyadenylation signals may also be used. Other initiation factors (eIF5 and eIF4E) have also been shown to use different poly (A) signals to generate multiple mRNAs encoding the same protein (Jaramillo, Pelletier et al. 1991; Si, Das et al. 1996). While the biological significance of this additional 3'UTR sequence is unclear, there is a growing indication that the additional sequence could be important for binding translational regulators that could either inhibit expression directly or recruit these factors to inhibitory complexes during embryogenesis and differentiation (Jackson and Standart 1990; Curtis, Lehmann et al. 1995). Additional experiments will need to determine how these differences in sequence and structural character influence recruitment of *ifg-1*mRNAs for translation, not to mention the subsequent changes in isoform expression and assembly of functional complexes during proliferation, differentiation, and cell death.

# CHAPTER 3: APAF-1/CED-4 IS UPREGULATED DURING DISRUPTION OF CAP-DEPENDENT TRANSLATION IN *C. ELEGANS*

## *Introduction*

Apoptosis is a naturally occurring, required and controlled mechanism used during the development of all multicellular organisms (Nagata 1997; Hickman and Helin 2002). It is required, for example, to remove extra cells during differentiation to promote limb formation, for T lymphocyte homeostasis in the thymus, and for proper neural tube formation (Oppenheim 1991; Anderson, Anderson et al. 1996). Programmed cell death (apoptosis) exhibits distinct morphological characteristics that distinguish it from other cell death programs such as necrosis. Initial cellular changes involve both nuclear and cytoplasmic condensation as several structural proteins and DNA become digested by several apoptotic proteases (Kerr, Wyllie et al. 1972; Fink and Cookson 2005). The cellular membrane next begins to bud off (blebbing) without disrupting integrity into small globules (apoptotic bodies) and detach from neighboring tissue. To prevent these shrinking cells from affecting other health cells, surface molecules are recognized by phagocytes (engulfment cells) that destroy the dying cell and prevent the release of pro-inflammatory contents. Cell corpse remnants are finally degraded by lysosomal digestion (He, Lu et al. 2009).

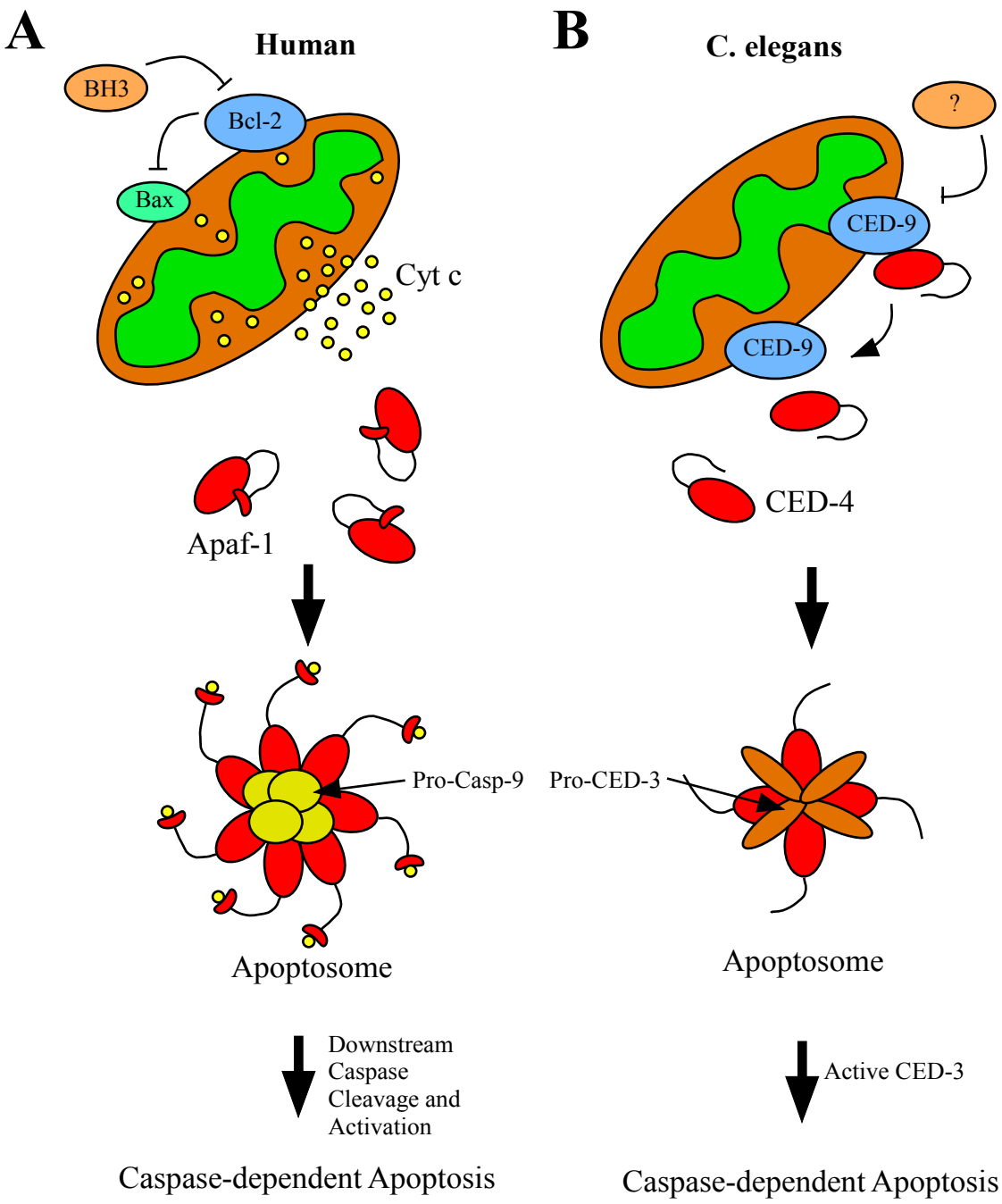
The destruction of cellular proteins such as nuclear lamins and actin during apoptosis is executed through the activation of certain cysteine aspartic acid directed proteases called caspases (Riedl and Shi 2004). Activation of caspases can involve either of two multi-step processes (extrinsic or intrinsic) that promote the maturation of the initiator caspases (protease activators) which further cleave effector caspase (executioner) zymogens to facilitate digestion

of cellular components (see Chapter 1). The extrinsic pathway mediates a ligand-dependent activation of caspases by sequential processesing using a single apical caspase (Caspase-8). By contrast, intrinsic apoptosis requires the assembly of a caspase maturation complex in order to execute cell death. Cellular disruption as a result of DNA damage or hypoxic stress can disrupt the integrity of the mitochondrion, releasing apoptogenic cytochrome c from the intermembrane space to the cytosol. Cytochrome c induces the formation of the apoptosome through oligomerization of the apoptotic protease activating factor 1, Apaf-1 (Figure 3.1). Upon the binding of dATP, the apoptosome complex adopts a heptameric "chakram-like" structure that allows pro-caspase 9 to associate, dimerize and autocatalytically cleave/activate (Li, Sonenberg et al. 2002; Salvesen 2002). Active caspase 9 dimers are then able to cleave downstream effector pro-caspases and initiate the apoptotic cascade (Acehan, Jiang et al. 2002).

Transcriptional and translational regulation of Apaf-1 is critical to the expression and formation of the apoptosome both during development and under stress conditions. Developmental studies in mice have shown that deletion of Apaf-1 causes disruption of morphogenetic (e.g. extra lens epithelial cells), histogenetic (e.g. uncontrolled cell number in brain), and phylogenetic (e.g. extra cranial palate) processes resulting in severe morphological defects (Cecconi 1999). This suggests that Apaf-1 is required for the temporal removal of cells to ensure that the correct architectural character is maintained during tissues and organ development. Under chronic stress or apoptosis-induced cultured cells reduces global output of proteins translated by the normal cap-mediated scanning mechanism (Graber and Holcik 2007). Under these conditions, only select groups of mRNAs are translated through an alternative cap-independent mechanism. The human Apaf-1 gene encodes four alternatively spliced transcripts,

**FIGURE 3.1 Diagram of apoptosome assembly.** (A) Intrinsic apoptosis (mitochondrial dependent) in human is induced during cell stress (hypoxia). Inhibition of Bcl-2 results in destabilization of the mitochondrial membrane releasing cytochrome c into the cytoplasm. The pro-apoptotic Apaf-1 adapter protein binds cytochrome c and pro-caspase-9 to form the apoptosome which causes downstream cleavage of executioner caspases. (B) *C. elegans* physiological germ cell apoptosis requires the release of the Bcl-2 homolog CED-9 from the outer mitochondrial membrane. CED-4 homodimerizes and associates with the inactive zymogen caspase-3 homolog pro-CED-3 to form the apoptosome and caspase-dependent cell death.

# Figure 3.1


A
Human
BH3
Bcl-2
Bax
Cyt c
Apaf-1
Pro-Casp-9
Apoptosome
Downstream
Caspase
Cleavage and
Activation
Caspase-dependent Apoptosis
B
C. elegans
?
CED-9
CED-9
CED-4
Pro-CED-3
Apoptosome
Active CED-3
Caspase-dependent Apoptosis

the largest of which produces the pro-apoptotic 1248 amino acid protein (Shi 2008). This Apaf-1 mRNA contains a 548 bp IRES in its 5' UTR that affords translation under cap-independent conditions. Such conditions prevail during later stages of apoptosis when the cell has committed to a dying fate, and the switch in translation initiation mechanism activates Apaf-1 protein synthesis. The 5' UTR contains hallmark features of IRESs, including polypyrimidine stretches, multiple AUG codons, and high secondary structure. The Anne Willis lab at the University of Leicester have shown that activation of the IRES requires several IRES transacting factors (ITAFs) such as the polypyrmidine tract binding protein (PTB) that likely acts to recruit initiation complexes during synthesis (Mitchell, Brown et al. 2001). During apoptosis, cleavage of the eIF4G isoforms p97 and eIF4GI cause the IRES-dependent translation of Apaf-1 which positively reinforces the apoptotic cascade (Henis-Korenblit, Shani et al. 2002; Nevins, Harder et al. 2003). As IRES-mediated translation is the predominant mode of synthesis of Apaf-1 protein, strict regulation of translation initiation mechanism is critical to promote apoptosome assembly and caspase activation.

Our studies in *C. elegans* suggest that the presence of a cap-independent isoform IFG-1 p130 allows for alternative translational mechanisms that may be used during periods of stress or apoptosis, much like the function of its human p97 counterpart. Two years ago we demonstrated that changing the balance between the cap-associated p170 and non-cap associated p130 increases the incidence of apoptosis in the *C. elegans* germline (Figure 3.2A, B) (Contreras, Richardson et al. 2008). As described above, the *C. elegans* Apaf-1 ortholog, CED-4, functions in the pro-apoptotic activation of the main caspase, CED-3. The CED-3 caspase is essential for the majority of somatic and germ cell deaths. Studies presented in this chapter address whether there is similar upregulation of the worm apoptosome protein CED-4 and a similar cap-

independent mechanism by which it is translated. Given the role of human eIF4G in the IRES-dependent translation of Apaf-1 during somatic apoptosis, it is likely there is an analogous previously undescribed paradigm involving IFG-1 induced increase of CED-4 expression during germ cell apoptosis.

## *Results*

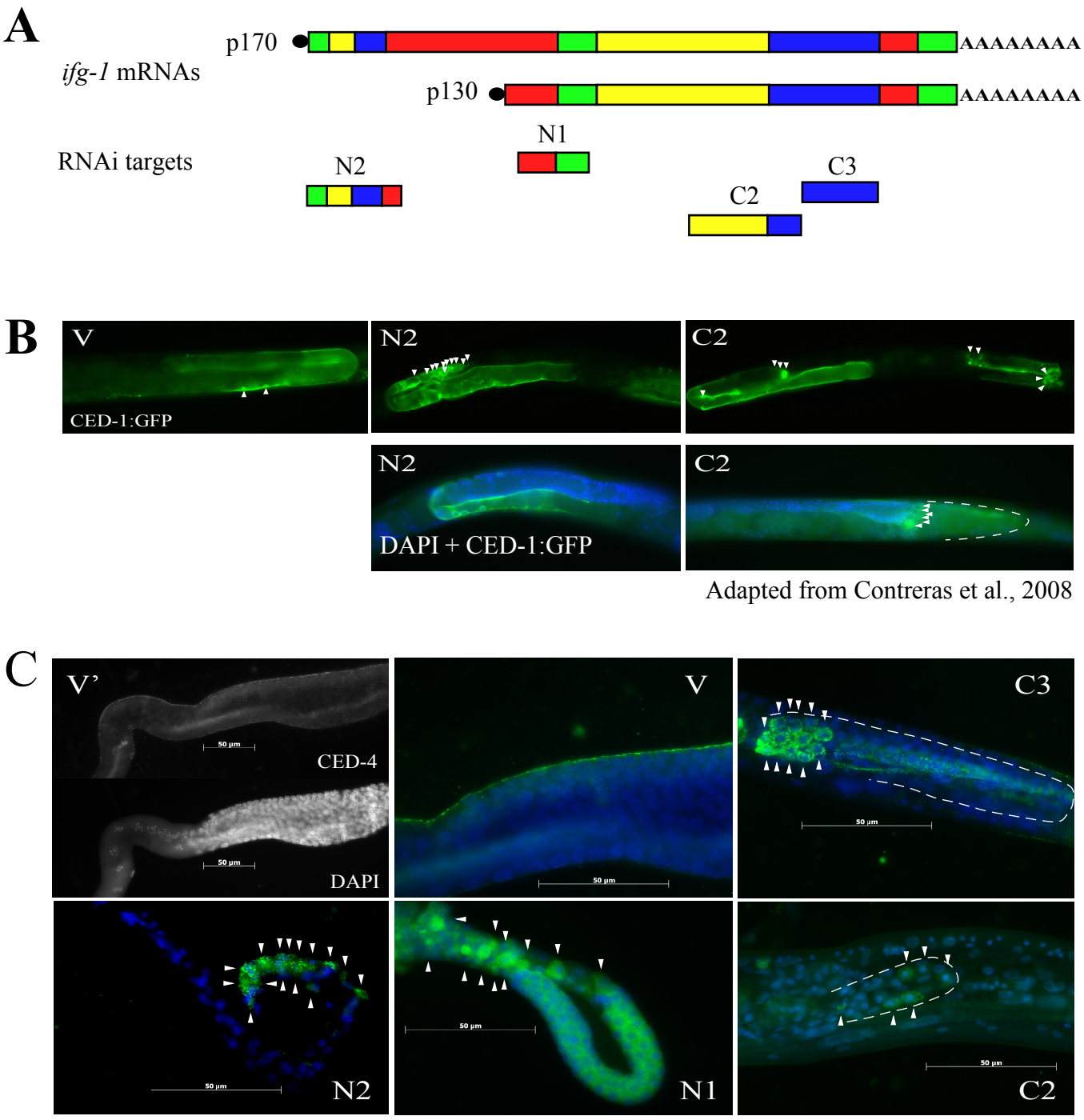
The pro-apoptotic protein CED-4 is required for both somatic and germ cell deaths in *C. elegans* (Hengartner and Horvitz 1994). To determine if CED-4 synthesis was induced in germ cells upon IFG-1 isoform depletion, we performed immunostaining of p170(RNAi)-treated gonads. Germ cell apoptosis was shown to be as much as 8-fold higher in p170 (RNAi)-treated worms than worms treated with vector alone. Dead cells were marked using a strain that expresses a CED-1 transmembrane receptor fused with GFP. CED-1 recognizes phosphotidylserine or "eat me" signals on the surface of corpses that allow neighboring sheath cells to engulf and remove the cellular detritus (Venegas and Zhou 2007). Since the engulfment process is a consequence of the execution phase of apoptosis, it is difficult to determine if these cell deaths were induced by activation of upstream factors in the pathway or as a non-specific byproduct of the cascade. Disruption of IFG-1 isoform representation in the *C. elegans* germline causes a dramatic induction of apoptosis within developing oocytes (Contreras, Richardson et al. 2008). *ifg-1* p170 mRNAs only were depleted using RNAi 5' target (N2), while both p170 and p130 were targeted by centralized N1, C2, C3 (Figure 3.2A). When worms were treated with control (RNAi) and gonads immunostained for CED-4, the protein was essentially undetectable in either the proximal or distal arms that displayed developing oocytes of all stages by DAPI co-staining (Figure 3.2C, V'). Closer inspection of individual post-mitotic oocytes at the reflex in

the gonad again showed no evidence of CED-4 protein (Figure 3.2C, V). By contrast, immunostaining of gonads dissected from *ifg-1*(RNAi) worms revealed that CED-4/Apaf-1 becomes strongly expressed in many of the post-pachytene oocytes. CED-4 expressing oocytes were prevalent in gonads from all four RNAi treatments (Figure 3.2 C, N2, N1, C2 and C3). Induction of CED-4 by N2 (RNAi) indicated that loss of p170 IFG-1 was sufficient to stimulate CED-4 synthesis. CED-4 accumulated in individual medium sized, post-mitotic oocytes in the proximal gonad arm, and suggests that these oocytes will die and be removed from the germline. This is consistent with the loss of viable, growing oocytes beyond the reflex in the gonads of IFG-1-depleted worms (Contreras, Richardson et al. 2008). Furthermore, CED-4 appeared to accumulate in punctate structures loosely surrounding nuclei of apoptosing germ cells, perhaps indicating that it assembles into large cytoplasmic complexes (apoptosomes). Similar CED-4 localization has been seen in dying cells during embryogenesis (Chen, Hersh et al. 2000; Tzur, Margalit et al. 2006). Interestingly, apoptosomes containing CED-4 appeared in large apoptosing oocytes. These oocytes still contained an intact nuclear membrane and increased cytoplasmic volume indicating that these gametes were in the early stages of apoptosis. This morphology differs greatly from CED-1:GFP expressing corpses, which appeared as small shrunken cells with decondensed nuclear material (Figure 3.2B). The expression of the CED-1 phagocytic receptor correlates with this phenotype and indicates a later stage of germ cell death. These results provide the first evidence for the involvement of a translation factor in germline apoptosis through the induction of CED-4 synthesis.

CED-4 is not detected in the germline under non-stressed conditions indicating that apoptosing oocytes must accumulate the apoptosome protein from *de novo* synthesis. However it is unclear how CED-4 is upregulated upon *ifg-1* (RNAi). It is possible that *ced-4* mRNAs may

**FIGURE 3.2 IFG-1 knockdown upregulates CED-4 in apoptosing cells.** (A) Diagram represents p170 and p130 mRNAs, RNAi targets, and regions of amplification. (B) Depletion of IFG-1 by RNAi causes increased germ apoptosis. DIC and fluorescence images showing CED-1:GFP decorated apoptotic corpses were modified from Contreras et al., 2008 for comparison with CED-4 immunostained dying germ cells. Note the increased number of corpses in *ifg-1* (RNAi) adults versus control V (RNAi) adults. (C) CED-4 immunostaining of *ifg-1*(RNAi) worms was performed, with the help of M Henderson and BD Keiper, on dissected gonads from (V', V, N2 and N1) or decapitated worms (where the gonad was very small; C2, C3) and then fixed and stained by immunofluorescence using an anti-CED-4 antibody. Co-staining with DAPI was conducted to observe nuclei of apoptosing cells. White arrowheads indicate individual oocytes that contain abundant CED-4 staining (apoptosomal complexes). High-magnification merged fluorescent images show the reflex portion of the RNAi-treated gonad s (V, N2, N1, C2, C3). The absence of CED-4 from the entire gonad of vector-treated control worms is also shown at low magnification (V').

# Figure 3.2




Adapted from Contreras et al., 2008

be translated preferentially during germ cell apoptosis similarly to Apaf-1 mRNA by IRES-dependent synthesis during somatic cell death. While a traditional IRES element has not been observed in *C. elegans ced-4* mRNAs, a careful examination of the transcript may provide an explanation of its induction by *ifg-1* (RNAi). Most *C. elegans* monocistronic mRNAs contain a trans-spliced leader at the 5' end that is co-transcriptionally added along with the addition of the poly (A) tail. In operons, aberrant 3' processing of upstream cistrons can affect downstream trans-splicing of downstream transcripts. The 2.2 Kb *ced-4* mRNA that encodes the full length 549 amino acid protein is derived from an operon transcribed as a contiguous polycistronic mRNA (Figure 3.3A). Further processing by trans-splicing produces a mature monocistronic mRNA transcript with a relatively short (50 nt) 5' UTR that is unlikely to provide internal ribosome entry. However, close examination of the intergenic region immediately upstream of the *ced-4* coding region revealed a long 483 nt sequence with two polypyrimidine regions that are both characteristics of IRESs. Interestingly, studies have shown that during heat shock, trans-splicing of polycistronic mRNAs becomes inefficient, leading to uncapped monocistrons with extended outruns (Liu, Kuersten et al. 2003). To determine whether some *ced-4* mRNA becomes aberrantly spliced and thereby maintains intergenic sequences (a form we will refer to as "*ced-4I*"), we analyzed the 5' end structures of mRNA from stressed worms. We performed a temperature shift of wild type worms from 20$^{o}$C to 25$^{o}$C to exert a similar heat shock effect on mRNA processing.

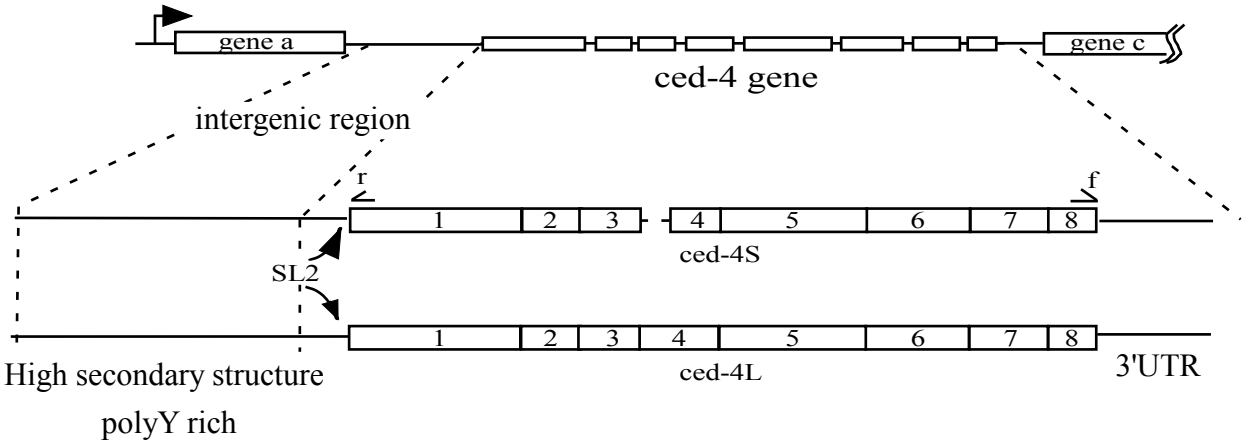
Longer forms of *ced-4* mRNA that are extended at the 5' end (*ced-4I*) from the stressed message population were identified through amplification of *ced-4* RNA sequences by cRT-PCR. Isolated total RNA was treated with TAP and the ends ligated with T4 RNA ligase to

circularize RNA molecules. cRT-PCR was conducted using diverging *ced-4* primers that flank exon 1 and the termination codon in exon 7. Given the location of the primer sets, the expected size for the amplification of the properly spliced *ced-4* transcript is minimally 289 bp. Amplification of TAP-untreated RNA from worms grown at 20$^{o}$C generated sizes of 200-250 bp and 325 bp (Figure 3.3B). Products of TAP-treated RNA yielded sizes of 250bp, 300bp, and 400-450bp. The size of the 300 bp fragment correlated well with the wild type end point for the mature *ced-4* transcript and was not detected in TAP-untreated samples. The 400-450 bp fragments also were absent in non-TAP treated samples suggesting that there are larger capped *ced-4* mRNAs. It is unlikely that the extra 100-150 bps are the result of an extended poly (A) tail or the use of alternative 3' ends since the predicted poly (A) signal (AAUAUA), which is located approximately 200 bp from the termination codon, is adjacent to the start of the next downstream gene. When total RNA from shifted (25$^{o}$C) worms was amplified in the absence of TAP, sizes detected for these uncapped mRNAs were 300 bp, 275 bp, 250 bp. While these products were clearly unique to the elevated temperature, they appeared to be too small to be from a *ced-4I* mRNA. When total RNA from 25$^{o}$C was treated with TAP, we observed discrete sizes of 125 and175 along with the previously observed albeit faint 300bp and 400-450 bp fragments from the 20$^{o}$C TAP-derived RNAs (Figure 3.3B, lane 4). Thus, while unique products corresponding to wild type *ced-4* and potentially longer *ced-4I* mRNAs were detected in TAP-treated samples there was no change in the distribution of products observed upon heat shock. It is possible that the elevated temperature was not high enough to sufficiently stress cells since previously described heat shock involved temperatures as high as 33$^{o}$C.

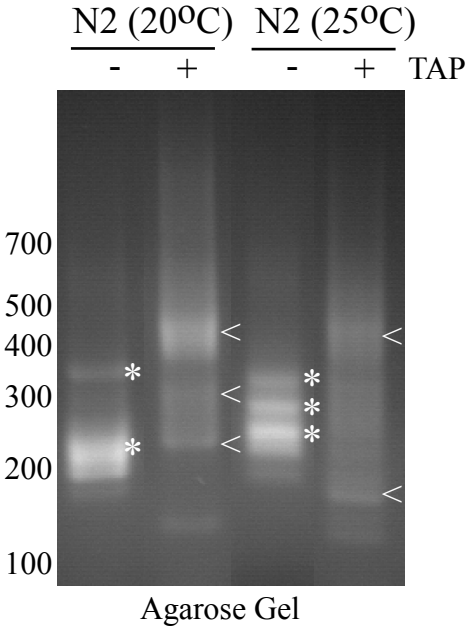
**FIGURE 3.3 Identification of a *ced-4* IRES-like mRNA (*ced-4I*).** (A) Schematic of C. elegans operon containing the *ced-4* gene. The *ced-4* gene encodes two alternatively spliced isoforms, CED-4S and CED-4L, that have opposing apoptotic functions. Monocistronic mRNAs are produced and labeled by trans-splicing with small RNA sequence called a spliced leader. SL2 leader sequences are almost always attached to operon based transcripts. Under heat shock, this splicing mechanism can be affected producing a longer uncapped mRNA. The upstream intergenic region between *ced-4* and the upstream gene is longer than usual and highly structured sequence that contains stretches of polypyrimidines. These sequences are characteristic of other IRESs. (B) cRT-PCR of total RNA to determine whether longer aberrantly spliced *ced-4* mRNAs were present. Worms were shifted from 20$^{o}$C to 25$^{o}$C and RNA isolated under TAP and non-TAP conditions as previously described prior to amplification. PCR products on agarose gel were stained with EtBr. Potential ced-4 wild type and *ced-4I* mRNAs are marked (carrots). Uncapped unique products are also shown that could be aberrantly trans-spliced products.

# Figure 3.3

A




B




Agarose Gel

In human cell lines the Apaf-1 IRES is activated during genotoxic stress (etoposide) induced apoptosis (Nevins, Harder et al. 2003). It is possible that upregulation of CED-4 through IRES-like mRNA translation is stress specific. To determine if a *ced-4* IRES-like transcript is created upon genotoxic stress, we treated *ced-1:gfp* worms with sublethal doses of copper. Copper is one of the few genotoxic agents that have been described to cause caspase- and apoptosome-dependent germ cell death in *C. elegans* (Wang, Wu et al. 2009). Worms were incubated for twelve hours with control media or with 50 uM $CuSO_4$ and scored for apoptotic corpses in the gonad by *ced-1:gfp* decoration. Control-treated worms displayed an average of 6.5 corpses per gonad lobe (Figure 16A). In contrast, treatment with 50 uM $CuSO_4$ moderately increased the number of germ cell deaths, ~8.1 per lobe (Figure 3.4B). The induction of germ cell corpses observed were not as dramatic as those observed by *ifg-1* (RNAi), but was consistent with previous findings (Wang, Wu et al. 2009) (Figure 3.4A). These germ cell deaths were also caspase –dependent because worms deficient in the caspase, CED-3, completely abrogated the apoptotic phenotype (data not shown). To determine if copper-stressed worms produced *ced-4I* mRNA, we once again performed cRT-PCR using circularized poly(A)+ RNA extracted from control and copper-treated worms. Amplified ends from control treated worms displayed three prominent sizes that included 225 bp, 175 bp, and 150 bp, as well as faint fragments of 400 and 750 bp (Figure 3.4C). Upon copper stress there were at least nine new mRNA products observed, including abundant bands of 400 bp, 325 bp, 300 bp, 200 bp, and 125 bp. In addition, there were also less abundant products observed at 600 bp, 900 bp, and 1000 bp. Smaller fragments (200bp and 125 bp) are too small to account for either wild type *ced-4* or *ced-4I*. Intermediate fragments (325 and 300) may correspond the wild type *ced-4* mRNA. Approximately 400 bp of intergenic sequence could be added to an aberrantly spliced *ced-4*

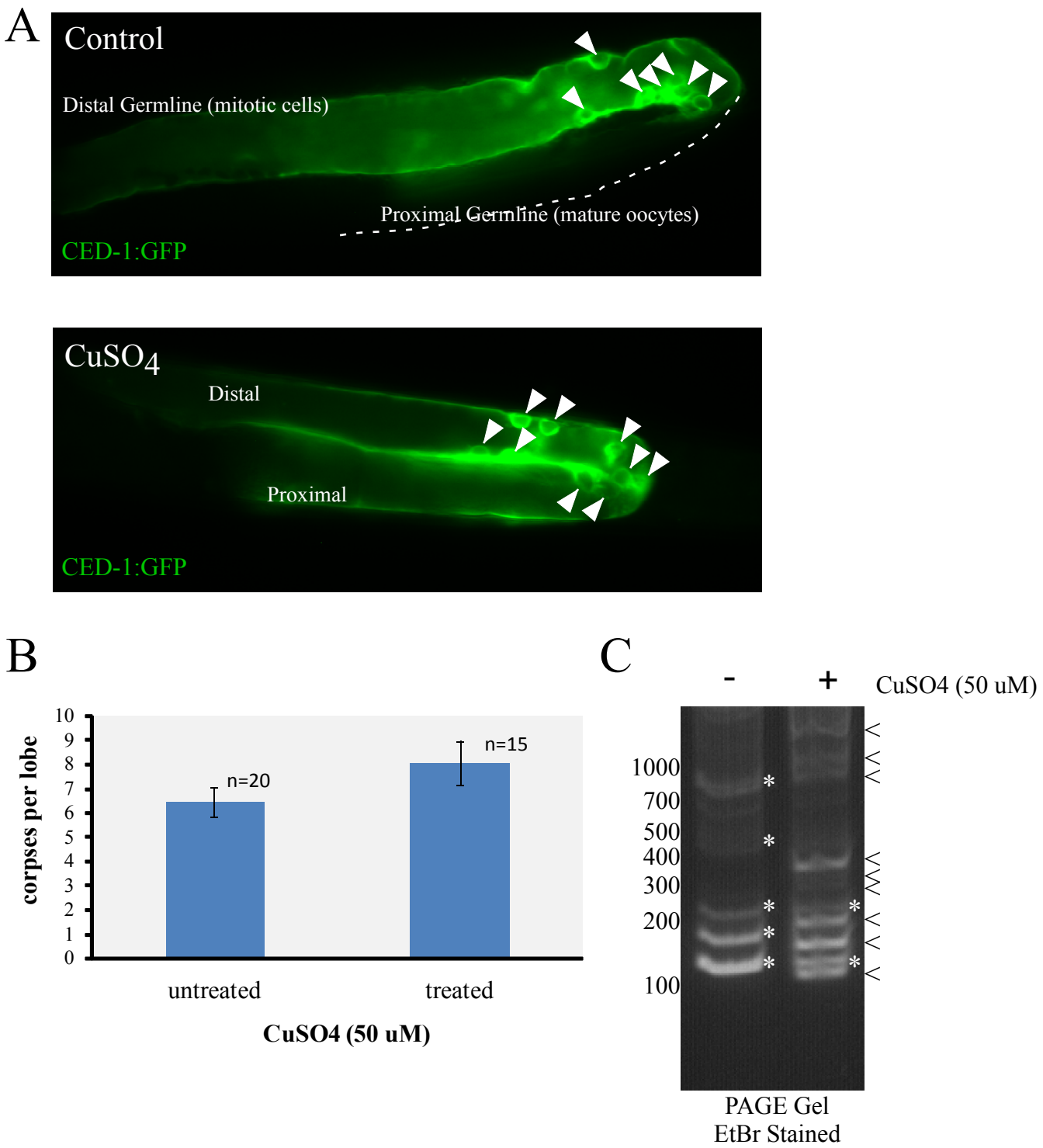
transcript. Therefore larger products (above 600 bp) may contain outronic sequences in addition to alternative polyadenylation signal usage. To our surprise the most pronounced fragment was a 400 bp amplicon, which is consistent with a 5' UTR that extends approximately 100 nt upstream of the wild type trans-spliced *ced-4* 5' end. Assuming those sequences arise from the intergenic region, it suggests that an uncapped *ced-4I* transcript is produced under genotoxic stress. Further characterization of *ced-4I* cRT-PCR amplicons through cloning and DNA sequencing will be required to determine how much of the intergenic region is added to the *ced-4* 5' end. Concomitantly, additional types of stress such as decreased cap-independent translation my also result in the production of *ced-4I*. It will therefore be necessary to address whether the depletion of the long IFG-1 p170 isoform correlates with an aberrantly spliced IRES-like mRNA. Recently, temperature shifted wild type worms and an *ifg-1* temperature sensitive mutant were analyzed for the presence of *ced-4I* (K Morrison and BD Keiper personal communication). In this case worms were shifted from the permissive (20$^{o}$C) to restrictive (25$^{o}$C) temperature followed by RNase protection of *ced-4* transcripts, using a probe that overlaps exon 1 and the 5' intergenic region. The results of these preliminary experiments also gave evidence of this 5'-extended *ced-4I* mRNA being a significant proportion of all *ced-4* transcripts. Collectively these results suggest that a both a capped and uncapped alternatively spliced, potential IRES-containing *ced-4* mRNA is generated upon induction of genotoxic induced apoptosis and temperature stress.

## *Conclusion*

Disruption of cap-dependent protein synthesis during cellular insult correlates with the IRES-dependent synthesis of many apoptogenic proteins (Coldwell, Mitchell et al. 2000; Henis-Korenblit, Strumpf et al. 2000). Increased apoptosome assembly as a result of upregulated Apaf-

**FIGURE 3.4 Identification of *ced-4I* in copper-treated worms.** (A) CED-1:GFP worms were exposed to sublethal doses of copper in order to generate genotoxic induction of *ced-4I*. (B) Bar graph depicts corpses counted following copper treatment. Values are in corpses per gonad lobe. Error bars represent S.E.M. (C) cRT-PCR analysis of poly(A+) purified RNA from copper treated worms. Unique products including the most abundant 400 bp fragment are marked (carrots).

# Figure 3.4


A
Control
Distal Germline (mitotic cells)
Proximal Germline (mature oocytes)
CED-1:GFP
CuSO4
Distal
Proximal
CED-1:GFP
B
corpses per lobe
10
9
8
7
6
5
4
3
2
1
0
n=20
n=15
untreated
treated
CuSO4 (50 uM)
C
-
+
CuSO4 (50 uM)
1000
700
500
400
300
200
100
PAGE Gel
EtBr Stained

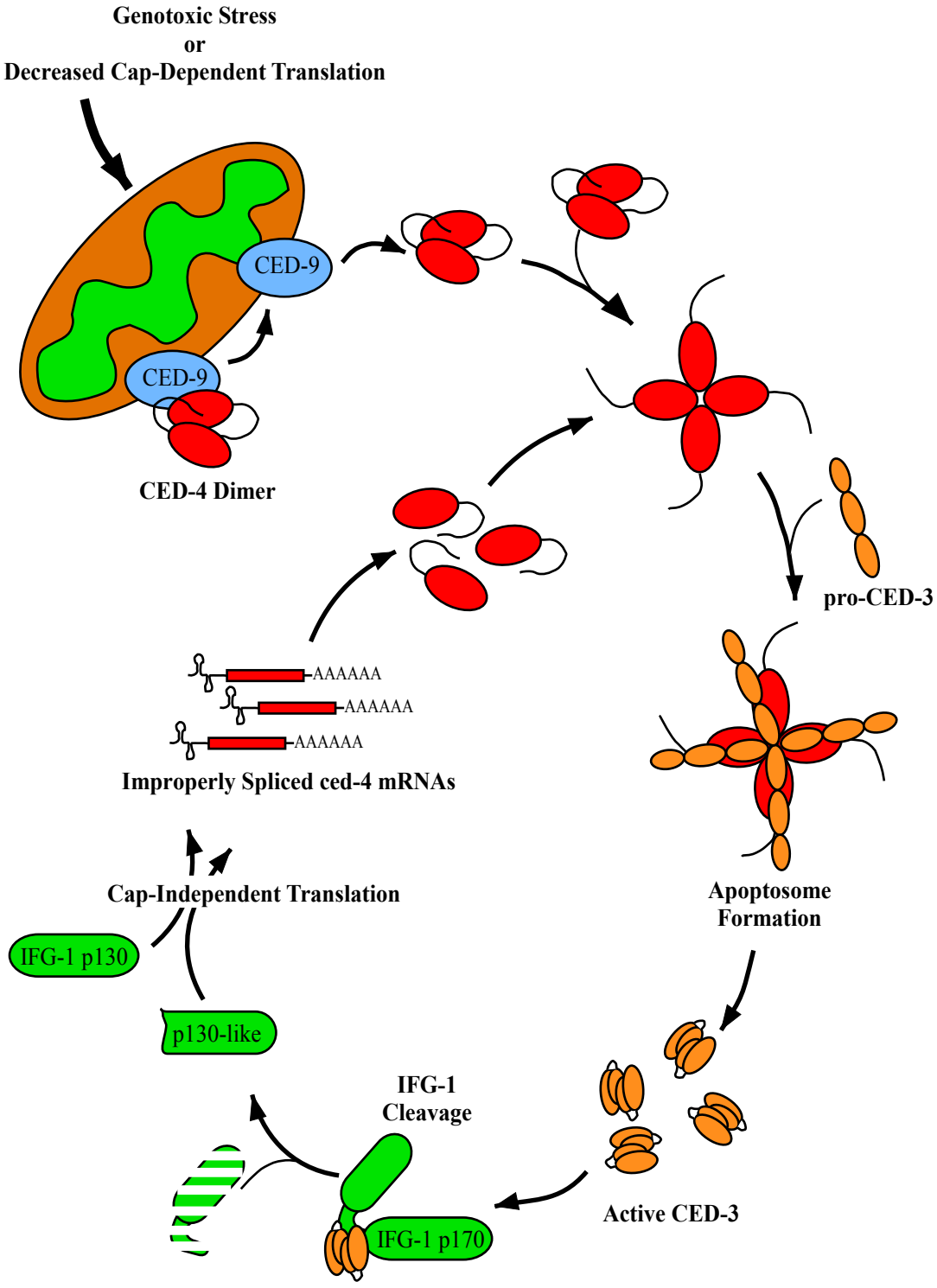
1 synthesis causes the activation of downstream caspase-3. Caspase-3 cleaves eIF4G and p97 to promote the cap-independent translation of IRES-containing Apaf-1 mRNA, resulting in a positive feedback loop that potentiates the caspase cascade. Here we show that, like its human ortholog Apaf-1, *C. elegans* CED-4 becomes upregulated upon disruption of cap-dependent protein synthesis. *De novo* synthesis of the apoptosomal protein results in the assembly of highly ordered structures that accumulate at the perinuclear surface of dying oocytes. Similar, but perhaps more tightly perinuclear CED-4 protein structures have been shown to associate directly with SUN-1 on the nuclear envelope in embryonic cells stressed to undergo apoptosis (Chen, Hersh et al. 2000; Tzur, Margalit et al. 2006). The appearance of cytoplasmic CED-4 foci in both cases suggests the productive assembly of apoptosomes in germ cells and embryonic cells that are destined to die. By analogy to Apaf-1 function in mammalian somatic cells, assembly of the apoptosome in oocytes signals the broader activation of executioner proteases such as caspase-3 (CED-3) to drive the completion of apoptosis. Consistent with this hypothesis, the temporal expression of CED-4 in medium to large oocytes occurs prior to changes in nuclear morphology, their shrinkage to "button-like" cells, and their engulfment via CED-1. Our results are the first evidence of increased CED-4 expression during germ cell death in apoptosome complexes that have been previously described to assemble throughout embryogenesis.

Data presented in Chapter 2 show that p130 is a cap-independent eIF4G isoform similar to human p97. These data, in conjunction with the data on the alternative *ced-4* mRNA forms (*ced-4I*) suggest that an isoform shift in translation initiation complexes may alter the balance of initiation mechanisms to favor synthesis of pro-apoptotic CED-4. Formally, no IRES element has yet been observed in the 5' UTR of the *C. elegans ced-4* mRNA, nor is there any direct

evidence that CED-4 synthesis proceeds by cap-independent translation. However, it is clear that CED-4 is induced by *ifg-1* p170 (RNAi). It is also of interest that the *ced-4* gene is located within an operon with a long upstream intergenic region that is unusual when compared with the common tendency of operon intergenic regions to be short (100-200 nt) (Blumenthal and Spieth 1996). In *C. elegans* splice leaders from specialized snRNPs are transferred to internal cistrons of polycistronic mRNAs upon 3' end formation of upstream cistrons (Liu, Kuersten et al. 2003). The *ced-4* intergenic region contains a high degree of secondary structure and stretches of polypyrimidines. Interestingly, the Blumenthal lab has shown that heat shock can prevent the trans-splicing of an SL2 leader, but not 3' cleavage of the upstream cistron, resulting in an uncapped mRNA containing an extended outron comprised of the intergenic sequences (Liu, Kuersten et al. 2003). We have discovered that upon genotoxic stress induced apoptosis, a novel uncapped *ced-4* mRNA (*ced-4I*) is generated that is not found in unstressed worms. The proposed activation of *ced-4I* may occur through caspase cleavage of IFG-1 p170 during germ cell apoptosis (Figure 3.5). Processing creates a p130-like fragment that initiates the cap-independent synthesis of CED-4. The IFG-1 fragment in addition to native p130 would then collectively reinforce the apoptotic cascade in unsalvageable germ cells. The unexpected induction of unique *ced-4* transcripts in the presence of such mild apoptotic insults is intriguing. There is a precedent for alternative usage of upstream 5' UTR sequences under stress. The mammalian X-linked inhibitor of apoptosis (XIAP) is a natural inhibitor of initiator and effector caspases (Riley, Jordan et al. 2010). XIAP mRNA translation is regulated during stress and apoptosis by a 162 nt IRES in its 1700 nt 5' UTR. However XIAP IRES activation has been shown to be induced during transient stress (hypoxia, ER stress, serum deprivation, and low level gamma irradiation). A recent report shows that there is an added complexity to its regulation

**FIGURE 3.5 Model for enhanced synthesis of CED-4 via short IFG-1 forms.** Stress from either genotoxic stress or decreased cap-dependent translation cause a change in translation mechanism utilizing short IFG-1 isoforms for cap-independent initiation. Formation of the apoptosome causes the activation of the caspase CED-3 which presumably cleaves IFG-1 p170 and generates a short p130-like fragment. This short product along with endogenous IFG-1 p130 facilitate the cap-independent translation of a *ced-4* IRES-like mRNA (*ced-4I*) resulting in additional synthesis of CED-4 protein that reinforces the caspase cascade.

# Figure 3.5


Genotoxic Stress
or
Decreased Cap-Dependent Translation
CED-9
CED-9
CED-4 Dimer
pro-CED-3
AAAAAA
AAAAAA
AAAAAA
Improperly Spliced ced-4 mRNAs
Apoptosome
Formation
Cap-Independent Translation
IFG-1 p130
p130-like
IFG-1
Cleavage
Active CED-3
IFG-1 p170

(Riley, Jordan et al. 2010). XIAP is normally expressed from a transcript containing a short 323 nt 5' UTR that is required under normal growth conditions to prevent spurious caspase activation. This short region is unable to support cap-independent translation. However, upon cellular stress, aberrant splicing generates an XIAP mRNA with a larger 1700 nt 5' outron that contains the IRES element, an observation nearly identical to what we have demonstrated for *C. elgans ced-4I*. Similar use of alternative mRNA isoforms has also been described for the cap-independent synthesis of p53 tumor suppressor (Ray, Grover et al. 2006). The translation of p53 from two distinct IRESs has now been shown to display different cell-cycle dependent activities. Thus the translational activation of IRES-containing *ced-4* transcripts may be required to promote rapid responses to stress-induced cellular perturbations in the germline.

Alternatively, the translation of *ced-4* may involve a different mechanism during *ifg-1* p170 (RNAi) induced germ cell apoptosis. The *ced-4* gene encodes two alternatively cis-spliced transcripts with opposing cell death functions. CED-4L (long) and CED-4S (short) are distinguished by an additional 24 amino acids encoded in exon 4 (Shaham and Horvitz 1996). CED-4L is a protein with antiapoptotic functions, whereas CED-4S is the pro-apoptotic protein that assembles the caspase-activating apoptosome. While a longer alternative spliced Apaf-1 isoform has been discovered, there is no evidence that this isoform serves a protective function . The balance between cap-dependent IFG-1 p170 and cap-independent IFG-1 p130 may therefore contribute to the preferential synthesis of a *ced-4* spliced isoform under certain conditions of stress.

It is worth noting that CED-4 protein is generally not detectable by immunostaining of the germlines (see Figure 3.2CB, V') as it is in early embryos and larvae, so that the CED-4 accumulation we observe in apoptosing oocytes must arise from *de novo* protein synthesis.

logical extension of this hypothesis is that in naturally occurring germline apoptotic events, initial entry into apoptosis might create a positive feedback loop (Figure 3.5) in which IFG-1 p130 either reinforces its own synthesis, like mammalian p97, or perhaps IFG-1 p130 and p170 become cleaved by CED-3. Cleaved IFG-1 proteins would subsequently reinforce the apoptotic pathway by inducing the synthesis of apoptotic factors such as CED-4I, and subsequent activation of the caspase, CED-3 (see Chapter 4) to potentiate the loop and ensure completion of programmed cell death.

# CHAPTER 4: eIF4G ISOFORMS p130 AND p170 REGULATE *C. ELEGANS* GERM CELL APOPTOSIS THROUGH APAF-1/CED-4 IN A CASPASE-DEPENDENT MANNER

## *Introduction*

During programmed cell death (apoptosis), cells commit to the structured disassembly of its metabolic framework.. Recent evidence suggests that signaling requires prominent translational control mechanisms for both the commitment phases and execution phase of cell death (Graber and Holcik 2007). *De novo* protein synthesis decreases initially in apoptosing cells, particularly from growth-related mRNAs. Apoptosing cells nevertheless upregulate the synthesis of several "death-related" proteins through an alternative mode of translation initiation. This switch in synthesis affords rapid responses to various types of stressors, allowing the cell to recover from injury, or submit to a path of suicide (Graber and Holcik 2007).

Apoptosis was first characterized in the simple worm *C. elegans*, and is marked by distinct morphological changes that mark the cell for destruction and removal (Ellis and Horvitz 1986; Elmore 2007). Many of these changes involve biochemical and structural modifications including a reduction in cytoplasmic volume, cytoskeletal rearrangements, nuclear fragmentation, and membrane blebbing (Elmore 2007). Apoptosis utilizes a series of specific proteolytic enzymes called caspases to induce the rapid execution of stressed and damaged cells. During intrinsic apoptosis cytotoxic signals cause the release of cytochrome c, which binds to the apoptotic protease activating factor (Apaf-1) to activate the formation of the apoptosome (Riedl and Salvesen 2007). The apoptosome activates a caspase cascade resulting in the processing of several protein synthetic and cellular repair proteins such as eIF2 alpha and PARP (Lazebnik, Kaufmann et al. 1994; Satoh, Hijikata et al. 1999).

In humans multiple isoforms of eIF4G are encoded by three separate genes (Imataka and Sonenberg 1997). Full length eIF4GI and eIF4GII are both expressed broadly in tissues and are cap-dependent (capable of establishing eIF4F complexes with eIF4E). The shorter p97/NAT1/DAP5 isoform, however, lacks the N-terminus and is cap-independent (capable of establishing eIF4F complexes without eIF4E). All three eIF4G proteins (I, II, and p97) are proteolytically processed by caspases. Studies have identified caspase-3 as the protease that cleaves eIF4GI into three distinct fragments by recognizing the sites $DLLD^{532}$ and $DRLD^{1176}$ (Clemens, Bushell et al. 1998; Bushell, Poncet et al. 2000). The processed fragments include an N-terminal fragment (N-FAG) containing the PABP binding site, a stable middle fragment (M-FAG) containing the eIF4E, eIF3, and ribosome associated binding site, and a C-terminal fragment (C-FAG) that contains the binding sites for eIF4A and the eIF4E kinase, Mnk-1 (Figure 1.3). M-FAG has been shown to enhance the translation of both pro-apoptotic Apaf-1 as well as the cap-independent p97. eIF4GII is also cleaved by caspase 3 into six fragments (Marissen, Gradi et al. 2000). However, its function is believed to be fully neutralized. The eIF4G-like p97 isoform is likewise processed into a smaller p86 fragment that also lacks eIF4E binding and is cap-independent. Despite the inability to associate with the mRNA cap, the cleaved product can still participate in protein synthesis and has been found in polyribosomal complexes (Henis-Korenblit, Strumpf et al. 2000; Lee and McCormick 2006). The resulting cleavages essentially disrupt mRNA cap-mediated translation initiation in the apoptosing cell while maintaining the capacity to facilitate ribosome association and translational complex formation (Ohlmann, Rau et al. 1996; Keiper and Rhoads 1997; De Gregorio, Preiss et al. 1999; Hellen and Sarnow 2001). This alternative mode of translation utilizes an IRES-dependent mechanism within the mRNA 5' UTR allowing ribosomal complexes to bypass cap recognition and facilitate synthesis of several

apoptotic proteins by internal translation initiation (Marissen and Lloyd 1998; Henis-Korenblit, Strumpf et al. 2000; Marissen, Gradi et al. 2000). The processed p86 and M-FAG fragments, of p97 and eIF4GI respectively, have both been implicated in the stimulation of IRES-mediated translation during cell death (Henis-Korenblit, Strumpf et al. 2000; Henis-Korenblit, Shani et al. 2002; Nevins, Harder et al. 2003; Holcik and Sonenberg 2005). IRES elements can be found in many cellular survival factors and oncogene mRNAs, as well as those encoding other proteins that control the onset of apoptosis (Bcl-2, XIAP, p97, and Apaf-1) (Holcik, Sonenberg et al. 2000). Therefore, loss of the modular functional domains of eIF4G from the initiation complex changes the mode of initiation and recruits a new type of mRNA for translation in response to changes in cell physiology. In the case of apoptosis, the change in translation mode allows the cell to operate synthetically during all states of physiological stress (cell cycle arrest, attempts at repair, and suicide of the unsalvageable cell).

Our lab recently discovered that *C. elegans* eIF4G (IFG-1) isoforms and by extension protein synthesis mechanism changes are involved in the apoptotic selection during germ cell development. Two major IFG-1 isoforms, p170 and p130, are encoded by a single gene ( *ifg-1),* but differ in their ability to associate with mRNA cap complexes (Contreras, Richardson et al. 2008). The shorter IFG-1 p130 lacks the N-terminal eIF4E binding region and has been suggested to participate in cap-independent initiation much like human p97. Disrupting the balance between p170 and p130 (RNAi-mediated p170 depletion) resulted in a dramatic increase in the number of oocytes undergoing apoptosis, upregulation of the Apaf-1 homolog CED-4, and the appearance of apoptosomes. However it was not determined how or even if the initiation of the caspase cascade resulted from this disruption. Here we report that propagation of the programmed cell death signal as a result of IFG-1 isoform imbalance requires a caspase-

dependent mechanism, and acts through the apoptosome factor Apaf-1/CED-4. Quite unexpectedly we found that forcing cap-independent synthesis in developing oocytes is not sufficient to drive apoptosis without the upstream signaling events. Upstream activation of germ cell death must therefore be suppressed by maintaining the balance between cap-dependent and –independent translation in the germline. The findings suggest that changes in protein synthesis mechanism are an upstream or parallel signal rather than a later consequence of the apoptotic fate. We further demonstrate that, like human eIF4G proteins, IFG-1 p170 is a substrate for the executioner caspase-3 homolog, CED-3. The cleavage products generated by CED-3 indicate similar targeting of the cap-dependent function of the long IFG-1 isoform. Furthermore we defined the precise cleavage site in IFG-1 to be just 65 amino acids downstream of the start site of the cap-independent p130 IFG-1 isoform. The significance of eIF4G integrity, the balance of isoforms maintained in developing germ cells, and the influences that cause apoptotic signaling in gamete development is discussed.

*Results*

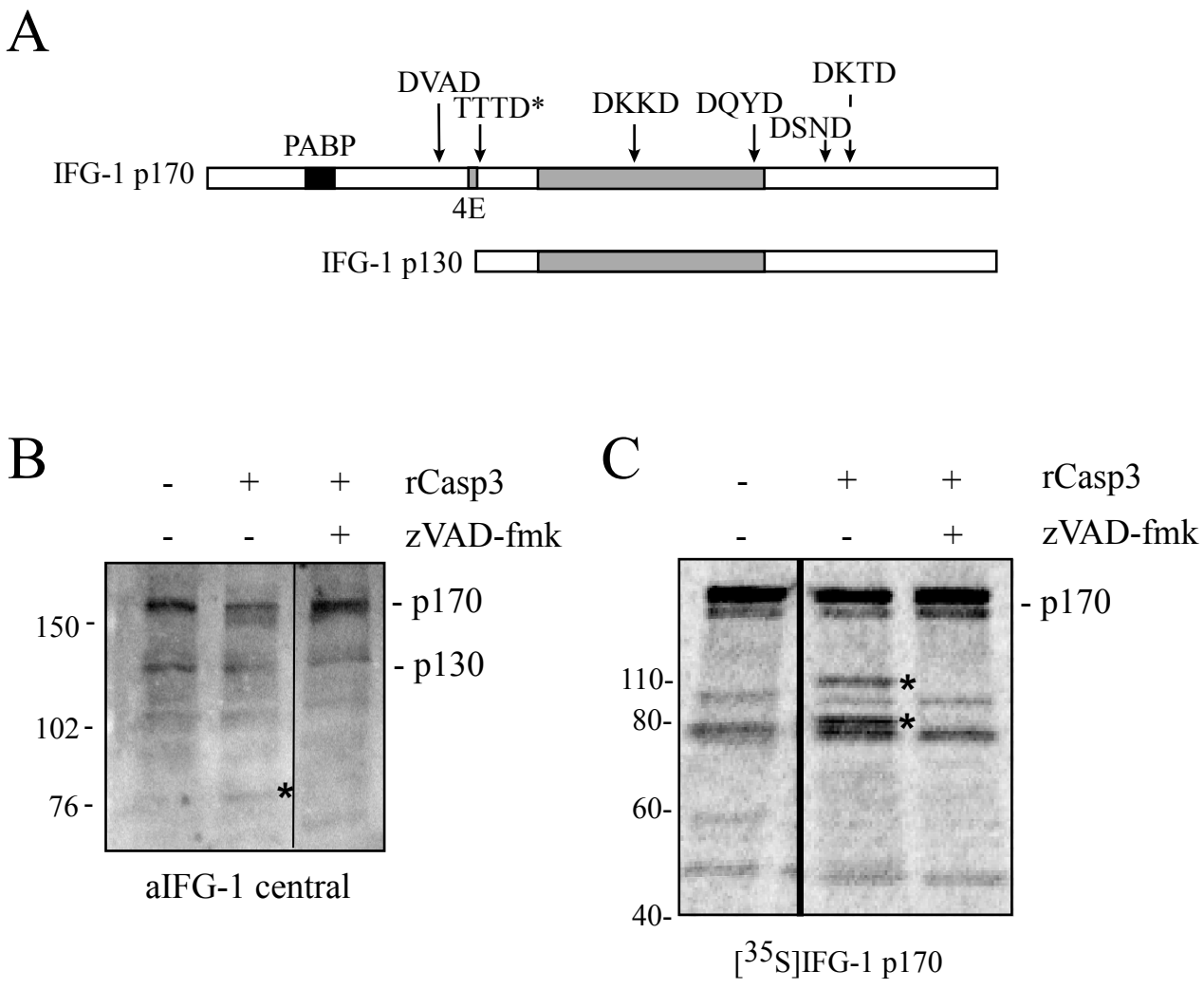
The depletion of the cap-dependent translation initiation complex induces extensive apoptosis in developing *C. elegans* oocytes (germ cells) and enhances the synthesis of the protease activating protein, Apaf-1 (CED-4) (Contreras, Richardson et al. 2008). Using RNAi to deplete the long eIF4G isoform (IFG-1 p170), the remaining short isoform (IFG-1 p130) can promote only cap-independent translation. This alteration of the initiation mechanism also presumably changes the pool of mRNAs being translated. In mammalian cells this shift in mechanism is accomplished naturally by proteolytic cleavage of eIF4GI and p97 by caspase-3 (Clemens, Bushell et al. 1998; Marissen and Lloyd 1998; Bushell, McKendrick et al. 1999). Activated caspase-3 recognizes the consensus peptide DXXD motif in eIF4GI (2 sites) and p97

(1 site) that liberates both proteins into central domain fragments called M-FAG and p86, respectively (see Chapter 1 diagram). These fragments bear domains that still bind eIF4A and eIF3, affording them enhanced activity to initiate cap-independent mRNA translation (Hundsdoerfer, Thoma et al. 2005). Cleavage abrogates association between eIF4G and the 5'end of mRNAs resulting in primarily cap-independent initiation events. However, to our knowledge there have been no demonstrations of direct caspase-mediated cleavage of non-mammalian (such as *C. elegans*) eIF4G proteins and their effects on the protein synthetic programs mediating physiological germ cell apoptosis.

To determine whether a similar caspase-3 mediated event occurs in *C. elegans*, we first assayed whether native worm eIF4G (IFG-1) was a substrate for mammalian caspase-3. Protein extracts from whole worm lysates were incubated with 100 ng recombinant human caspase-3 (Sigma) in the presence or absence of the pan-caspase inhibitor, z-VAD-fmk. Western blotting showed that IFG-1 p170 is depleted in the presence of caspase-3. Caspase-3 depleted p170 generated a small fragment approximately 80 kDa in size, though this proved hard to resolve suggesting further proteolysis (Figure 4.1B). Additionally, the small fragment indicated that IFG-1 p170 is susceptible to cleavage near the central domain to generate two similarly sized products. Interestingly, there appeared to be no change in the levels of IFG-1 p130 in the presence of caspase-3. These results indicated that IFG-1 p170, like its human counterpart, is a substrate for the mammalian executioner caspase-3. However, cleavage of native IFG-1 p170 by human caspase-3 is quite inefficient in worm extracts. Because *C. elegans* IFG-1 protein is quite labile and subject to indirect proteolysis by worm or bacterial proteases, its detection by western blot requires numerous use of protease inhibitors. This may interfere with the activity of caspase-3. Conversely, residual non-specific proteases prevent obvious accumulation of intact

**FIGURE 4.1 IFG-1 p170 is a substrate for caspase-3.** (A) Diagram depicting potential canonical caspase recognition sites within *C. elegans* IFG-1 (schematic drawn to scale). C. elegans caspase actual cleavage site and potential cleavage sites based on canonical caspase-3 recognition motifs are indicated (*). Relative positions of cleavage sites to major translation interacting partners (PABP, eIF4E, and conserved eIF4A/eIF3 binding region) are also shown. (B) Western Blot analysis of native IFG-1 cleavage using 100ng caspase-3 (Sigma). Immunoblot was probed with IFG-1 central domain antibody. Cleavage products are indicated (*). N2 extract was incubated at 37$^{o}$C for 2 hours with 100ng of purified caspase-3 in a total reaction of 25 ul. (C) Cleavage of *in vitro* synthesized IFG-1 p170 by caspase-3. $^{35}$S-labeled full length IFG-1 (1-1156) was incubated with purified human recombinant caspase-3 for 2 hours at 37$^{o}$C. Samples were stopped with 4X SDS loading buffer, loaded on a 4-20% Novex Tris-Glycine Midi gel (Invitrogen) and radioactive signals detected by autoradiography. The pan-caspase inhibitor z-VAD-fmk was used to prevent processing.

# Figure 4.1


A
DVAD
TTTD*
DKKD
DQYD
DSND
DKTD
PABP
IFG-1 p170
4E
IFG-1 p130
B
- + + rCasp3
- - + zVAD-fmk
150 -
102 -
76 -
- p170
- p130
*
aIFG-1 central
C
- + + rCasp3
- - + zVAD-fmk
110-
80-
60-
40-
- p170
*
*
[35S]IFG-1 p170

products in these extracts. These problems were overcome by producing synthetic [$^{35}$S]-labeled IFG-1 p170 in reticulocyte lysate. To verify that mammalian caspase-3 directly cleaves *C. elegans* eIF4G in an extract devoid of any other *C. elegans* or *E. coli* proteins, we generated recombinant IFG-1 p170 and performed proteolysis in a rabbit reticulocyte lysate. Control reactions containing bovine serum albumin failed to elicit any cleavage of *in vitro* [$^{35}$S]- labeled IFG-1 p170 (Figure 4.1C, lane 1). However, there were discrete products of approximately 100 and 80 kDa generated in caspase-treated reactions (Figure 4.1C, lane 2). Addition of the inhibitor z-VAD-fmk completely inhibited cleavage indicating that the processed fragments were caspase-dependent (Figure 4.1C, lane 3). The sharp migration of recombinant [$^{35}$S]-labeled products may indicate post-translational modifications to the full length native protein that are likely absent from the *in vitro* synthesized IFG-1. These results show that the worm eIF4G is a substrate for the human executioner caspase-3 and suggests that IFG-1 may be similarly processed by the *C. elegans* main effector caspase, CED-3.

CED-3 is essential for the majority of developmentally associated apoptotic deaths in the worm (Shaham, Reddien et al. 1999; Chan, Yee et al. 2000). Like mammalian effector caspases, CED-3 is a highly specific protease that cleaves proteins after a tetrapeptide sequence containing an aspartate residue in the P1 position (Taylor, Brumatti et al. 2007). Based on its effector function and substrate specificity, CED-3 has been grouped into the class II family of caspases with its closest human homologs, caspase-3 and -7 (Thornberry 1997). It is synthesized as a catalytically inactive proenzyme of approximately 32 kDa. Upon activation it is autoprocessed to form a heterotetramer composed of large subunit, p17 and small subunit p15/p13. How CED-3 contributes to the control of apoptosis in the germline to date remains unclear because very little

is known about its natural substrates and the alteration of cellular protein synthesis in developing gametes (Taylor, Brumatti et al. 2007).

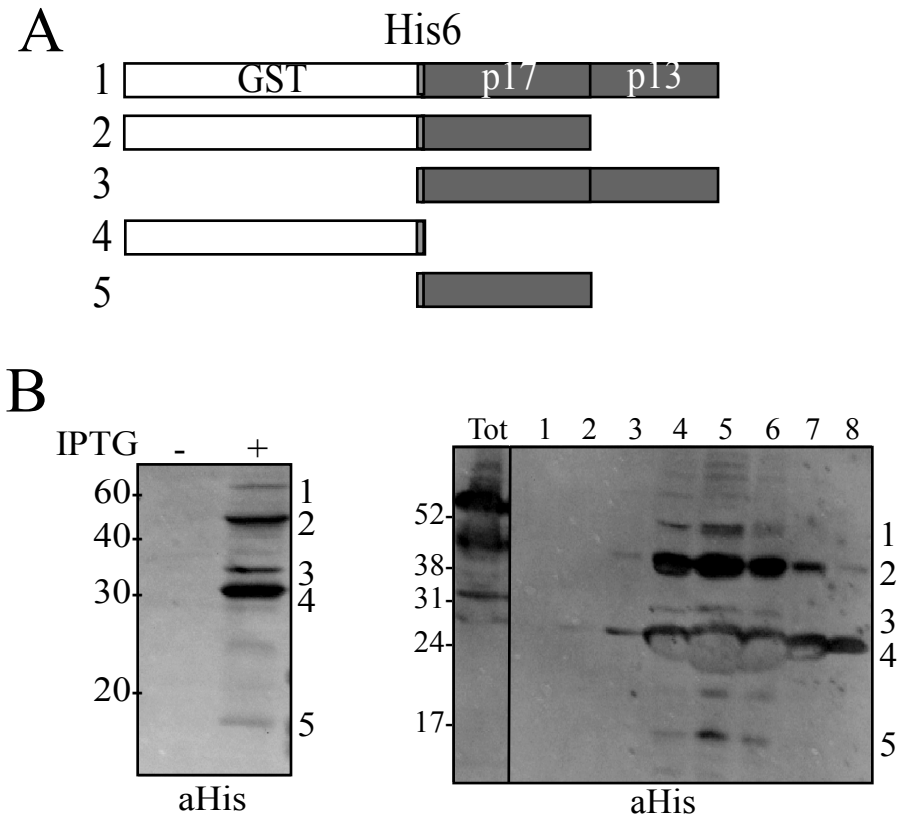
Given the similar substrate specificities between human caspase-3 and CED-3, we considered it likely that the worm caspase would cleave IFG-1 p170 but needed to demonstrate it empirically. We therefore generated active recombinant *C. elegans* CED-3 (rCED-3) using previously described purification conditions (Taylor, Brumatti et al. 2007). The catalytic portion (amino acids 221-503) was fused to N-terminal GST and 6X His tags and expressed in *E. coli* (Figure 4.2A). Expression of the protein in bacteria revealed robust autocatalytic processing into well-defined subunits (Figure 4.2B). Western blotting of bacterial lysate showed very little full length GST-$His^{6}$-$CED\text{-}3^{201\text{-}503}$, but considerable accumulation of processed intermediates. Similar intermediates, which include the p17 and p13 subunits required for activity, were observed when rCED-3 was purified by Ni-NTA affinity chromatography (Figure 4.2B). Proteolytic activity was also confirmed in partially purified rCED-3 using the synthetic tetrapeptide substrate Ac-DEVD-pNA and revealed a 7- fold increase in activity above background (Figure 4.2C). These data demonstrate a rCED-3 that displays similar autocatalytic activity and substrate specificity to other purified effector caspases like caspase-3 (Thornberry 1997).

We next addressed whether native IFG-1 can be cleaved by the *C. elegans* recombinant executioner, CED-3. Cell free cleavage assays were performed on endogenous IFG-1 using worm lysates and subsequently analyzed for proteolysis using an antibody directed against the central domain which detects both IFG-1 isoforms. rCED-3 cleaved IFG-1 p170 to generate a fragment of approximately 135 kDa (Figure 4.3A). Interestingly, this size migrated closely to the naturally expressed cap-independent IFG-1 p130. In order to more carefully define the sites

**FIGURE 4.2 Purification of active recombinant CED-3 (rCED-3).**

(A) Schematic depicting recombinant CED-3 (rCED-3) processed intermediates. The full length (58 kDa) protein consists of the catalytic amino acids 221-503 fused to GST and His6 tags. Autocatalysis of CED-3 into individual subunits (p17 and p13) that are required to create the mature heterotetramer complex are numbered 1-5. rCED-3 expresson was induced in *E. coli* using 100 uM isopropyl β-D-1-thiogalactopyranoside (IPTG) and lysates analyzed by SDS-PAGE and immunoblotted with a His6 monoclonal antibody (Genscript). The tagged proteins were subsequently purified by Ni-NTA affinity chromatography and elutions subjected to western blotting with His6 antibodies. (B) Caspase activity for rCED-3 was determined using chromogenic substrate Ac-DEVD-pNA (Promega) and measured at a wavelength of 405 nm. Purified enzyme (0.1-0.15mg) was added to the substrate (0.2 mM) and rate of proteolysis monitored over a 4 h period. Activity is expressed as nmol pNA/mg protein/h. Caspase activity was compared against a non-enzymatic control containing equivalent amounts of a bovine serum albumin (BSA). Data is representative of three independent preparations of rCED-3. Error bars represent S.E.M.

# Figure 4.2


A
His6
1
GST
p17
p13
2
3
4
5
B
IPTG
-
+
60
40
30
20
1
2
3
4
5
aHis
Tot
1
2
3
4
5
6
7
8
52
38
31
24
17
1
2
3
4
5
aHis
C
Caspase Activity
(nmol pNA/mg protein)
Control
rCED-3
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
Time (h)

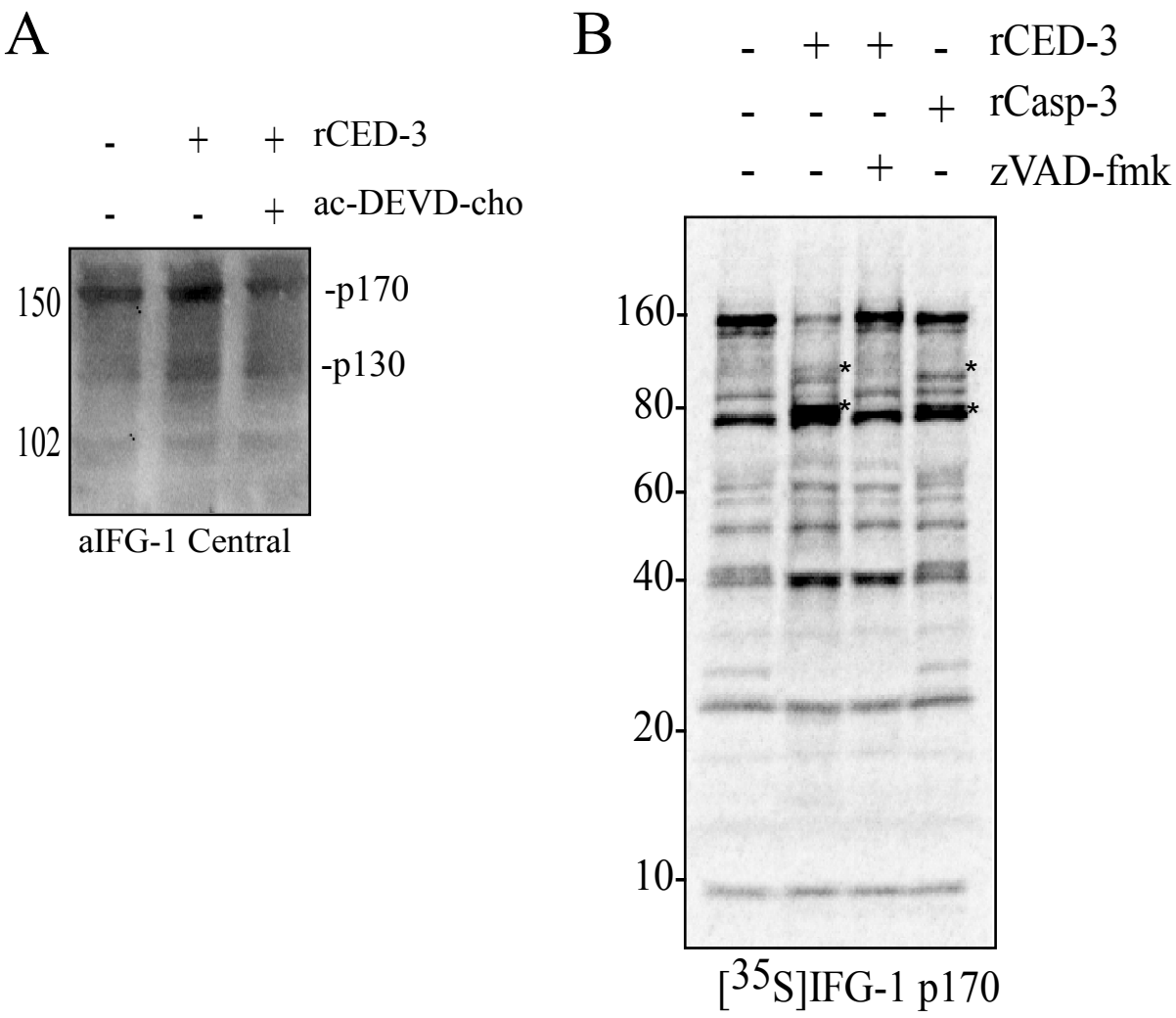
of proteolytic cleavage by CED-3, cleavage assays were conducted using radiolabeled full length IFG-1 p170 incubated with rCED-3. Approximately 80% of full length IFG-1 was processed by rCED-3. Two major fragments of approximately 80 and 70 kDa were observed representing C- and N-terminal fragments respectively (Figure 4.3B). However our results indicate that under conditions of extensive cleavage the smaller 70 kDa product likely gets further processed (Fig 3B, inset). Cleavage of the 150 kDa full length IFG-1 and appearance of both products was prevented in the presence of z-VAD-fmk, indicating further that the cleavage of IFG-1 is dependent on the worm caspase. Furthermore, our rCED-3 was also able to cleave *in vitro* synthesized CED-9, a known anti-apoptotic substrate that had been previously described (data not shown) (Xue and Horvitz 1997). The major product resulting from IFG-1 p170 cleavage was similar to that generated by digestion with human caspase-3. The product size suggests a conserved substrate recognition motif that may be shared among the effector caspases in eIF4Gs from species as divergent as mammals and nematodes. These results show that CED-3 specifically cleaves IFG-1 p170, and further suggest that a caspase-dependent mechanism may modulate the protein synthetic state of cells during apoptosis in *C. elegans* analogous to the role of caspase-3 in mammalian cells.

We have shown that *in vitro* synthesized IFG-1 p170 is processed by both recombinant human caspase-3 and *C. elegans* CED-3, indicating the presence of a major cleavage site that bifurcates the protein. Potential protease recognition sites for caspase-3 (DXXD) in IFG-1 p170 were identified spanning the length of IFG-1 and include $DVAD^{334}$, $DKKD^{671}$, $DQYD^{784}$, $DSND^{890}$, and $DKTD^{931}$ (Figure 4.1A). We next addressed if any of these motifs were recognized by rCED-3. To narrow down the potential cleavage sites, we designed constructs for generating truncated *in vitro* synthesized [$^{35}$S]-labeled IFG-1 proteins (Figure 4.4A). Full length

**FIGURE 4.3 IFG-1 p170 is a substrate for rCED-3.**

(A). Western blot of IFG-1 cleavage by rCED-3. Equal amounts of soluble extract were mixed with approximately 150 ug of partially purified enzyme. IFG-1 central domain antibody was used to detect p170 and p130 80 kDa product is marked in lane 2 and is absent in the caspase inhibitor lane. (B) Cleavage of *in vitro* radiolabeled IFG-1 p170. $^{35}$S-labeled full length IFG-1 (1-1156) was incubated with partially purified rCED-3 for 2 hours at 37$^{o}$C. Extent of IFG-1 processing is depicted on autoradiographic scan. A 100 kDa and 80 kDa fragments are visible and labeled. IFG-1 p170 cleavage fragments are marked (*).

Figure 4.3

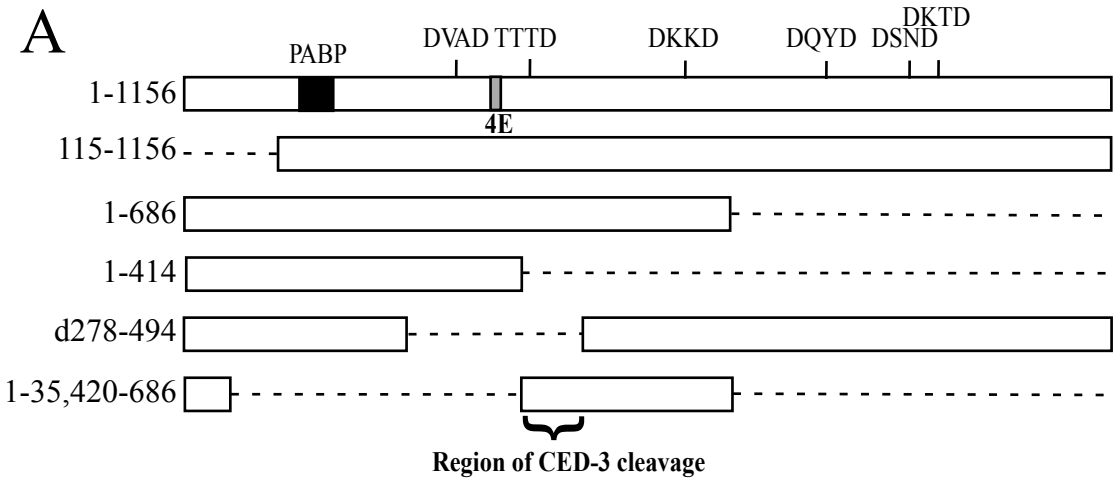

A
- + + rCED-3
- - + ac-DEVD-cho
150
102
-p170
-p130
aIFG-1 Central
B
- + + - rCED-3
- - - + rCasp-3
- - + - zVAD-fmk
160
80
60
40
20
10
[35S]IFG-1 p170

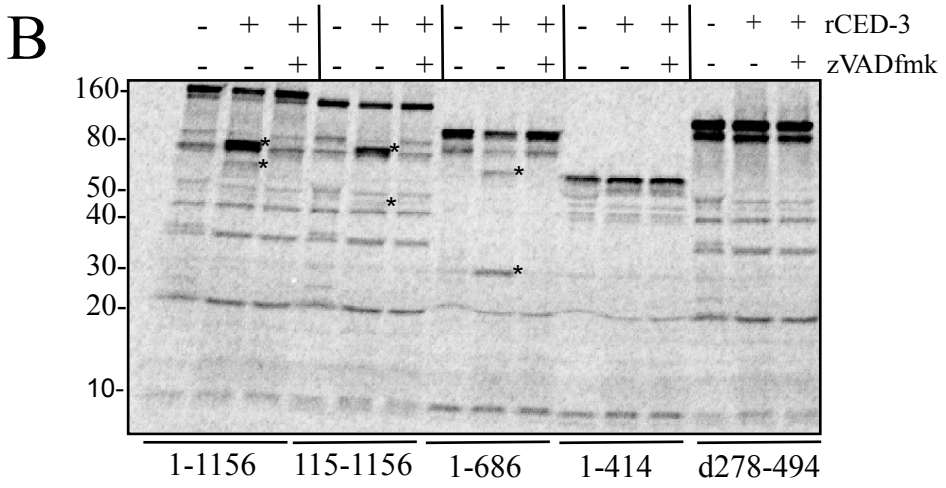
(1156 aa) IFG-1 is processed into 80 and 70 kDa fragments upon cleavage by rCED-3 (Figure 4.4B, lane 2). As previously stated, the appearance of the 70 kDA fragment is likely caused by less extensive secondary processing. Of the five potential caspase consensus sites, most were toward the C-terminal end of IFG-1. We generated N-terminally truncated IFG 115-1156 and C-terminally truncated IFG 1-686 and 1-414 to identify the region containing the major site of cleavage. When radiolabeled IFG 115-1156 was incubated with rCED-3, the product sizes observed were 80 and 50 kDa respectively (Figure 4.4A, Lane 5). The 80 kDa fragment migrated identically to the 80 kDa product from cleaved full length IFG1-1156 (Figure 4.4, lane 2). By contrast, no 70 kDa fragment was detected. Rather a smaller digested product (50 kDa) consistent with the absence of the 114 N-terminal amino acids was observed. The cleavage pattern therefore indicates that the 70 kDa fragment generated from full length IFG-1 is an N-terminal product and the 80 kDa fragment must be a C-terminal product, since the latter is present in both IFG 1-1156 and IFG 115-1156 reactions. The result further suggests that a more central cleavage site rather than one near the C-terminus is being used as the primary rCED-3 site.

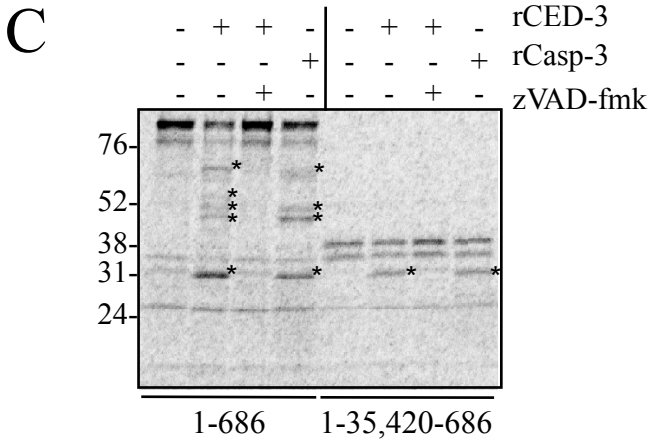
The N-terminal domain of eIF4Gs causes the proteins to migrate aberrantly on SDS-PAGE due to the unique composition of amino acids forming the hinge region of the scaffold (Bradley, Padovan et al. 2002). This is also true of *C. elegans* IFG-1 since the protein has a predicted size of 120 kDa but migrates at approximately 170 kDa (Contreras, Richardson et al. 2008). To optimize resolution of electrophoretic mobility, we next analyzed rCED-3 cleavage of C-terminally truncated IFG-1 (IFG 1-414 and IFG 1-686). Interestingly, incubation with active enzyme produced cleavage of IFG 1-686 only (Fig. 4A, lane 8). Cleavage of IFG 1-686

**FIGURE 4.4 IFG-1 p170 caspase cleavage region is downstream of eIF4E site.** Diagram of IFG-1 deletion proteins used for in vitro determination of CED-3 recognition site. Full length protein is shown with potential cleavage motifs are shown relative to eIF4E and PABP binding sites. Deletion of protein sequence is indicated by dashed line. Region of CED-3 proteolysis is also indicated. (B,C) Analysis of the cleavage pattern of radiolabeled IFG-1 proteins following in vitro treatment with rCED-3. Pattern of fragments (*) indicate that the CED-3 site of cleavage is in the central domain just downstream of eIF4E site

# Figure 4.4


A
PABP
DVAD TTTD
DKKD
DQYD
DSND
DKTD
1-1156
4E
115-1156
1-686
1-414
d278-494
1-35,420-686
Region of CED-3 cleavage


B
rCED-3
zVADfmk
160
80
50
40
30
20
10
1-1156
115-1156
1-686
1-414
d278-494


C
rCED-3
rCasp-3
zVAD-fmk
76
52
38
31
24
1-686
1-35,420-686

generated major sizes corresponding to 70 and 30 kDa. The 70 kDa fragment comigrated with the 70 kDa band from IFG 1-1156 (Figure 4.4B, lanes 2 and 8), confirming that this as an N-terminal product. Since the IFG-1 C-terminus generally migrates more accurately with predicted sizes, the second major fragment of 30 represents a truncated portion of the C-terminal 80 kDa product observed in digested IFG 1-1156. Notably, IFG 1-414 was fully resistant to active rCED-3 (Figure 4.4B, lanes 10-12), demonstrating that no cleavage site is present upstream of position 414. Based on these results, the major rCED-3 cleavage site for IFG-1 must be located centrally between amino acids 414-686. Furthermore, the CED-3 cleavage is unlikely to involve the consensus caspase sites, $DVAD^{334}$, $DQYD^{784}$, $DSND^{890}$, or $DKTD^{931}$ as all of these lay outside of the identified CED-3 cleavage region (Figure 4.4A, 414-686). Remarkably, caspase-3 also cleaved IFG 1-686 with a similar product pattern, however, the major fragments observed were 30 and 45 kDa and less of the N-terminal 70 kDa product suggesting a more prominent secondary cleavage event in the N-terminal fragment. IFG 1-686 encodes approximately 80 kDa but migrates at 110 kDa due to aberrant migration of the N-terminus. Thus, the 45 kDa product indicated that there was bifurcation of the protein into roughly equivalent pieces. This pattern of cleavage was consistent with secondary processing by caspase-3 at the canonical site, $DVAD^{334}$ which is more consistent with caspase recognition. These results also suggested that there is a clear difference in substrate specificity between the worm and human effector caspases.

The data thus far point to a major processing site for IFG-1 by rCED-3 located centrally in the protein (aa 414-686). This was confirmed by analyzing potential digestion of radiolabeled IFG d278-494, which eliminated 192 amino acids in the identified region. No rCED-3 cleavage products detected from IFG d278-494 (Figure 4.4A, lanes 13-15). Importantly, the resistance of internally truncated IFG d278-494 to rCED-3 also indicated that secondary cleavage sites are

indeed minor in IFG-1. The absence of processing observed for IFG-1 p170 lacking amino acids 278-414 and the efficient cleavage of IFG 1-686 suggested that the CED-3 recognition site must reside in an 81 amino acid stretch (414-494). Interestingly, this small region contains none of the consensus DXXD motifs within IFG-1. While CED-3 shares a similar DXXD motif with caspase-3, recent reports have shown that non-classical sites are utilized by CED-3 due to the relative promiscuity of the enzyme for residues in the P4-P2 positions (Chan, Yee et al. 2000; Taylor, Brumatti et al. 2007). A search for such non-conventional caspase processing sites with a P1 aspartate but variation among the P4-P2 amino acids revealed six potential aspartic acids including QLAD$^{415}$, FGLD$^{419}$, RVSD$^{427}$, TTTD$^{456}$, QQRD$^{467}$, and SSKD$^{494}$ in this short stretch. To determine which of these might be the major cleavage site, rCED-3 was incubated with radiolabeled IFG-1 1-35, 420-686. This small (36 kDa) substrate lacks most of the N- and C-termini of IFG-1 p170 allowing for more accurate characterization of the central region and its cleavage products (Figure 4.4C). It also encodes only four of the six non-canonical sites. Upon rCED-3 cleavage, a single product was detected that migrated at approximately 30 kDa (Figure 4.4C, lane 6). The product co-migrated with the 30 kDa fragment generated from digested IFG 1-686, indicating it must be a C-terminal product. Again the cleavage was caspase specific as evidenced by similar processing by human caspase-3 and inhibition by z-VAD-fmk (Figure 4.4C, lanes 7 and 8). Based on predicted product sizes, it was determined that the P1 aspartate residue must proximal to the fusion point of IFG 1-35, 420-686. The tetrapeptide sequence, RSVD$^{427}$ and TTTD$^{456}$were identified as the only motifs whose position could yield a product of 30 kDa. These data indicate that the major CED-3 cleavage site is located within the central domain of IFG-1.

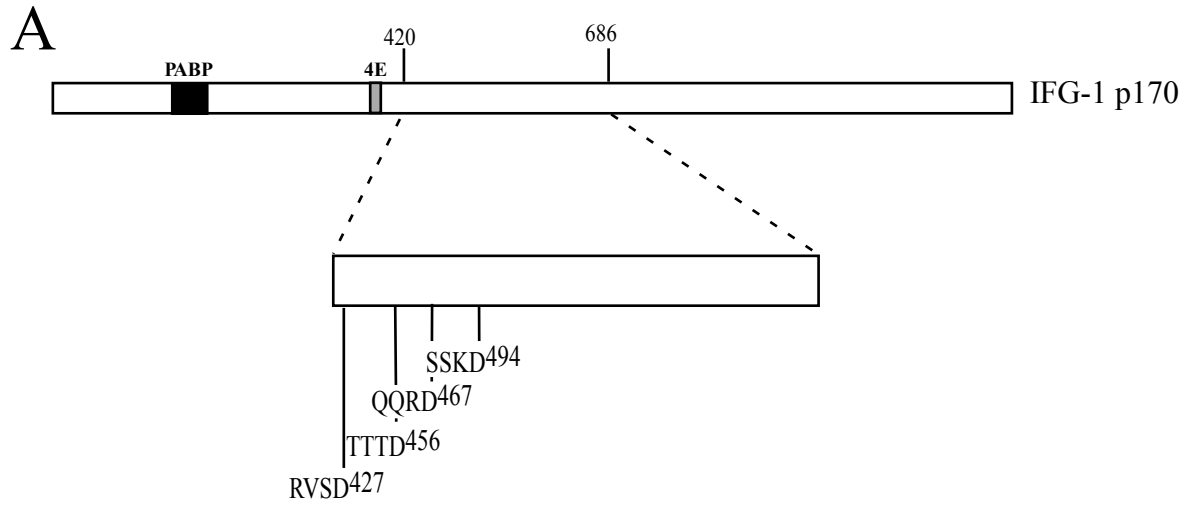
Like human eIF4GI, the *C. elegan*s eIF4G (IFG-1 p170) is a substrate for caspase-3 and its homolog CED-3. Truncation mapping of IFG-1 p170 cleavage suggested that the major processing event occurs at either of two non-canonical sites, RVSD$^{427}$ or TTTD$^{456}$ (Figure 5.5A). Unique among eIF4Gs cleaved by caspase-3, the two cleavage motifs in IFG-1 p170 are both located downstream of the predicted eIF4E binding site. To determine which of these sites is used, we performed site-directed mutagenesis to independently change the aspartates to alanine. *In vitro* cleavage of full length IFG-1 (1-1156) D427A and D456A mutants by recombinant human caspase-3 was then performed and extent of processing assayed by SDS-PAGE. rCED-3 efficiently digested IFG-D427A into previously observed fragments (100 kDa and 80 kDa) from wild type IFG-1 (Figure 4.5B). Thus susceptibility of IFG D427A to human caspase-3 indicates that RVSD$^{427}$ is not the major cleavage site. Cleavage of IFG D456A, however, was resistant to processing and yielded only a single fragment of 100 kDa. The C-terminal 80 kDa fragment was not present indicating that the mutated site prevented cleavage at the major IFG-1 p170 site, TTTD$^{456}$. Interestingly the minor 100 kDa product was enriched during cleavage indicating that this site is more efficiently utilized by human caspase-3 in the absence of the primary processing site. This secondary site for human caspase-3 has not been further mapped but is consistent with cleavage at N-terminal DVAD$^{334}$.

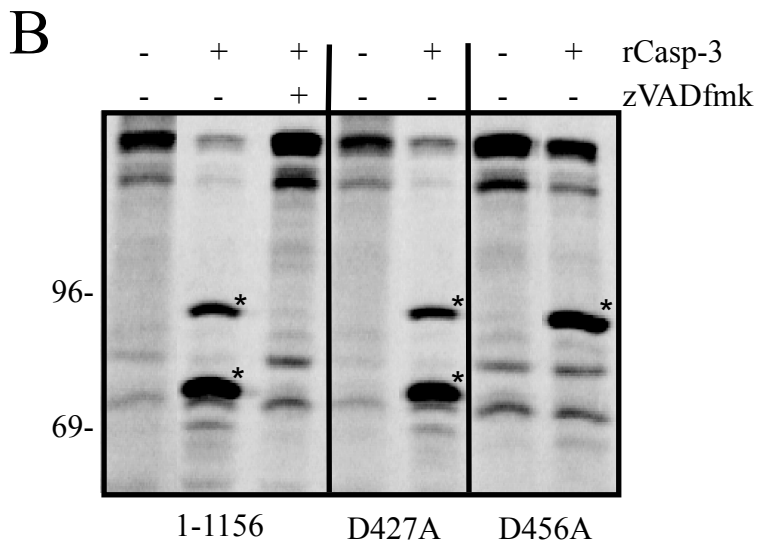
Because CED-3 is the only known physiologically relevant caspase for cleavage of *C. elegans* IFG-1, we tested whether the site-directed mutant IFG D456A was similarly resistant to worm rCED-3 enzyme. Like its human counterpart, CED-3 was unable to generate the major IFG-1 N- and C-terminal fragments when TTTD$^{456}$ was mutated to TTTA$^{456}$ (Figure 4.5C). Interestingly, rCED-3 did not efficiently cleave the secondary site, as did human caspase-3, suggesting again that the worm executioner protease has a higher specificity for the primary site

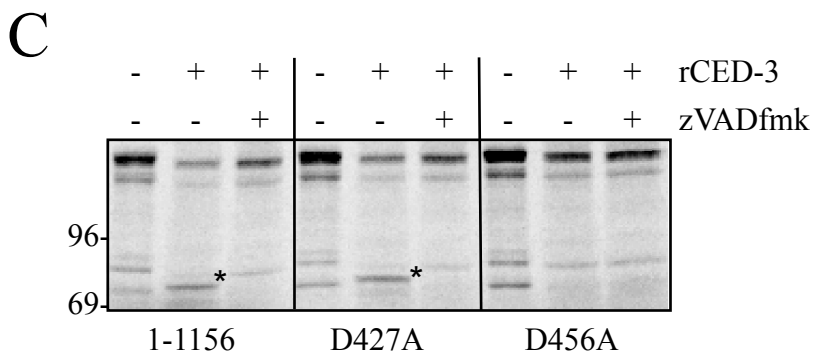
**FIGURE 4.5 The tetrapeptide motif TTTD$^{456}$ is the major site of CED-3 cleavage.**

Schematic showing the central portion of IFG-1 and the potential sites required for CED digestion. Four non-canonical caspase motifs are shown that contain a P1 aspartate. (B,C) Processing of full length $^{35}$S-IFG-1 mutants with caspase-3 and CED-3 reveals site of cleavage. Two sites, RVSD$^{427}$ and TTTD$^{456}$, were mutated by changing the P1 aspartate (D$^{427}$ and D$^{456}$) to alanine. The addition of rCED-3 or rCaspase-3 causes cleavage of full length IFG-1 (1-1156) and RVSD mutant (D427A) but not the TTTD mutant indicating that D$^{456}$ is the site of cleavage.

# Figure 4.5


A
420
686
PABP
4E
IFG-1 p170
SSKD494
QQRD467
TTTD456
RVSD427


B
- + + - + - + rCasp-3
- - + - - - - zVADfmk
96-
69-
1-1156
D427A
D456A


C
- + + - + + - + + rCED-3
- - + - - + - - + zVADfmk
96-
69-
1-1156
D427A
D456A

in worm IFG-1. Collectively these results confirm the motif $TTTD^{456}$ as the major caspase processing site in IFG-1 p170. Cleavage at this single position separates the cap-associating N-terminus from the ribsome-binding C-terminus of IFG-1.

Data presented here identify IFG-1 p170 as a substrate for the main effector caspase in *C. elegans*, CED-3. But the means by which cleaved IFG-1 p170 or the cap-independent IFG-1 p130 induce the apoptotic cascade in the germline is unclear. Over half of all oocytes produced during meiosis in the *C. elegans* germline naturally undergo programmed cell death (Gumienny, Lambie et al. 1999). These deaths occur in a defined region of the worm gonad that can be readily assayed by apoptotic markers (Figure 4.6). The cell death abnormal (*ced*) pathway includes several genes responsible for both the execution and elimination of cells (Hentgartner 1997). To determine whether IFG-1 p130 is a downstream byproduct of the caspase cascade, or might be an upstream initiator of apoptosis in germ cells, we used genetic analysis to determine which of these events comes first. Strong loss-of-function (lf) mutations in the core apoptotic genes, *ced-3* (n2452) and *ced-4*(n1162) have been shown to cause increased survival of germ cells (Hengartner, Ellis et al. 1992; Shaham and Horvitz 1996; Shaham, Reddien et al. 1999). Deficient worms were crossed with a *ced-1:gfp* marker strain to confirm the absence of dying cells. IFG-1 p170 isoform was subsequently depleted (relative to p130) using p170-specific RNAi to mimic caspase-3 cleavage and disrupt cap-dependent translation. Following depletion, the extent of apoptosis in the germline was assayed by observing the number of oocyte corpses decorated with CED-1:GFP. Wild type worms treated with control RNAi sequences unrelated to *ifg-1*exhibited 2-5 corpses which indicated normal levels of physiological apoptosis. These corpses appeared as small fluorescent orbs in the characterized region of germ cell death (Figure

**FIGURE 4.6 Schematic of the *C. elegans* germline.** (A) Diagram of one lobe of an adult hermaphrodite gonad. Each lobe contains maturing haploid oocytes that are mitotically driven in the distal end, progress through meiotic prophase I proximally, become fertilized in the spermatheca, and deposited in a common uterus. Over half of all germ cells will undergo apoptosis as part of a natural clearance mechanism to ensure the fertile competence of mature oocytes. The region of apoptosis is shown and can be easily assayed by DAPI, CED-4 immunohistochemistry, and CED-GFP decoration of dying cells.

Figure 4.6

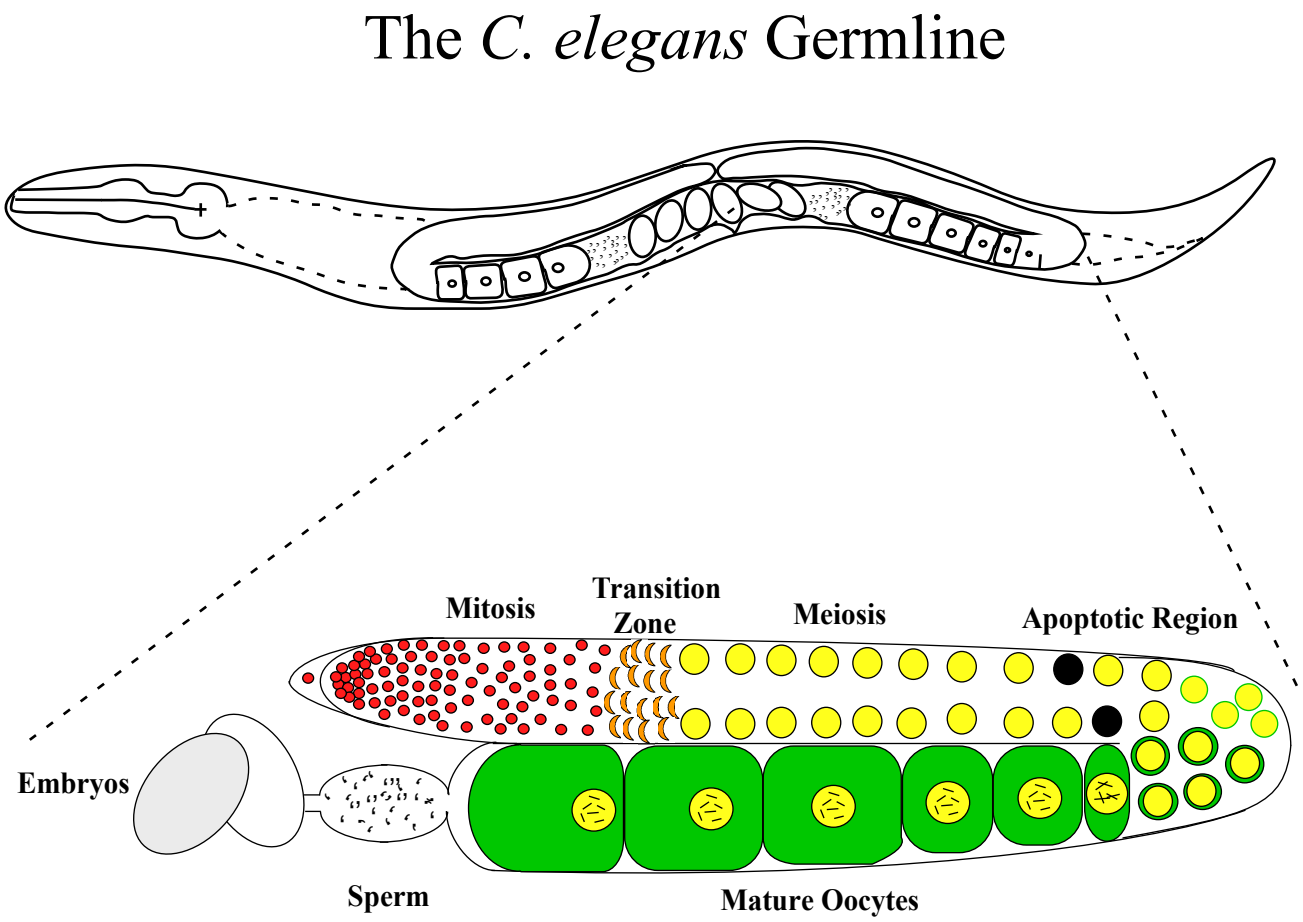
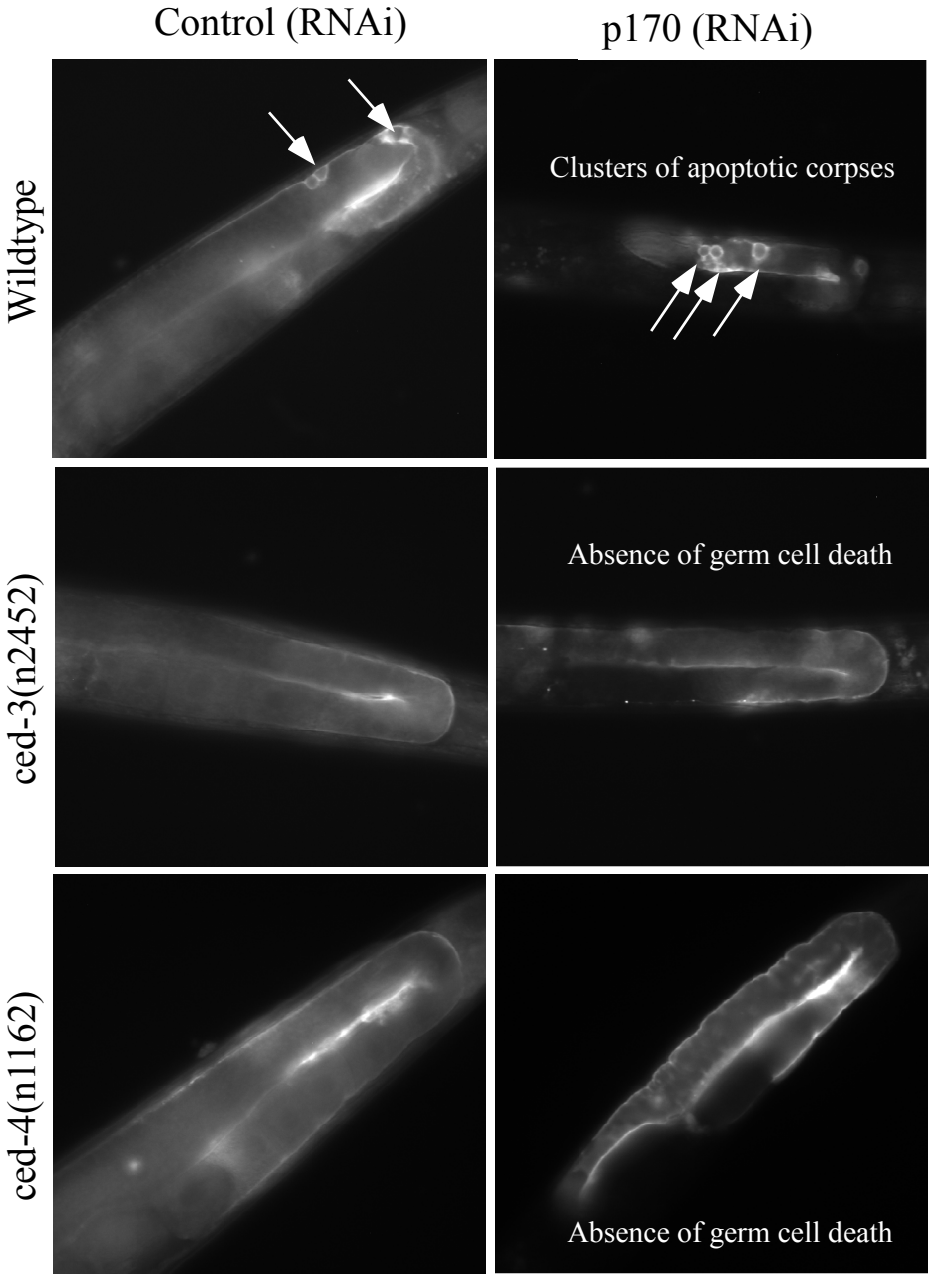

The *C. elegans* Germline
Mitosis
Transition Zone
Meiosis
Apoptotic Region
Embryos
Sperm
Mature Oocytes

**FIGURE 4.7 *p170(RNAi)*-induced apoptosis is upstream of the apoptosome and caspase.** Fluorescent images depicting extent of germ cell apoptosis in either a wild type, **ced-3** (loss-of-function) or *ced-4* (loss-of-function) genetic background. These mutants are deficient in apoptosome and caspase activity. Apoptotic corpses were counted following treatment with p170(RNAi) that disrupts cap-dependent translation. Germ cell corpses are shown with white arrows. Apoptosis was assayed by counting CED-1:GFP- labeled corpses. Germ cell corpses were absent in *ced* mutants indicating that increased apoptosis as a result of *ifg-1* (RNAi) is dependent on the caspase cascade.

# Figure 4.7

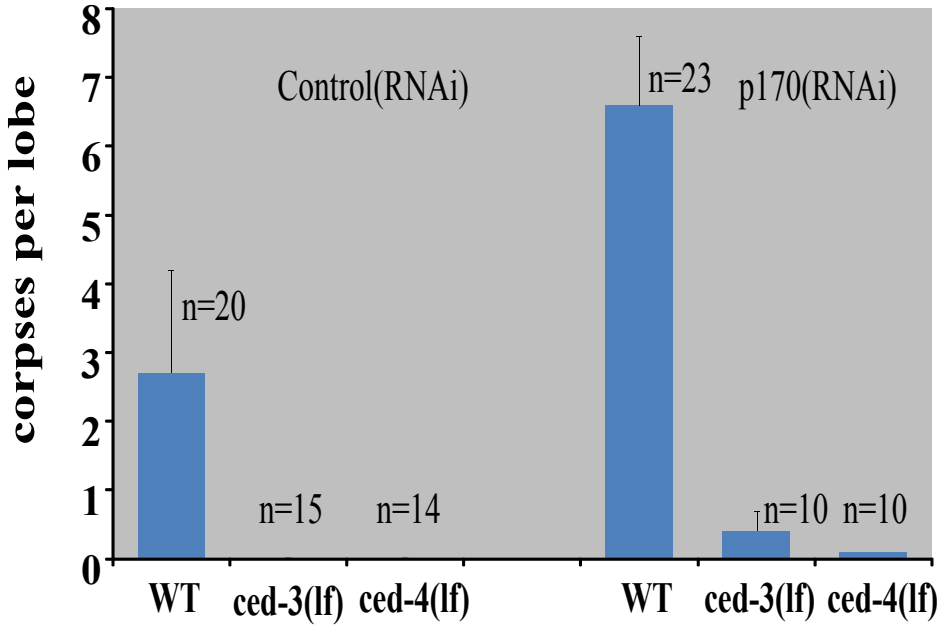

Control (RNAi)
p170 (RNAi)
Wildtype
Clusters of apoptotic corpses
ced-3(n2452)
Absence of germ cell death
ced-4(n1162)
Absence of germ cell death

4.7). Knockdown of IFG-1 p170 in wild type worms induced clusters of germ cell corpses (~2.5 fold) consistent with previously reported observations (Figure 4.8). Surprisingly, worms lacking the executioner caspase (*ced-3*(lf)) exhibited no observable corpses despite the depletion of IFG-1 p170. In addition, p170 (RNAi)-treated worms produced both mature oocytes and fertilized embryos but no viable progeny, in contrast to p170 (RNAi)-treated wild-type worms (data not shown). Worms depleted of IFG-1 p170 possessed few mature oocytes and virtually no fertilized embryos (Contreras, Richardson et al. 2008). Thus, the germ cell death caused by *ifg-1* (RNAi) is suppressed by the caspase activity. These data also indicate that CED-3 partially restores an oocytes competence for fertilization but not progression through embryogenesis. It is likely that IFG-1 p170 is therefore involved in other oogenic processes which are caspase-and apoptosis-independent.

We next addressed whether the apoptosome protein Apaf-1/CED-4 was required for IFG-1-mediated apoptosis. Similar depletion of IFG-1 p170 by RNAi was conducted in *ced-4*(lf) worms. Again, germ cell apoptosis, was not observed in RNAi-treated *ced-4*(lf) worms (Figure 4.7). Additionally several embryos were detected in the uterus of *ced-4*(lf) worms lacking p170. The combined genetic data show that *ifg-1* p170/p130 functions upstream of both *ced-3* and *ced-4*. Concomitantly, germ cell apoptosis as a consequence of IFG-1 p170 depletion is dependent on the apoptosome both its assembly (Apaf-1) and its stimulation of proteolytic activity (caspase). Cell deaths induced as a result of disrupting the balance between cap-dependent and cap-independent translation lead to *de novo* apoptosome (CED-4) formation and subsequent caspase (CED-3) activation. As such, IFG-1 p170 appears to protect oocytes from programmed cell death through cap-dependent synthesis. The order of these events is consistent with our previous observation of increased CED-4 synthesis prior to the morphological changes preceding

**FIGURE 4.8 Caspase and apoptosome mutants do not exhibit germ cell apoptosis following *ifg-1* (RNAi).** Bar graph depicts quantitative evaluation of germ cell apoptosis in cell death-deficient mutants. Error bars represent S.E.M. Corpses per gonad were significantly increased in wild type worms treated with p170 (RNAi) than ced-3(lf) or ced-4(lf) under similar conditions indicating that the observed apoptosis required both the apoptosome and caspase.

Figure 4.8


corpses per lobe
0
1
2
3
4
5
6
7
8
Control(RNAi)
p170(RNAi)
n=20
n=15
n=14
n=23
n=10
n=10
WT
ced-3(lf)
ced-4(lf)
WT
ced-3(lf)
ced-4(lf)

oocyte shrinkage and engulfment (Contreras, Richardson et al. 2008). We propose that eIF4G isoforms play a more active role in the decision-making process between growth and apoptosis in cells.

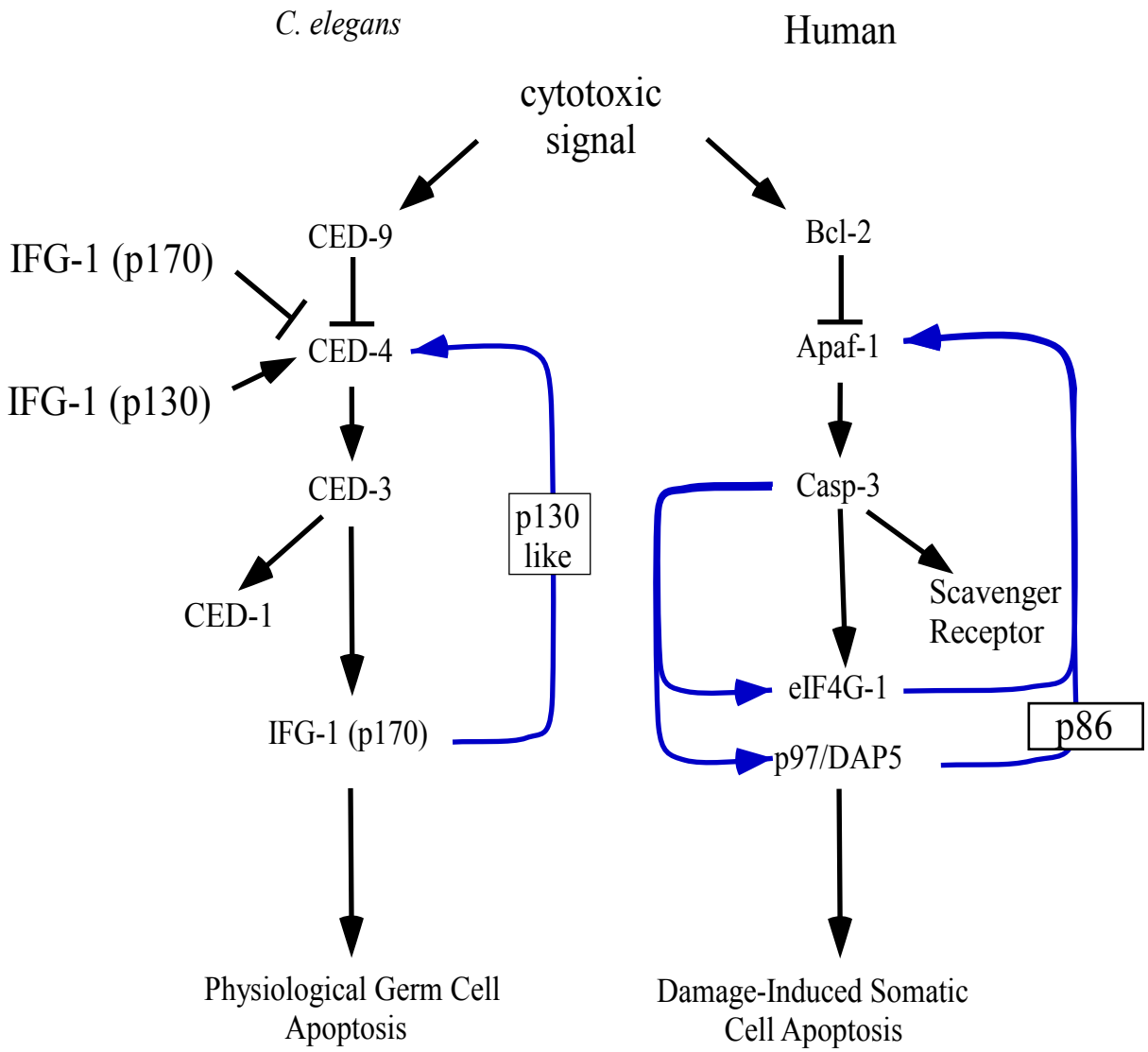
### *Conclusion*

The decision to commit cellular suicide, like many other events requiring changes in gene expression, depends heavily on new protein synthesis. In the initial phases of apoptosis induced by somatic cell damage, the quantity of protein synthesis activity is substantially reduced to allow for cellular recovery (Deckwerth and Johnson 1993; Zhou, Li et al. 1998; Scott and Adebodun 1999). However, long term exposure to insults causes a switch in translation mode to synthesize pro-apoptotic factors like Apaf-1 that push the cell to a ”point of no return” (Holcik and Sonenberg 2005). Thus, the organization and mode of protein synthetic events play a critical role in the timely response needed to protect cells during distress.

Apaf-1 is essential in the cytochrome-c mediated stimulation of pro-caspases, a crucial event in the apoptotic cascade (Bao and Shi 2007). The caspase-mediated cleavage of the translation initiation factor eIF4GI and p97 promote Apaf-1 translation via the IRES element. The cleaved products of eIF4GI and p97 retain the highly conserved MIF4G domain that mediates ribosome recruitment and association with mRNA. These fragments mediate ribosome complex formation and initiate IRES-driven translation. Synthesis of pro-apoptotic proteins are increased, while hindering that of many proteins involved in cell cycle and growth (e.g. VEGF, c-myc, cyclin D1), which rely heavily on cap-mediated translation. Cleavage of eIF4G thus directs a switch from a cap-dependent to cap-independent mode of translation and creates a positive feedback loop in the caspase cascade through Apaf-1 synthesis that reinforces a cell’s commitment to die (Figure 4.9). Remarkably, the physiological signals that trigger normal

**FIGURE 4.9 Proposed mechanisms for activation of *C. elegans* apoptotic pathway.** Diagram shows the comparison between eIF4G isoforms and the apoptotic cascade in human and *C. elegans*. Insults that damage cells in human cause the formation of the apoptosome and activation of downstream caspases like caspase-3. The consequential cleavage of eIF4G and p97 generates fragments which enhance the cap-independent translation of IRES-containing Apaf-1 mRNAs. This ensures a continuous reinforcement of the pathway. In *C. elegans*, Apaf-1/CED-4 is upregulated in dying germ cells as a result of changing the balance between cap-dependent and –independent IFG-1 isoforms. This upregulation is propagated upstream of the apoptosome proving these changes in translation mechanism are not all a consequence of apoptosis. As germ cell become unsalvageable, the increased activity of the CED-3 caspase processes IFG-1 p170 into a p130-like fragment that supports endogenous p130 in the enhanced cap-independent translation of *ced-4 mRNAs.*

Figure 4.9


C. elegans
Human
cytotoxic signal
CED-9
IFG-1 (p170)
CED-4
IFG-1 (p130)
CED-3
CED-1
IFG-1 (p170)
p130 like
Physiological Germ Cell Apoptosis
Bcl-2
Apaf-1
Casp-3
Scavenger Receptor
eIF4G-1
p97/DAP5
p86
Damage-Induced Somatic Cell Apoptosis

developmental cell deaths (e.g. in embryos and the germline) are less well understood. Clearly, their sibling cells readily utilize protein synthetic mechanisms to carry out growth and differentiation. It stands to reason that cells selecting a natural developmental suicide fate should also do so by protein synthetic changes. Here we have delineated such mechanism changes at the level of the translation initiation complex.

We show that *C. elegans* translation factor eIF4G (IFG-1) is a substrate for the cysteine protease CED-3, and that loss of p170 integrity activates germ cell apoptosis in a caspase-and apoptosome-dependent manner. While the apoptotic cascade is highly conserved between humans and nematodes, the initial signals that induce the pathway during physiological developmental death are poorly understood. In worms as in human, initiating apoptosis leads to the activation of caspases and the digestion of several structural and signaling proteins in the cytoplasm, among them, the isoforms of eIF4G. Caspase-3 has been shown to directly cleave eIF4GI into three distinct fragments (Clemens, Bushell et al. 1998; Bushell, Poncet et al. 2000). While the proteolysis of eIF4G has been extensively characterized, little is known about the respective function of each cleavage product in apoptotic progression. We have shown that both human caspase-3 and CED-3, the worm executioner caspase can also cleave the worm eIF4G IFG-1 p170) *in vitro* into discrete N- and C-terminal fragments. Proteolysis was inhibited by a pan-caspase inhibitor z-VAD-fmk indicating that IFG-1 p170 is a caspase-specific substrate. These results show that the *C. elegans* IFG-1 may be a target during the initial phases of apoptosis.

The convincing demonstration of CED-3 cleavage of *C. elegans* substrates *in vivo* has remained elusive to us and others because of the difficulty to generate a significant homogeneous population of developmentally apoptosing cells relative to the healthy cells remaining in the

whole animal (Taylor, Brumatti et al. 2007). As an alternative, we showed *ex vivo* cleavage of native IFG-1 p170 using recombinant CED-3 and human caspase-3. IFG-1 p170 is processed into a 135 kDa product that migrated similarly to the native cap-independent IFG-1 p130 isoform. p170 digestion was prevented by the caspase-3 specific inhibitor, Ac-DEVD-CHO indicating that the cleavage event was due solely to the nematode CED-3. To further characterize the exact site of cleavage of IFG-1 by CED-3, we performed *in vitro* cleavage of radiolabeled truncations that placed the site in a region containing no caspase consensus (DXXD) motifs. Site-directed mutations revealed a single non-conventional sequence at $TTTD^{456}$ that was responsible for generating the discrete C-terminal and N-terminal fragments. Other studies have reported the recognition of non-consensus sites in worms and *Drosophila* substrates (Chan, Yee et al. 2000; Taylor, Brumatti et al. 2007; Creagh, Brumatti et al. 2009).

Interestingly, human recombinant caspase-3 appears to recognize not only the primary site ($TTTD^{456}$) but a second major site at $DVAD^{334}$ that was only weakly recognized by rCED-3. This cleavage pattern was similar to that for human eIF4GI in that the C-terminal/central domain cleavage product is predicted to contain the putative eIF4E binding region. By contrast *C. elegans* CED-3 cleavage of $^{35}S$-labeled IFG-1 p170 bifurcated the protein into two fragments in a similar manner to viral protease digestion (2A and L) of eIF4G during picornavirus infection (Lloyd 2006). It is interesting that a single cleavage event removing the eIF4E site as a mechanism for nematode apoptotic protein synthesis so closely parallels mammalian viral infection. Numerous studies have demonstrated that cleavage of eIF4G in this manner disrupts eIF4F assembly and promotes the cap-independent new synthesis of viral proteins (Lamphear, Yan et al. 1993; Lamphear, Kirchweger et al. 1995; Ohlmann, Rau et al. 1996). The location of the CED-3 cleavage site in IFG-1 p170 was just 65 amino acids downstream of the predicted

start site (AUG) for the naturally occurring cap-independent IFG-1 isoform, p130. We confirmed the latter by sequencing the non-trans-spliced 5' end of *ifg-1* p130 mRNA. These data indicated that perturbation of IFG-1 p170 by CED-3 may yield a p130-like protein that promotes cap-independent translation during the onset of germ cell apoptosis.

More informative than the molecular details of CED-3 specificity for a particular cleavage site in IFG-1 is the biological regulation of physiological germ cell apoptosis brought on by this event. Until now, protein synthesis changes during apoptosis have been viewed more as an "effect" rather than a "cause" (Henis-Korenblit, Strumpf et al. 2000; Henis-Korenblit, Shani et al. 2002). There has been no evidence to date linking eIF4G cleavage to the incidence or onset of apoptosis. We have shown that the worm Apaf-1 homolog CED-4 is also upregulated by shifting the balance between the cap-dependent p170 and cap-independent p130 isoforms of IFG-1(Contreras, Richardson et al. 2008). This study provides evidence that the *C. elegans* eIF4G acts as an upstream activator of caspase-mediated apoptosis, rather than a consequence during the early development of germ cells. Genetic evidence indicates that an IFG-1-mediated switch occurs upstream of or parallel with the core apoptotic machinery, Apaf-1/CED-4 and caspase-3/ CED-3 (Figure 4.9). As cap-mediated protein synthesis diminishes, there is a coordinate increase in cap-independent initiation and the synthesis of death associated factors like CED-4 through the p97-like p130 IFG-1 isoform. An artificial shift in the initiation mechanism by p170 (RNAi) enhanced the number of apoptotic events in the germline (Contreras, Richardson et al. 2008). Subsequent upregulation of CED-3 activity caused further proteolytic processing of IFG-1 p170 resulting in more p130-like, cap-independent eIF4G isoform, thereby reinforcing the apoptotic cascade through enhanced translation of CED-4 and other death-associated factors. Mammalian cell studies bear out the consequences of such a

change in protein synthesis mechanisms. The utilization of mRNAs containing IRES elements allows cells to respond rapidly to changes in growth conditions during apoptosis (reviewed in Spriggs, 2008). While such IRES elements have yet to be discovered in *C. elegans*, there is a precedent for large-scale changes in the pool of translating mRNAs required for gametes in the gonad (Evans and Hunter 2005).

A remarkable cascade of molecular events allows cells to control their proliferation, coordinate differentiation, and minimize damage through an organized cell death program. Apoptosis is necessary for the disassembly and removal of cells that either disrupt neighboring cell function or are unnecessary for the architecture of tissues (Graber and Holcik 2007; Taylor, Brumatti et al. 2007). It has become clear that the demolition process is not a static series of identical steps for every situation, or response to all stressors. The integrity of IFG-1 p170 is maintained in germ cells to prevent entry into the apoptotic cascade and allow them to complete oogenesis. This study adds protein synthesis mechanism change to the list of natural signals that induce a cell to begin the process of apoptosis. While the circuitry of protein synthesis regulation during germ cell apoptosis requires further investigation, it is clear that a fine-tuned balance of translational control is necessary for the developmental competence of early germ cells and their maturation to egg and sperm.

# CHAPTER 5: CONCLUSIONS

## *IFG-1 Isoforms Promote Changes In Germ Cell Fate*

The control of gene expression can occur at multiple levels over the course of differentiation in a eukaryotic cell. However, regulation of pre-existing mRNA translation affords the cell the necessary flexibility to respond rapidly to environmental stimuli. This level of fine control can affect the total synthetic output as well as the tissue specific expression of select mRNAs to meet the physiological demands of a changing cell. More and more evidence indicates that the dynamic interactions facilitating eIF4F assembly influence the polyribosomal recruitment of mRNAs that are critical for physiological changes that specify whether a cell proliferates, differentiates, or dies. The data presented in this dissertation provide further support for the effect of translational control on cell fate determination. The balance of two structurally distinct isoforms of *C. elegans* eIF4G (IFG-1), p170 and p130, is required to suppress the expression of apoptogenic Apaf-1/CED-4 and maintenance of oocyte survival during gametogenesis. Suppression of apoptosis is genetically parallel or upstream of apoptosome formation and caspase activation. IFG-1 isoforms differ in the N-terminus and appear to be generated by the use of alternative splicing and promoter usage, much like human eIF4GI. The structural differences further correlate with the lack of IFG-1 p130 association with mRNA cap binding complexes indicating that it is a cap-independent isoform. The increased expression of CED-4 during depletion of the cap-dependent IFG-1 p170 suggests that Apaf-1/*ced-4* transcripts are preferentially recruited for translation during periods of stress, even in germ cells. Genotoxic induction of germ cell apoptosis cause the expression of a novel IRES-like *ced-4I* mRNA that may contribute to *de novo* induction and apoptosome formation during IFG-1 p170 depletion.

These results suggest a model in which a translation factor is involved in activating the apoptotic cascade through the upregulation of a novel stress-induced *C. elegans* Apaf-1 transcript. Cleavage of IFG-1 p170 by CED-3/caspase provides a caspase-mediated reinforcement of germ cell apoptosis that may occur through a p130-like product. The cleavage site of IFG-1 is located 65 amino acids downstream from the mapped p130 translational start site. This suggests that a conserved pathway exists in *C. elegans* involving the cap-independent induction of germ cell apoptosis.

IFG-1 is expressed broadly throughout *C. elegans* with increased expression in the pharynx, intestine, and germline. The ubiquitous expression pattern is similar to human eIF4GI and –II isogenic forms which are enriched in brain and the reproductive system (Gradi, Imataka et al. 1998). Expression patterns of other eIF4G isoforms in various species reveal further differential expression during development as isoforms have been identified during specific stages of spermatogenesis in *Drosophila*, as well as fertilization in sea urchin (Franklin-Dumont, Chatterjee et al. 2007; Oulhen, Salaun et al. 2007). Because the *ifg-1:gfp* transgene expression construct contained the full gene under the endogenous promoter, it is difficult to address isoform localization during gametogenesis and early embryogenesis. Western blot data shows that expression of IFG-1 isoforms is not dramatically different through all larval stages of development. Both isoforms are also expressed in both somatic and germline tissues. Beyond these observations, further analysis with isoform specific expression constructs may identify more precise spatial and temporal expression of each, p170 and p130, during development.

*Different ifg-1(IFG-1) Isoform Structures Affect mRNA Complex Assembly*

One of the characteristics that distinguish human eIF4GI, eIF4GII, and p97 is the ability to associate with the mRNA cap binding protein, eIF4E. eIF4GI and eIF4GII binding regions for the PABP and eIF4E have been mapped to the N-terminal domain (Mader 1995, Imataka 1998) (Mader, Lee et al. 1995; Imataka, Gradi et al. 1998). It has been shown that the N-terminus facilitates binding to mRNA cap-complexes (Gradi, Imataka et al. 1998). p97 lacks the sequences necessary for mRNA cap-mediated association but does contain the conserved 'recruitment core' that has been shown to bind other initiation factors and ribosomes (Imataka and Sonenberg 1997; De Gregorio, Preiss et al. 1999; Lee and McCormick 2006). While full length eIF4GI and eIF4GII participate in the translation of all capped mRNAs, p97 appears to only associate with select IRES-containing mRNAs during periods of low or compromised cap-dependent translation (Lewis, Cerquozzi et al. 2008). Western blotting using two different IFG-1 antisera has shown that *C. elegans* p170 and p130 differ in their N-terminus. We further show that IFG-1 p170 associates with m7GTP bound mRNA cap-complexes. This association indicates that the long isoform can bind one or more of the five isoforms of the worm eIF4E, IFE 1-5. The IFEs have been shown to be differentially expressed and contribute to a variety of cellular processes including neuronal development, gametogenesis, and meiotic recombination (Amiri, Keiper et al. 2001; Henderson, Cronland et al. 2009; Song, Labella et al. 2010). It would therefore be interesting to know how IFG-1-IFE isoform initiating complexes recruit mRNAs and if different regulatory mechanisms exist to control tissue specific expression or assembly of such alternative eIF4 complexes. On the other hand, IFG-1 p130 does not associate with mRNA cap complexes indicating that it likely participates in cap-independent translation. Some fraction of both IFG-1 p170 and p130 were found in polysome complexes suggesting that both probably participate in translation initiation, but do not remain bound during elongation. While the

association of p130 in these complexes does not confirm its function in initiation, it does correlate with structural evidence that the short IFG-1 isoforms contain the 'core recruitment' domain for ribosome, eIF4A, and eIF3 binding. In human, p97 loading onto ribosomes indicates that cells utilize the cap-independent isoform to synthesize either pro-survival or pro-apoptotic proteins during mitosis, ER stress, and apoptosis. There appeared to be no difference in the amount of either IFG-1 isoform on polysomes due to the fact that complexes analyzed were from unstressed worms. Thus disruptions to cellular homeostasis in *C. elegans* may need to be performed in order to address whether there is a preferential recruitment and assembly of IFG-1 p130 containing ribosomal complexes.

The primary sequence and structure of human eIF4Gs have been extensively characterized yet the functional significance of isogenic forms is unknown. The eIF4GI gene contains three promoters which generate numerous transcripts that are further alternatively spliced. Furthermore the expression of mRNAs is regulated by an upstream IRES in the 5' UTR. So there are obviously multiple levels of transcriptional and translational mechanisms controlling eIF4G expression (Byrd, Zamora et al. 2005; Coldwell and Morley 2006). IFG-1 p170 and p130 are derived from a single gene. Northern and RNase protection confirm that two independent transcripts encode each isoform. More precise mapping of the 5' ends show that capped *ifg-1* p170 mRNAs all contain the same translational start and are trans-spliced to a SL1 splice leader. Approximately 70% of *C. elegans* mRNAs contain a 22 nt (SL1 or SL2) RNA tag that is co-transcriptionally added to the 5' end (Zorio, Cheng et al. 1994). SL1 leaders are trans-spliced to the 5' end of either monocistronic mRNAs or the first cistron in a polycistronic transcript. The 5' end of the IFG-1 p130 mRNA, however, revealed no trans-splicing to SL1 or SL2 and multiple transcriptional starts. All p130 5' ends were upstream of the same in-frame AUG

located at the 3' end of exon 4. This was consistent with the endpoint observed during RNase protection of p130 transcripts . Furthermore we also detected alternative cis-splicing immediately upstream of the translational start. Thus the regulation of the short isoform may involve an analogous transcriptional and translational complexity to human eIF4GI. While the transcriptional origin of *ifg-1* p130 mRNA is unclear, the identification of multiple endpoints and the absence of trans-spliced leaders suggest alternative ("internal") promoter usage. Interestingly, characterization of the 3' ends for p170 and p130 showed heterogeneity of the 3' UTR indicating that alternative polyadenylation signals may also be employed to regulate *ifg-1* expression.

### *Evolution of eIF4G Functional Domains*

It is clear that eIF4G isoform heterogeneity can lead to different functional and binding capacities. However little is known about the selective constraints that led to the creation of multiple eIF4G gene loci from a single ancestral copy in various animal species. IFG-1 exists as multiple transcripts cloned from a single gene while several species of varying physiological complexity contain multiple genes for eIF4G. Yet despite this complexity (e.g. *C. elegans* vs human), there are similarities in gene structure and isoform organization. For example, human eIF4GI and p97 are encoded by two distinct genes. Both proteins contain similar ribosomal binding capacities but confer functionally divergent mechanisms (cap-dependent vs. cap independent). These mechanisms appear to be shared between human isoforms and IFG-1 p170 and p130. Thus the degree of structural and functional homology between *C. elegans* and human allude to a unifying paradigm that may involve multiple duplication events throughout the evolution of eIF4G. Interestingly, we have discovered that the *C. elegans* sister species,

*Caenorhabditis briggsae*, also contains at least two isoforms of IFG. However unlike *C. elegans*, these two isoforms (cbIFG-1 and cbIFG-2) are derived from paralogous genes (see Appendix, Figure C.1A). Furthermore, IFG-2 primarily encodes the highly conserved ribosome/eIF4A/eIF3 recruitment domain required for cap-independent complex assembly. Western blotting of *C. briggsae* extracts using the *C. elegans* IFG central domain antibody reacted with three major proteins indicating that there is a shared distribution of isoforms between the two species (Figure C.1B). These results suggest that partial duplication and shuffling events may have been responsible for a truncated IFG leading to partitioning of function. While it is not known whether these isoforms are functionally active, the phylogenetic relationship between *C. briggsae* and *C. elegans* IFG orthologs may shed light on the early divergence of eIF4G from the last common ancestor.

How alterations to eIF4G structure via duplication came about, and the corresponding effect on their overall function during translation is a mystery. One plausible explanation may involve selective pressures and initiation factor dependency underlying protein biogenesis in early eukaryotes. It has recently been suggested that IRES-dependent translation may have once been the predominant mode of protein synthesis due to its relatively simplified requirements for recruitment of mRNAs (Hernandez 2008) . Characterization of viral IRESs have shown that ribosome association is possible with minimal or no initiation factors (Pisarev, Shirokikh et al. 2005). In addition, studies have suggested that some cellular IRESs may even contain Shine-Delgarno-like ribosomal RNA (rRNA) complementarities that may further facilitate ribosome recruitment (Zhou, Edelman et al. 2001; Akbergenov, Zhanybekova et al. 2004). While the origin of initiation factors is vague, the eIF4A DEAD box helicase is found in all three ancestral domains (Archeae, Bacteria, and Eukarya) and is believed to be one of the earliest RNA-binding

factors. Most of the early RNA factors like proto-eIF4A served in the splicing of heavy intron-containing RNAs as a result of foreign genetic incorporation during the endosymbiotic origin of eukaryotes (Hernandez, Altmann et al. 2009). Unlike eIF4A, common ancestral proteins for eIF4E and eIF4G are difficult to identify indicating that their proto-functions may not have existed at the last common ancestor. It is believed that a proto-eIF4G appeared during eukaryogenesis and only contained the conserved ribosomal recruitment (MIF4G) domain (Hernandez 2008). The separation of the genome from ribosomes (protein synthesis) by the nuclear membrane, the loss of Shine-Delgarno motifs , the increased complexity of the mRNA 5' UTR, and the appearance of proto-eIF4E (then an RNA shuttle protein) enabled proto-eIF4G to evolve modular domains that conferred scanning and cap-dependent translation (Hernandez, Altmann et al. 2009). The current modular incarnation of eIF4G probably evolved from the proto-form that primarily consisted of the core-recruitment domain to the current multi-domain translation factor as a result of chimeric duplication which incorporated surrounding genetic information. Interestingly, *C. briggsae* IFG-2 contains extremely long 5' sequences which are highly divergent from the 5' end of cbIFG-1 or even *C. elegans* IFG-1 indicating that chimeric duplication may have been the result of these extraneous sequences. Co-transcriptional modifications of mRNAs through the addition of the 7-methylguanosine cap and poly (A) tail ensured a more stable means of protecting genetic information between the nuclear and cytoplasmic compartments. A modular scaffold was more efficiently used to bring together the protective cap binding protein and the ribosome in the newly separated cytoplasm. Thus it is believed that cap-dependent translation was only used when eIF4G evolved the appropriate binding regions in addition to the already present recruitment domain (Hernandez, Altmann et al. 2009). It is therefore highly likely that utilization of the core recruitment domain contained in

abbreviated eIF4Gs were in fact the first adapters of translation initiation. Today these adapters mediate cap-independent initiation when cap-dependent translation is compromised acting as "fail safe" , rapid response modulators during the cell cycle (G2/M transition), stress (serum starvation) , and apoptosis (DNA damage) (Pyronnet, Pradayrol et al. 2000; Nevins, Harder et al. 2003).

### *IFG-1 Isoforms Activate Apoptosis through a Translation Switch*

The structural differences between IFG-1 p170 and p130 indicate that *C. elegans* can use cap-independent translation during periods of compromised translation. Depletion of the cap-mediated p170 isoform results in increased germ cell apoptosis. This is in stark contrast to depletion of both isoforms which caused a reduction in overall somatic growth (Contreras, Richardson et al. 2008). Since the worm germline contains cells in a common cytoplasm a careful balance between initiation mechanisms must, therefore, be maintained during transitions of mitosis (cell cycle), early meiotic prophase (differentiation), and late meiotic pachytene (germ cell apoptosis). This rationale suggests that IFG-1 isoform specific complexes alternate as protein synthetic requirements change with the fate of the cell. We have discovered that the pro-apoptotic Apaf-1 homolog CED-4 is upregulated in the absence of IFG-1 p170. In human cells, Apaf-1 translation is mediated by an IRES in its long 5' UTR by cap-independent p97 during etoposide induced apoptosis (Nevins, Harder et al. 2003). Adding further complexity, induced IRES-driven Apaf-1 synthesis depends on the severity of the cellular insult indicating a hierarchical response during apoptosis. In addition, this hierarchy of gene expression can be modulated during apoptosis in a biphasic manner. For example, low level gamma irradiation and serum starvation stimulate the translation of anti-apoptotic XIAP to suppress the caspase cascade

and allow cellular recovery from transient insults (Holcik and Sonenberg 2005). However XIAP synthesis is not enhanced by chronic apoptotic stress (Nevins, Harder et al. 2003). Instead, prolonged exposure to severe insults or extreme apoptotic conditions can cause an irreversibly damaged cell to switch its synthetic output to pro-apoptotic proteins such as Apaf-1. Furthermore Apaf-1 synthesis has been shown to be upregulated in a caspase-dependent manner upon treatment of cells with the apoptosing inducer, etoposide (Nevins, Harder et al. 2003; Warnakulasuriyarachchi, Cerquozzi et al. 2004). Adaptive changes in the expression of new proteins can therefore determine alternate or even sequential fate decisions in cells during period of stress.

It is unclear if a similar mechanism occurs in *C. elegans* but it is apparent that there is *de novo* translation of *ced-4* mRNA in apoptosing germ cells. Further evidence demonstrates that *ced-4* may be induced by an alternatively spliced uncapped mRNA containing a long outronic (5' intragenic) sequence. Since *ced-4* is one of four cistrons in an operon, the outron likely is generated by an aberrant trans-splicing of the polycistronic mRNA. Thus *ced-4I* IRES-like transcripts may be utilized during periods of stress. Interestingly upon genotoxic stress, worms exhibited an increase in an uncapped *ced-4I* transcript that was not observed in control treated worms. There is also preliminary evidence that the expression of the normally spliced monocistron and *ced-4I* are both increased when wild type and mutant worms containing a temperature sensitive mutation in *ifg-1* is shifted to higher temperatures (K Morisson and BD Keiper, personal communication). These results provide support for an apoptotic program that utilizes different *ced-4* mRNAs during compromised protein synthesis. Interestingly the caspase inhibitor XIAP is translated in human cells undergoing apoptosis from two distinct mRNAs that change in the length of the 5' UTR following stress (Riley, Jordan et al. 2010). Curiously,

studies from the Blumenthal lab show that aberrantly spliced *C. elegans* uncapped transcripts accumulate during heat shock (Liu, Kuersten et al. 2003). Little is known about the stability of these uncapped mRNAs. However de Gregorio, et al have shown that the central domain of eIF4G is competent to stimulate the translation of uncapped mRNAs through a 5' end-dependent scanning initiation mechanism (De Gregorio, Preiss et al. 1999). These results provide support for a model depicting upregulation of cap-independent translation initiation of an uncapped *ced-4I* mRNA by IFG-1 p130. Further investigation of *ced-4* mRNAs must be done to demonstrate the actual mechanism leading to CED-4 synthesis when IFG-1 p130 is the predominant form of eIF4G present. If CED-4 is generated from translational activation of an IRES element in the upstream intergenic region, then its activity could be measured by the gold standard for IRES characterization, dicistronic expression assay. These constructs use a two reporter system whereby the upstream cistron (first reporter) is translated by cap-dependent synthesis while the downstream cistron (second reporter on the same mRNA) is under the control of the IRES element in the space separating the two open reading frames. More immediately, it should be feasible to determine the efficiency of *ced-4I* translation by analyzing the co-sedimentation of the mRNA with polysomes in a sucrose gradient profile analysis of stressed worms. Given the unique additional sequences present in *ced-4I* as compared to the wild type properly spliced *SL2-ced-4*, the putative IRES-containing mRNA can be individually detected by real-time PCR.

However the enhanced CED-4 expression in apoptosing germ cells may have an alternative and less complex explanation that does not invoke a 5' extended ced-4I mRNA. It is equally feasible that increased *ced-4* translation is due to the preferential recruitment of alternatively cis-spliced transcripts, *ced-4L* and *ced-4S*. Two isoforms of CED-4 protein are derived from alternatively spliced mRNAs and exhibit opposing functions in apoptotic signaling.

CED-4S has been shown to be pro-apoptotic, while CED-4L is anti-apoptotic (Shaham and Horvitz 1996). Recent evidence has shown that human Apaf-1 likewise contains at least four additional alternatively spliced mRNAs in normal cells and an additional variant discovered in prostate cancer cells (Ogawa, Shiga et al. 2003). However the functional significance of these isoforms is unknown. It is logical to speculate that mRNA utilization may differ for the *ced-4* isoforms during physiological germ cell apoptosis. A shift between cap and non-cap-associated IFG-1 isoforms might facilitate their coordinated synthesis in oocytes destined to die. Further investigation is required to determine whether CED-4 regulation occurs via relative activities of IFG-1 p170 and p130 isoforms. None the less, the addition of the *ifg-1* gene into pathway of activation of the apoptosome suggests that a highly conserved regulatory event during apoptosis is modulated by a protein synthesis initiation mechanism.

Our findings seem to support the IFG-1 upregulation of the apoptosome formation leading to activation of the caspase cascade. Human eIF4G's participation in the apoptotic cascade has, until now, been relegated to activation of IRES containing apoptogenic factors following cleavage of its modular domains (Clemens, Bushell et al. 2000). However, we can now show through epistatic genetics that germ cell apoptosis induced by *ifg-1* disruption is dependent on genes in the apoptotic signaling cascade that encode the apoptosome protein CED-4 and the caspase CED-3. These results indicate a novel requirement for translation factor eIF4G upstream of the apoptotic pathway. The placement of IFG-1 in the signaling pathway is consistent with the observed *de novo* synthesis of CED-4. Moreover, germ cell apoptosis is likely suppressed by IFG-1 p170, whereas p130 acts as a cap-independent inducer of apoptosis. Ultimately, then, the physiological trigger for naturally occurring germ cell deaths may require a change in the balance of protein synthesis initiation mechanism.

Finally, our studies demonstrate that IFG-1 p170 is a substrate for the main *C. elegans* caspase, CED-3. It was already known that all human eIF4G proteins are substrates for caspase-3, but it had not been demonstrated in lower animals. Cleavage disrupts cap-dependent initiation by separating the eIF4E and PABP-binding domains from the ribosome recruitment domain. However among the human proteins there are distinct patterns of processing for each isoform. eIF4GI is cleaved into three fragments. The middle fragment (M-FAG) has been shown to enhance the translation of Apaf-1. Curiously, M-FAG still contains the eIF4E binding site. It is believed that the losses of the eIF4G domains that enhance cap-binding lead to transient and low translational activity (Morley, Coldwell et al. 2005). By contrast, eIF4GII is cleaved into many small fragments that completely abolish its function. The cap-independent p97 is further processed to a p86 form that is found associated on polyribosomal complexes during apoptosis. The *C. elegans* IFG-1 p170 is cleaved downstream of the predicted eIF4E binding site, suggesting that a cap-independent apoptotic form results (p130-like) that may be utilized during germ cell apoptosis. Site-directed mutagenesis identified the caspase-3 non-consensus motif, $TTTD^{456}$ as the site of cleavage. This motif is 65 amino acids downstream of the predicted translational p130 start site, providing support for a p130-like product that likely reinforces the apoptotic cascade through CED-4 synthesis. Thus *C. elegans* employs multiple regulatory mechanisms to sustain a translational switch from cap-dependent growth to cap-independent apoptosis.

The work described herein shows for the first time an activating role for a translation factor in the determination of a physiological cell death fate. The notion of eIF4G as a housekeeping protein factor responsible only for general cellular protein synthesis is no longer valid. Growing evidence indicates that eIF4G participates in alternative initiation mechanisms

and is differentially expressed during cellular perturbations and differentiation. While eIF4G overexpression continues to garner interest in cancer pathology, its role in naturally occurring apoptosis has been left by the proverbial wayside (Silvera, Formenti et al. 2010). This neglect likely comes from the complexity of the eIF4G genes and the difficulty of functional isoform studies in whole animals. We have successfully combined the simplified genetics of *C. elegans* with fundamental protein biochemistry to delineate the functional relationship between IFG-1 isoforms and the highly conserved caspase cascade. These results provide evidence for a modal response to stress and aberrant translation through two structurally distinct IFG-1 isoforms, p170 and p130. This switch in isoform specific initiation probably includes an evolutionary relic (IRES-dependent protein synthesis) that now serves as a translational rheostat to allow cellular adaptation to changing environmental effectors.

# CHAPTER 6: EXPERIMENTAL PROCEDURES

## *Maintenance of Caenorhabditis Strains*

*C. elegans* strain N2 var. Bristol or *C. briggsae* strain AF16 were grown at 20$^{o}$C (unless otherwise stated) on NGM plates with *E. coli strain* OP50 or HT115 containing plasmids that synthesize dsRNA unless otherwise noted (Brenner, 1974). Loss of function strains *ced-3* (n2452) and *ced-4* (n1162) were obtained from the *Caenorhabditis* Genetics Center.

## *Creation of ced-1:gfp, ced-3 and ced-4 Homozygous Strains*

Males from strains carrying the *ced-3* (n2452) and *ced-4* (n1162) were crossed into *ced-1:gfp* expressing MD701 (*bcls39v [$P_{lim-7}$ ced-1::gfp and lin 15(+)]*) hermaphrodites which was kindly provided by Dr. Barbara Conradt (Dartmouth University). *ced-3* and *ced-4* homozygous worms were obtained by self crossing F2 progeny carrying the deletion and picking sixteen individuals to fresh seeded NGM plates for observation at 20$^{o}$C. The mutant alleles of the resulting F3 homozygous strains, KX84 (*ced-3* loss-of-function) and KX87 (*ced-4* loss-of-function) were monitored for the absence of GFP positive germline corpses. A minimum of 20 gonads were analyzed for each strain. In addition to the absence of germ cell corpses, *ced-3* genomic PCR using primers, forward 5'-AGTTCACCGTGACAGCGTCTCTTC-3' and reverse 5'-CGATTACGACTTGAACTGTATCCGA-3' (wild type allele) or reverse 5'-CATTGCAAATCTCGTTTACACAAGA-3' (mutant allele) were used to monitor the deletion. Worms were frozen at -20$^{o}$C and then lysed in Single Worm Lysis Buffer (SWLB) [1X AmpliTaq Gold PCR Buffer, 1 mM $MgCl_2$, 0.45% NP40, 0.45% Tween 20) supplemented with 200 ug/ul Proteinase K. Samples were then lysed by pre-incubating in the thermocycler for 60 minutes at 60$^{o}$C and 10 minutes at 95$^{o}$C. Thermocycling conditions used to amplify ced-3

sequences were: 94°C for 9 minutes, (94°C for 30 seconds, 51°C for 30 seconds, 72°C for 2 minutes), and repeated 43 cycles with a final 10 minute extension at 72°C. The absence of corpses only was used to monitor for the presence of the *ced-4* (n1162) allele.

Developmental stage synchronization of worms was performed by treating gravid (egg-bearing) hermaphrodites with a hypochlorite solution [1.5%] supplemented with 1M NaOH that destroys the outer hypodermal layer. Released eggs containing a tough chitinous shell are protected and therefore allowed to develop normally upon rinsing with M9.

*Creation of ifg-1:gfp Transgenic Strains by Injection*

Transgenic DNA corresponding to the full length *ifg-1* gene (M110.4) fused to *gfp* was linearized and injected as a "complex" array into worm gonads (see Appendix A for a complete description of "complex" arrays). The injection solution contained linearized *pPD ifg-1:gfp* and selectable marker at [1 ng/ul], along with N2 worm genomic DNA [60 ng/ul]. Approximately 50 young *unc-119 (ed3)* adults were injected with the previously described solution under the following conditions. Injections were performed by transferring hermaphrodites to mineral oil on 1.8% agarose pads. Solution was injected at 20-30 psi into the rachis (common cytoplasm shared by germ cells in the distal gonad). After injection, worms were allowed to recover in M9 for 10-15 minutes and then transferred to bacterially seeded NGM plates. Worms were maintained at 20°C and observed for F1 transformants expressing GFP and carrying the selection marker which restores sinusoidal swimming to *unc-119 (ed3)* worms. GFP expressing F1 worms were picked to clone plates and allowed to self-fertilize. F2 progeny that continued to generate GFP positive offspring were considered a heritable strain and frozen at -80°C until integration of the transgene into the genome. Integration of lines with the highest expression of GFP were

performed by UV irradiation and subsequently backcrossed to remove any extraneous deleterious mutations (BD Keiper, personal communication).

*Plasmid Constructions*

The coding sequence (CED-$^{3221-503}$) for the catalytic region was amplified from N2 total RNA using forward 5'-GCCCATATGCACCACCACCACCACCACGTCGATGCACCAACCATAAGCC-3' which includes a 6X His tag and reverse 5'-GCCAAGCTTTTAGACGGCAGAGTTTCGTGCTT-3' primers. The PCR fragment was subcloned into pGEX4T-1(GE Healthcare) to generate pGSTH6CED-3$^{221-503}$.

*ifg-1* deletion plasmids for *in vitro* caspase cleavage assays were encoding various portions of the full length protein (1-1156). The full length IFG-1 p170 protein was generated using the complete *ifg-1* open reading frame (nt 1-3494) from the plasmid pSK *ifg-1* LONG. IFG 115-1156 contained nt 366-3494. Restriction digestion of the *ifg-1* LONG cDNA generated the following deletion constructs: IFG 1-414 (nt 24-1265), IFG 1-686 (nt 24-2083), IFG d278-494 (nt 24-857, 1509-3494) and IFG 1-35, 420-686 (nt 24-128, 1281-2083). PCR oligonucleotide site-directed mutagenesis was carried out to create IFG D427A using primers, forward 5'-GCTCAGCTAGCTGATTTCGGATTGGATATCAAAACGATGCGACTTTCTGCTAATAAG-3' and reverse 5'-AAGAGAGCGAGATGTTTTTG-3'. Construction of IFG D456A was generated with complementary oligos containing the directed nucleotide mismatch and used to generate two *ifg-1* fragments with overlapping ends. The double primer sets included forward 5'-ATGTCAAACGCTGTTAGTAGGG-3' and reverse 5'-TTCCAGCTGTGGTTGTTCTTC-3'

for the 5' prime amplicon and forward 5'-AAGAACAACCACAGCTGGAAC-3' and reverse 5'-TCACCAGTAACGGTCCACAA-3' for the 3' amplicon. The resulting fragments were then mixed and amplified further using nested primers, forward 5'-TCAGACACAACCACCACTAC-3' and reverse 5'-AAGAGAGCGAGATGTTTTTG-3'. The final PCR products for both IFG D427A and IFG D456A were digested and subcloned into pSK Long to create caspase resistant constructs. All constructs were verified by DNA sequencing (Iowa State).

*Expression and Purification of Recombinant CED-3*

Recombinant CED-3 (rCED-3) was expressed in *E. coli* DH5a cells and grown in a 5 L culture of 2X YT until OD595=1.0-1.5 as previously described (Taylor, 2007). Proteins were affinity purified using Ni-NTA sepharose (Invitrogen) and eluted in CED-3 reaction buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.5 mM sucrose, 5% glycerol) with 250 mM imidazol. Elutions were supplemented with DTT and glycerol to a final concentration of 2 mM and 20% respectively. Protein concentrations were determined by Bradford method using a bovine serum albumin (BSA) standard curve. Caspase activity was quantified using the colorimetric substrate Ac-DEVD-pNA (Promega). 10 ul of purified protein was incubated with the substrate (0.2 mM) at 37$^{o}$C and absorbance monitored at 405 nm over a 4 hour period. Activity was calculated using a pNA standard curve.

*Recombinant Caspase-3/CED-3 Cleavage Assays*

Recombinant enzymes were pre-incubated at 37$^{o}$C for 30 min – 1 h prior to all cleavage assays. Protein substrates were labeled with [35S]-methionine (Perkin Elmer Life Sciences) using TnT T3 coupled reticulocyte lysate system (Promega). Small reactions (10 ul) were prepared containing 0.5 ug of DNA and 0.8 ul of [35S]-methione (10 uCi/ul). *in vitro*

synthesized product was incubated with 10 ul of recombinant CED-3 at 37$^{o}$C for 2 h. Control reactions were mixed with non-catalytic BSA prepared in CED-3 reaction buffer. Reactions we stopped by adding equal volumes of 4X SDS buffer. Cell free reactions were performed using frozen N2 worm pellets ground in the presence of liquid nitrogen and 2X extract buffer (100 mM HEPES pH 7.4, 0.2% CHAPS, 100 mM NaCl, 2 mM PMSF, 50 ug/mL leupeptin, 10 mM DTT) supplemented with a Halt protease inhibitor cocktail (Thermo Scientific). Insoluble material was pelleted 30 min at 14,000 x g. CED-3 (10 ul) was immediately incubated with soluble worm extract for 2 h at 37$^{o}$C. Both *in vitro* and *ex vivo* reactions were also incubated with either the pan-caspase inhibitor z-VAD-fmk (Promega) or caspase-3-specific inhibitor, Ac-DEVD-CHO (Sigma).

*Purification of Cap-Binding Proteins from C. elegans.*

The mRNA cap-binding complex was purified from snap-frozen worms ground in liquid nitrogen by affinity chromatography on m$^{7}$GTP-Sepharose (Pharmacia) as previously described (Amiri, Keiper et al. 2001).

*Western Blot Analysis*

All protein samples analyzed by immunoblot were performed as follows. Samples were boiled for 10 min at 100$^{o}$C in 4x SDS sample buffer and protein lysates resolved by electrophoresis on either 12% (29:1), 8% (30:0.8), or 6% (30:08) polyacrylamide SDS-PAGE. Lysates from in vitro CED-3 cleavage assays were loaded on a Novex© 4-20% Tris-Glycine Midi Gel (Invitrogen). Proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes and incubated with a 1:2000 dilution of appropriate primary antibody in TST (10 mM Tris-HCl ph 7.4, 150 mM NaCl, 0.05% Tween 20) containing 5% dry non-fat milk.

Blots were washed extensively and incubated 1:2000 goat anti-rabbit IgG or 1:10,000 rabbit anti-mouse IgG secondary antibody conjugated to horseradish peroxidase. Detection was by ECL+ kit (GE Healthcare/Amersham) using a Typhoon 9410 Fluorescence/Phosphorimaging scanner at the ECU PhIFI Core Facility.

*RNA Isolation, RNA Analysis, and cRT-PCR*

Total RNA purification was performed using the Trizol method as previously described (Contreras, Richardson et al. 2008). Poly(A+) RNA was isolated using the Oligotex Direct mRNA Isolation kit according to manufacturer's instructions (Qiagen). cRT-PCR was conducted as described by Mullen and Marzluff, 2008. Approximately 70 ug of total N2 RNA was treated with 25 units of RQ1 DNase (Promega) in a 100 ul total volume and incubated for 30 min at 37$^{o}$C. RNA was extracted with a 1:1 volume of phenol:chloroform:isoamyl alcohol and precipitated with 70% ethanol. RNA was resuspended in 20 ul of RNase free H2O. Purified total RNA (10 ug) was treated with 2.5 units of tobacco acid pyrophosphatase (Epicentre) in a 20 ul reaction and incubated for 1 h at 37$^{o}$C. Following a second phenol:chloroform extraction and ethanol precipitation, nucleic acids were resuspended in 10 ul of H2O. Four micrograms of either TAP-treated (+) or –untreated (-) RNA was incubated with 20 units of T4 RNA ligase (Epicentre) and 20 units of RNasin (Promega) in 400 ul reaction for 16 h at 16$^{o}$C. A final extraction and precipitation was performed in the presence of 10 ug of glycogen and the circularized RNA resuspended in 12 ul $H_2O$. One microgram of ligated RNA was then amplified by RT-PCR using Superscript One Step RT-PCR with Platinum Taq (Invitrogen) using primary *ifg-1* primers, forward 5'-TCGAGCTATTTGACGGGCAA-3' and reverse 5'-TGTGGTTGGTTGTATTGTT-3' (p170) or reverse 5'-GCGCATCGTTGGAGGATACA-3'

(p130). Nested PCR was performed using 1 ul of cDNA with GoTaq DNA polymerase (Promega) and primers, forward 5'-CCTACCGATTTTATGTCTTATTGGG-3' and reverse 5'-CCCTACTAACAGCGTTTGACAT-3' (p170) or reverse 5'-ATCTCTTTGCTGCTGACCGCCA-3' (p130). The products were subcloned directly into pCRII TOPO TA vector (Invitrogen) and *ifg-1* positive clones sequenced. For Southern blotting, PCR products were resolved on 1.7% agarose gel and blotted on NitroPlus 2000 membrane (MSI Separations) and hybridized overnight at 58$^{o}$C. After stringently washing the membrane with 2X SSC at 60$^{o}$C, hybridization signals were analyzed with Typhoon 9410 imager and ImageQuant TL software. The antisense probes used were transcribed from the plasmids containing nt 24-2083 and nt 3092-3494 of *ifg-1* full length cDNA. As a control, plasmid pF170 containing full length *ifg-1* open reading frame was digested with EcoRV and EcoRI to generate multiple fragments for comparison against cRT-PCR products.

cRT-PCR of *ced-4* mRNAs from total RNA derived from temperature stressed wild type worms and poly(A+) RNA from $CuSO_4$-treated *ced-1:gfp* worms was performed as previously described. Primary ced-4 primers used were forward 5'-GAAGACTTCCCAAAGTTCATGCA-3' and reverse 5'-GCCTTCTAAATAAGTCAATGCGTCA-3'. Nested PCR was performed using primers, forward 5'-GCACCAGAAATTCTATGACTCCCT-3' and reverse 5'-GTGGATGAGCCTCGTGTGTG-3'. The PCR products were analyzed on a 6% polyacrylamide gel and stained with ethidium bromide. Amplified products are currently being subcloned and sequenced as previously described.

*Copper-Induced Apoptosis Assay*

Assays were performed as previously described (Wang 2009). Briefly, MD701 (*ced-1:gfp*) synchronized eggs were harvested and incubated at 20$^{o}$C until young adulthood. Worms were collected (250 ul packed worms) by washing off worms in M9 and then resuspended in a 1:1 volume of M9 following centrifugation at 5000-6000 x g. A 24 well cell culture plate was prepared with either 1 mL of K-Medium [52 mM NaCl and 32 mM KCl] or K-Medium supplemented with $CuSO_4$ to a final of 50 uM. An equal volume of packed worms were added to each well and incubated for 12 hours at 20$^{o}$C in the presence of OP50 bacteria. Worms were washed twice in M9 and resuspended in a 1:1 volume of sterile water supplemented with 2 mM Vanadyl Ribonucleoside Complex. Samples were dripped in liquid $N_2$ and pellets stored at -80$^{o}$C.

### *CED-4 Immunostaining*

CED-4 localization in RNAi-treated germlines was performed with the help of Melissa Henderson by immunostaining using the anti-CED-4 (cN-21) goat polyclonal antibody (Santa Cruz Biotechnology, Inc.) as previously described (Contreras, Richardson et al. 2008).

### *RNA Interference and Microscopy*

RNA interference by feeding was performed as previously described (Contreras, 2008). Briefly, knockdown of IFG-1 p170 was conducted using the plasmid containing 398bp (nt 24-479) of the *ifg-1* cDNA. Double stranded RNA was expressed in HT115 (DE3) and a single bacterial colony was incubated overnight at 37$^{o}$C in 2X YT media containing 100 ug/mL ampicillin. A ten-fold diluted aliquot was added to 2 mL cultures and grown for 8 h, then seeded onto NGM plates containing 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and ampicillin. Plates were subsequently incubated at 22oC overnight. Adult hermaphrodite F1s

were immobilized in 30 nM sodium azide plus M9 buffer and the presence of CED-1:GFP expressing germ cell corpses examined using a Zeiss Axiovert 200M Differential Contrast (DIC) microscope equipped with a Axiocam MRM2 CCD camera, FITC/GFP optics, and F-fluar 40X objectives.

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# APPENDIX A: STUDYING CELL FATE USING AN IFG-1 CONDITIONAL STRAIN

## *Introduction and Results*

IFG-1 p170 and p130 appear to provide distinct functional roles during translation and development. We have shown that disrupting the balance between these isoforms causes dramatic effects on germ cell development and fertility. Knockdown of IFG-1 p170 results in significant degeneration of the germline specifically cells undergoing differentiation (Contreras, Richardson et al. 2008). This defect was marked by fewer mature oocytes competent for fertilization leading to the low brood sizes. Furthermore, the loss of meiotically differentiated cells was attributed to increased apoptosis. Genetic experiments indicated that the main apoptotic machinery (*ced-3* and *ced-4*) is required for these events. Interestingly, the loss of these apoptotic genes partially restores oocyte competency for fertilization (more uterine embryos observed) indicating that the short IFG-1 p130 isoform likely performs non-apoptotic functions during germ cell development.

The depletion of IFG-1 by RNAi employed several targets spanning the length of the ifg-1 mRNA. There were clearly phenotypic differences between worms treated with p170 (RNAi) which caused germ cell defects and central (RNAi) which affected somatic growth. However the germline phenotypes often were too severe (rapid deterioration from increased apoptosis) to make any detailed observations on when IFG-1 is most critical for later germ cell or embryonic development. The use of conditional genetic knockouts affords more careful characterizations of stage-specific defects. For example, developmental decisions that require essential genes (where homozygous deletion of alleles results in lethality) can be studied using temperature-sensitive mutants that maintain viability of the organism at a permissive temperature (Hirsh and Vanderslice 1976). Temperature shift experiments using a higher restrictive temperature can

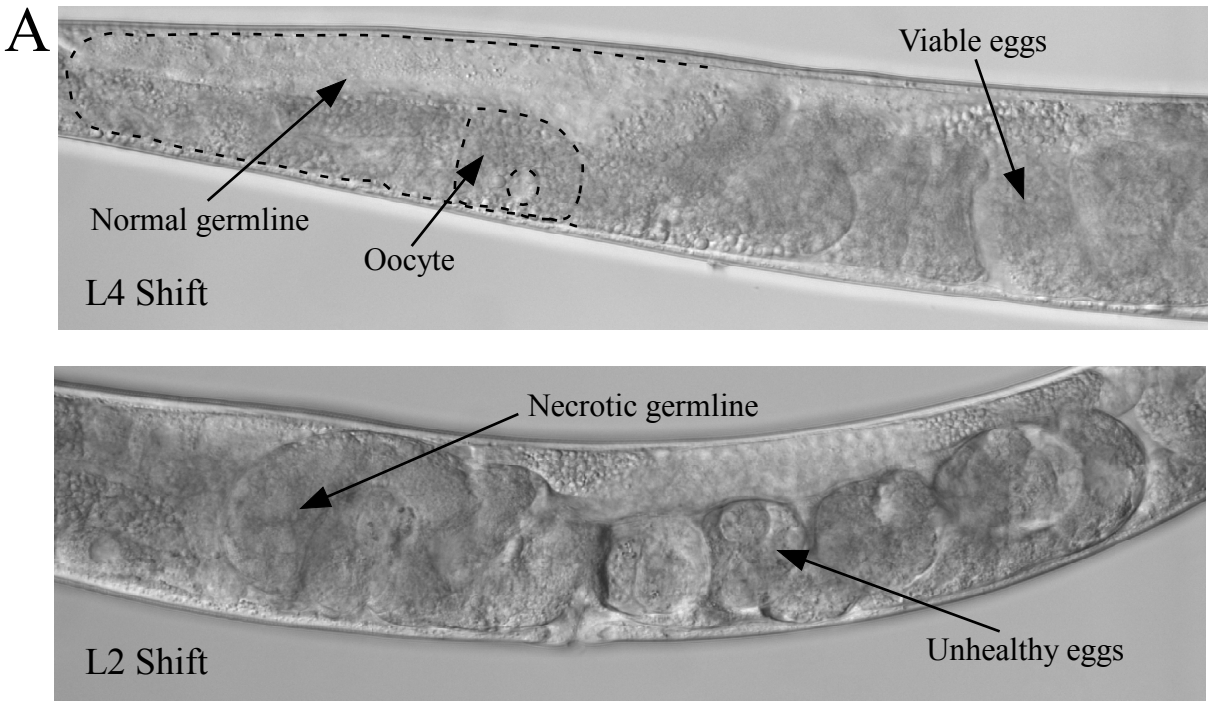
then be performed that control the temporal expression of the gene in order to observe what physiological processes are most critically affected. We recently received an *ifg-1* mutant strain that contains a transposon insertion in the gene that creates aberrant spliced *ifg-1*mRNAs that presumably encode non-functional proteins. The *Drosophila* Mos1 transposon was originally inserted in intron 5 of *ifg-1* by the French lab of Laurent Segalat and homozygosed by Melissa Henderson to generate the KX34 (*ifg-1:mos homozygous*) strain (Henderson 2009). Northern blotting and RT-PCR have shown that elevated temperatures caused aberrant splicing as a result of an alternative splice acceptor site within the transposon sequence, and confirmed the presence of wild type as well as improperly spliced transcripts. While initial immunoblotting showed a dramatic reduction in isoforms (Figure A.1B), subsequent expression analysis, using the central domain antibody, routinely revealed only a modest decrease in IFG-1 p170 and no effect on IFG-1 p130. These inconsistencies indicated that there were "leaky" effects of the Mos1 insertion on expression. It was ultimately determined that decreased IFG-1 expression leads to temperature-sensitive sterility (Henderson 2009). M. Henderson showed that when L4 larvae were shifted to the restrictive temperature (25$^{o}$C), they developed normal germlines, but their offspring suffered severe degeneration of the germline occurred resulting in sterile F1 progeny. This "maternal effect sterility" (grandchildless) phenotype and temperature sensitivity has been described for other essential genes i.e., germline sex-determination factors (Capowski, Martin et al. 1991). The deterioration of the germline observed at the restrictive temperature also correlated with decreased IFG-1. However it remained unknown what stage of germline development is sensitive to disruption of IFG-1 expression.

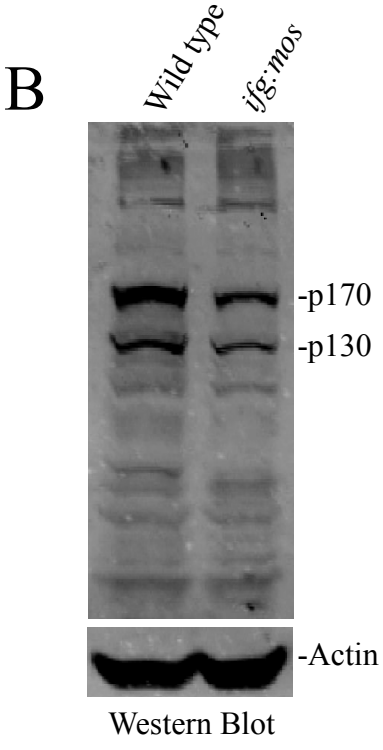
Considering the massive degeneration observed by M. Henderson upon shifting late larval/adult hermaphrodites to 25$^{o}$C, it is possible these defects represented the sum of

compounding disruptions to germline development from depletion of both isoforms. In order to address what developmental stage is most critically required for both p170 and p130, temperature shift experiments were performed. Worms containing both the *ifg-1*mutation and the *ced-1:gfp* apoptotic reporter gene were used to determine whether germline deterioration in adults is caused by disruption of IFG-1 during either the clonal expansion of germ cells (L2 larval stage) or progression of differentiating oocytes (L4 larval stage). When L4-staged worms were shifted to 25$^{o}$C and incubated until 24 hours post-adult stage, the germline appeared relatively normal (Figure A.1A, L4). There were no dramatic effects on mitotically dividing (distal) cells, differentiating (proximal) cells, or mature oocytes. In order to determine whether there was an increase in germ cell apoptosis, we scanned germlines for the presence of GFP-labeled corpses. No increase in apoptotic corpses were observed in these worms. However, when L2-staged worms were shifted to the restrictive temperature and allowed to develop until adulthood, there was significant degeneration of the germline (Figure A.1A, L2). There appeared to be fewer numbers of mature oocytes which coincided with the observed germ cell deterioration. Curiously, there were also no detectable corpses in the germline. The lack of observed CED-1:GFP decorated corpses and the presence of large cellular debris in the germline may signify a more progressive stage of cell death that is indicative of necrosis. These results suggest that there is a stage-dependent response to disruption of both IFG-1 p170 and p130. Since there were no detectable effects on somatic development under both shift conditions, we can conclude that modest loss of IFG-1 is likely not critical to terminally differentiated cell types but is important during dynamic states of proliferative growth and differentiation.

**FIGURE A.1 Larval L2 stage of development is sensitive to depletion of IFG-1.** (A) DIC images of KX38, *ifg:mos:ced-1:gfp*, adults. Worms were shifted from the permissive (20$^{o}$C) to the restrictive (25$^{o}$C) as either L2 or L4 larvae. Worms shifted as L4 contain a normal distal and proximal germline (dashed outline). Both mature oocytes and viable embryos were detected. L2 shifted worms, however, show massive degeneration of the germline with severe necrosis and unhealthy embryos. (B) Western blot shows decreased IFG-1 p170 and p130 in KX38 adults grown at the restrictive temperature. Detection of IFG-1 isoforms, using the central domain antibody, shows moderate loss of each isoform.

# Figure A.1


A
Viable eggs
Normal germline
Oocyte
L4 Shift
Necrotic germline
Unhealthy eggs
L2 Shift


B
Wild type
ifg-mos
-p170
-p130
-Actin
Western Blot

The evidence presented here suggests that the L2 larval stage is sensitive to aberrant splice-mediated disruption of *ifg-1* whereas, the L4 stage is not. Perhaps the expression of IFG-1 is also affected more dramatically at an earlier stage of development. In order to detect changes in IFG-1

expression, western blotting was conducted using synchronized wild type (*ced-1:gfp*) and mutant (*ifg-1:mos::ced-1:gfp*) adults that had been shifted as eggs from the permissive temperature to 25$^{o}$C. There appeared to be a significant decrease in both IFG-1 p170 and p130 suggesting that shifting KX38 (*ifg-1:mos::ced-1:gfp*) mutants earlier does have an effect on the amount of IFG-1 available during adulthood(Figure A.1B). However further quantitation of each isoforms expression relative to actin as well temperature shifts at each developmental stage (L1-L4) will have to be performed before making any conclusions regarding IFG-1 expression patterns in *ifg-1:mos* mutants.

*Conclusion*

The data presented here show that early developmental stages are most sensitive to aberrant splice-mediated depletion of both IFG-1 p170 and p130. There was also an observed decrease in both isoforms when a single generation of worms were incubated at the restrictive temperature from eggs to adulthood. M. Henderson showed a significant decrease in levels of p170 and no loss of p130 by western blot using adults shifted at 25$^{o}$C further indicating that the stage shifted may be important when addressing IFG-1 p130 effects on development. While the cap-dependent/-independent isoform switch in germ cell development cannot be directly assayed, the *ifg-1:mos* strain can be used to identify what stages during germ cell proliferation and differentiation that are most dependent on IFG-1. For example, shifting worms from the

permissive to restrictive temperature and back down again may show that the stem cell niche of proliferating cells or the translationally controlled gradient used to coordinate the transition from mitosis to meiosis are affected during transient disruptions to IFG-1 expression. Conditional depletion of IFG-1, therefore, should continue to be a beneficial tool in analyzing the state of mRNA recruitment and protein expression during germ cell development.

# APPENDIX B: CREATING IFG-1 ISOFORM-SPECIFIC STRAINS

## *Introduction*

The regulation of gene expression specifically during oogenesis in *C. elegans* is shown to be linked to multiple isoforms of a translation initiation factor (Keiper, Lamphear et al. 2000; Amiri, Keiper et al. 2001; Henderson, Cronland et al. 2009; Song, Labella et al. 2010) . However, the physiological properties of the two IFG-1 isoforms still remain unclear. RNAi of p170 or both isoforms suggests distinct roles during development of the oocyte. Even with very efficient depletion of *ifg-1* mRNA by RNAi, the complete and immediate loss of IFG-1 protein from treated worms is unlikely. To observe worms lacking all IFG-1 forms, we needed a chromosomal deletion of the *ifg-1* gene. The *ifg-1*(ok1211) strain, heterozygous for a deletion in exons 4 through 7, was obtained from the *C. elegans* Knockout Consortium. This deletion removes almost 2 kb of the *ifg-1* gene, including over half of the coding capacity of both p170 and p130 mRNA forms (Contreras, Richardson et al. 2008). This deletion is presumed to be null. When the offspring from heterozygous worms (-/+) were grown and observed for 96 hours, their morphological development was parallel to wild type. However, homozygous (-/-) worms lacking a functional *ifg-1* gene arrested as L1/L2 larvae. These homozygotes remained as arrested yet live swimming larval staged worms in excess of 96h. Interestingly, this phenotype was similar to the population of C2 (RNAi)-treated worms that experienced identical larval stage arrest. These data seem to suggest a requirement for IFG-1 during early larval stages prior to germline proliferation. The information presented here describes experiments aimed at determining the contribution of each IFG-1 isoform to gametogenesis and embryogenesis.

## *Construction of ifg-1 Rescue Plasmids*

Construction of the IFG-1 p170-only rescue plasmid was performed using Splicing by Overlapping Extension PCR (SOE-PCR). The *ifg-1* endogenous promoter (1220 bp) was amplified from pCR 2.1 *ifg* gene using primers, forward 5'-ATGGCCCAGGCATTGATGATG-3' and reverse 5'-AATCTTTCAGTTTGAGGATCCACTGAAAAGAGTTTTAATA-3'. A second amplicon containing *ifg-1* coding sequences was amplified from pSK *ifg-1* Long using primers, forward 5'-GTGGATCCTCAAACTGAAAGATTATGTCAAACGCTGTTAGTAGGG-3' and reverse 5'-GAGCAGTAAGGTTTGGCAGC-3'. Amplification from both PCR products was next performed using primers, forward 5'-ATGGCCCAGGCATTGATGATG-3' and reverse 5'-CGCATCGTTTTGATATCCAATCCG-3'. The resulting product was digested with BglII and NheI and ligated into pSK *ifg-1* Long cut with BamHI and NheI to create pSK *ifg-1* minigene3. In order to construct the 3' end containing FLAG (DYKDDDDK) encoding sequences, SOE-PCR was similarly used. The *ifg:flag* amplicon was created by amplifying pSK *ifg-1* Long using primers, forward 5'-ACCAAACTGGGCAAACAAAG-3' and reverse 5'-TCACTCGAGTTTATCATCATCATCTTTATAATCGTTGCCCGTCAAATAGCTCG-3'. The second PCR product containing flag and let858 3'UTR was amplified from pPD117 using primers, forward 5'-GATTATAAAGATGATGATGATAAACTCGAGTGATCGACGCCAACGTCGTTG-3' and reverse 5'-GCGTCGACAAGCGAGGACAATTCTCATC-3'. Primary PCR products were combined and a second product created using primers, forward 5'-TCGAGACTGTAGGCGTGATG-3' and reverse 5'-GCGTCGACAAGCGAGGACAATTCTCATC-3'. This secondary PCR product was digested with restriction enzymes XbaI and SalI and inserted into pSK *ifg-1* minigene3ΔX, which lacks a

5’ XbaI site, to generate pF170. pF130 ( which does not contain the correct N-terminal start site, but generates a similar p130 protein), was constructed by cutting pF170 with BamHI and NheI followed by a blunt-end ligation. To generate pFGENE (a complete gene version containing a C-terminal Flag tag), the plasmid pPD95 containing the full length *ifg-1* gene was digested with BamHI and XbaI and inserted into pF170 cut with the same enzymes.

In order to more effectively express the *ifg-1-flag* construct in the germline, ifg-1 promoter sequences were replaced by those of pie-1. A germline-specific *ifg-1* expressing construct was created by digesting pF170 with BamHI and XhoI and ligating the fragment into pSS4-GFP: *ife-1* cut with the same enzymes. This removes the *ife-1* cDNA leaving only the *pie-1* promoter and 3’UTR regulatory sequences. The ligation of this intermediate plasmid (pPie170b) resulted in a loss of *ifg-1* termination sequences as well as approximately 400 bp of the pie-1 3’UTR. Reconstitution of these sequences was performed by amplifying the desired sequence using a reverse primer with a different but compatible restriction site for unidirectional insertion of the PCR product into pPie170b. The primers used were forward 5’-CTCGAGTAGACTAGTGGGCTAATTTTGCCGTATTTTCCATATTTTGTTTTCTA-3’ and reverse 5’-GTCGACGGCTACATC-3’. The PCR product was digested with XhoI and SalI, and then ligated into pPie170b cut with XhoI only to create pPie170 final. Finally a pPie130 plasmid containing the correct start site is currently being cloned by amplifying pSK-*ifg-1* Long with primers, forward 5’-GGGATCCATGGTTACTATTCGCGAAATTGAA-3’ and reverse 5’-GCGCATCGTTGGAGGATACA-3’. PCR products have been digested with BamHI and NheI to generate a 67 bp fragment that will be inserted into pPie170 cut with the same enzymes.

*Results*

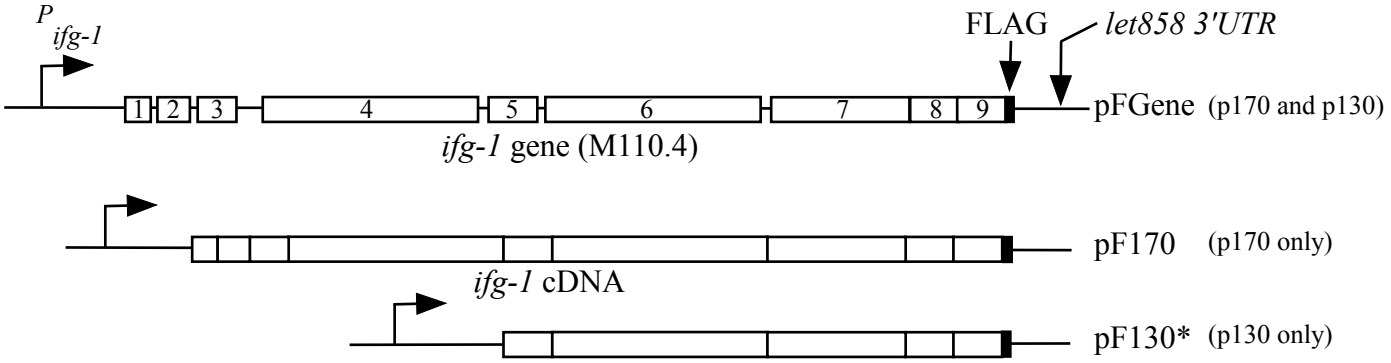
In order to further determine how p170 and p130 influence worm development, cDNA based minigene constructs were prepared to drive the specific expression of each IFG-1 isoform in the *ifg-1* (ok1211) null mutant. The chimeric constructs generated using splicing by overlapping extension PCR (SOE-PCR) and standard subcloning techniques contain 1.2 kb of the *ifg-1* promoter sequence linked to the *ifg-1* cDNA and a C-terminal Flag expressing epitope tag (Figure B.1A). The advantage of these transgenes is that they preclude alternative splicing of *ifg-1* thereby producing only the isoform of interest. Rescue DNA was directly injected into heterozygous *ifg-1* (-/+) hermaphrodite gonads. The distal gonad is marked by proliferating mitotic and differentiating early meiotic immature oocytes surrounded by a semi-permeable membrane that are dispersed in a common cytoplasm or syncytium (Hall, Winfrey et al. 1999). Therefore, injections in this region allow for maximum distribution of the minigene DNA prior to complete enclosure and growth of the oocyte in the proximal gonad. DNA was injected directly as two different arrays, simple or complex, into worm gonads. Simple arrays exist as extrachromosomal copies of repetitive DNA containing the gene of interest and a selectable marker (Evans and Hunter 2005). These arrays have the advantage of generating many transgenic progeny and transmitting the genetic material through many generations without variability in expression (at least somatically). *ifg-1* constructs were injected initially at 100-200 ng/ul with either of two markers *rol-6* (rolling phenotype) or *myo-3:gfp* (body wall fluorescence). It must be noted that the injections of *ifg-1 p130* constructs did not contain the correct 5' start site and likely would not have created a true p130 rescue strain. A strategy has since been devised to generate a p130 rescue construct with the correct start site.

No transgenic lines were established for p170 or p130 and any F1 transformants (identified by either selectable marker) that were isolated did not transmit the array to F2

**FIGURE B.1 *ifg-1* constructs developed for rescue of deletion mutant phenotype.** (A) Diagram of the three primary *ifg-1* rescue plasmids designed. The full length gene or cDNA was fused to FLAG-encoding sequences under the *ifg-1* endogenous promoter. Let858 3'UTR was add to enhance expression. The plasmid (pF130*) does not contain the correct N-terminal start site. (B) Schematic of *pie-1* expressing *ifg-1* constructs. pPie170 contains *ifg-1* full length cDNA under *pie-1* promoter and 3'UTR to generate germline rescue of *ifg-1* null mutants. The pPie130 construct (dashed) which is currently being subcloned contains the correct p130 start site.

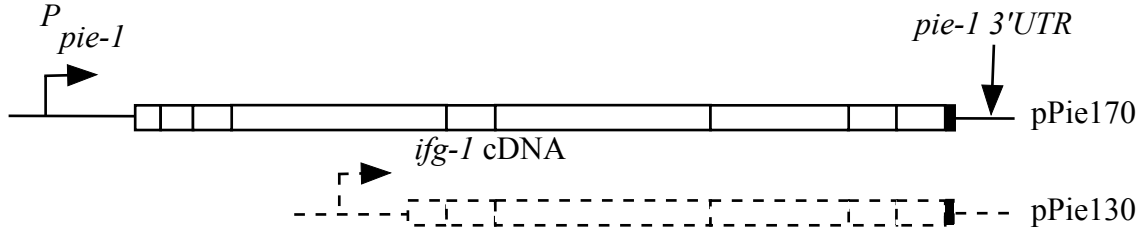
# Figure B.1

A




***ifg-1* rescue constructs under endogenous promoter**

B




***ifg-1* rescue constructs under *pie-1* promoter**
**(germline-specific expression)**

progeny. In a few cases, there were F1s that did not produce any F2s and were thus sterile. This fertility defect may be the result of multiple copies of the array that are toxic at high doses. A lower concentration (10-50 ng/ul) was used to improve fertility but no transformants were isolated. The lack of heritability among transformants containing simple arrays is partly due to strong silencing of repetitive arrays in the germline (Kelly, Xu et al. 1997). Transgenes experience mitotic instability in germ cell nuclei through an as yet undescribed mechanism. In order to increase the likelihood of germline transmission, we next injected DNAs as complex arrays. These arrays incorporate fragmented *C. elegans* genomic DNA along with the transgene and selectable marker. The fragments limit the formation of tandem repeats that cause chromatin silencing in germ cells (Kelly and Fire 1998). *ifg-1* transgenes were injected at no more than 1ng/ul into either *ifg-1* heterozygous mutants or wild type worms. Injecting into wild type worms was performed so that integrated transformants could later be crossed with *ifg-1* mutants to create a stable isoform specific strain. Additionally, it was surmised that the genetic background of *ifg-1* mutants, which contain not only a pharyngeal *gfp* balancer but a second dumpy marker, was perhaps disrupting transformation. The dumpy (short and compact worms) marker is extremely useful and allows for easy identification of homozygous *ifg-1* (+/+). Given the relatively complex genetics of these mutants, it is possible that transgenic expression was suppressed. Worms carrying some dumpy mutant backgrounds are known to suppress the expression of *rol-6* (roller) (Evans and Hunter 2005). Injections of complex arrays again produced no transgenic lines and few F1 transformants (all somatic expressing). These results indicate that high doses of *ifg-1* extrachromosomal arrays may be toxic and germline silencing likely contributed to the lack of *ifg-1* expression under its endogenous regulatory elements.

Germline transformation in *C. elegans* is usually peformed using transgenes under the control of *pie-1* regulatory regions. PIE-1 is a germline transcriptional repressor that is highly expressed in all germ cells from early larval to adult stages (Seydoux and Strome 1999; Tenenhaus, Subramaniam et al. 2001; Merritt, Rasoloson et al. 2008). In addition, transgenic expression in the germline is enhanced as this gene is less susceptible to silencing. In order to minimize germline silencing of IFG-1:FLAG expression, the p170 coding region was inserted into a cassette containing the *pie-1* promoter and 3'UTR which significantly affects the pattern of expression in the germline (Figure B.1B) (Merritt, Rasoloson et al. 2008). Injection of *pie-1* driven *ifg-1 p170:flag* in wild type and *ifg-1* mutants is currently underway but has not yet proven successful in generating heritable transformants. It is however worth mentioning that M. Henderson generated several *pie-1* driven eIF4E (IFE-1) transgenic lines that were demonstrated to express Flag-tagged protein and have now been integrated. The non-integrated strains appear to only rescue the oocyte-specific defects indicating that there is partial restoration of germline function (M. Henderson and B.D. Keiper, personal communication). These results provide encouraging evidence for the potential for at least partial rescue of germ cell arrest in null *ifg-1* mutants using a $P_{pie\text{-}1}$*:ifg-1:flag* constructs.

In addition to DNA-based transformation, we also employed an RNA approach to isoform-specific rescue of *ifg-1* deletion mutants. *ifg-1* constructs were used as templates *for in vitro* synthesized transcripts containing an Anti-Reverse Cap Analog (ARCA). ARCA caps unlike endogenous $m^7$GpppG caps, can only be incorporated in the forward orientation thereby allowing for 100% cap-dependent translation from mRNAs (Grudzien-Nogalska, Stepinski et al. 2007). Individual ARCA-modified p170 and p130 mRNAs (incorrect start) were injected either together (1:1) or individually at a concentration of 500 ng/ul into the germline of *ifg-1* (-/+)

heterozygous mothers. Homozygous null (-/-) larvae were isolated and morphology monitored over 96 hours. To validate germline expression from injected RNAs, BFP expressing RNAs were also injected and fluorescence observed over the same amount of time. The appearance of weak fluorescence in some oocytes indicated that the germline was directing BFP expression from injected RNAs. However, there were no changes in the terminal phenotype of *ifg-1* (-/-) larvae. The germline and size of the worms were also unchanged. Therefore, no rescue by *ifg-1* mRNA injection was observed. It is possible that these RNA-based injections were not sufficient to support the synthetic consumption required for late larval development. This may be due to a loss in stability of the injected RNAs over the length of embryonic and larval development. While no change in phenotype was detected, this technique may yet prove useful. In chapter 2, we discovered that *ifg-1* may contain a cryptic promoter that is responsible for creating the short *ifg-1 p130* mRNA. If this is correct, our longer *ifg-1 p170* construct may also be able to generate IFG-1 p130. Injections of individual mRNAs would preclude the possibility of generating *ifg-1* p130 mRNA from a p170 template.

### *Conclusion*

The heritability of transgenic information from one generation to the next has been marked with many issues rooted in germline transmission. Many problems arise during silencing in germ cells and early embryonic blastomeres, improper expression patterning as a result of 3'UTR dysregulation, and the high copy number of transgenes leading to either mosaic expression or toxicity (Kelly, Xu et al. 1997; Merritt, Rasoloson et al. 2008). One or all of these obstacles may be the reason for the inability to produce a stable *ifg-1* line. Recently new techniques have been used to transfer low copy, easily integrated transgenes into worms. The predominantly used alternative to injection, gene bombardment, utilizes the forced projection of

DNA bound gold particles into the gonad. As a result, a higher number of integrated transformants are generated because DNA is able to enter the nucleus of germ cells directly (Praitis, Casey et al. 2001). In addition bombardment has been shown to increase germline expression as a result of decreased silencing. It may therefore be more beneficial to create *ifg-1* lines through ballistic transformation. Bombardment however still has the potential to project multiple copies of the transgene leading to sterility because of increased toxicity. Since we have preliminary evidence that high doses of *ifg-1* DNA cause increased embryonic arrest, a single copy-based approach may be more appropriate for the expression of IFG-1. Mos1-mediated Single Copy Insertion (MosSCI) employs the Mos1 transposase under a germline specific promoter which is co-injected with the transgene. Excision causes double-stranded breaks at the targeted locus as well as the transgene allowing for homologous recombination and insertion of the array into the selected chromosome (Frokjaer-Jensen, Davis et al. 2008). Thus a stable single copy *ifg-1* line may provide a more accurate characterization of individual isoform spatial and temporal expression patterns through either partial *pie-1* driven germline rescue or full rescue under the endogenous promoter.

# APPENDIX C: COMPARISON OF eIF4G ISOFORMS IN *C. ELEGANS* and *C. BRIGGSAE*

## *Introduction*

The results and ideas discussed in this dissertation address a biphasic translation mechanism that requires cap-dependent and –independent isoforms of eIF4G during stress and apoptosis. These isoform-specific mechanisms now appear to be conserved between human and *C. elegans*. However, nothing is known about why structurally distinct eIF4G isoforms are similarly required for different modes of translation in such evolutionary divergent species. Comparative studies of eIF4G diversity using human, *C. elegans*, and other species should therefore be considered in order to better understand the functional significance of isoforms in multimodal forms of translational control over evolution.

## *Results*

We have begun addressing the functional significance of other isoforms by searching for eIF4G orthologs in other closely related species. The relative simplicity of gene organization shared between *C. elegans* and *C. briggsae* has recently been utilized to perform comparative gene function studies (Coghlan, Stajich et al. 2006). *Caenorhabditis briggsae*, like its close cousin, is a soil nematode that has been used in the study of othologous genes because of its nearly identical morphology, behavior, and genome size (Stein, Bao et al. 2003). We searched for a *C. briggsae* ortholog of the single *C. elegans ifg-1* gene using a nematode database, Wormbase, which curates genomic information as well as syntenic alignments between species. Two distinct genes were discovered for *C. briggsae*. Expressed sequence tags (ESTs), used to identify mRNAs, were also identified suggesting that multiple transcripts were derived from these two genes (Figure C.1A). These gene products, like *ifg-1* mRNAs, differed in their 5' sequences. The largest *C. briggsae* mRNA, termed *cbifg-1*, contains eight exons (3489 coding

bp) encoding 1163 amino acids with a predicted protein size of 130 kDa. This is consistent with *C. elegans ifg-1* p170 which encodes 1156 amino acids and a predicted size of 129 kDa (Contreras, Richardson et al. 2008). An alignment of *ifg-1* p170 and *cbifg-1* mRNAs showed highly conserved sequences shared between both species. These conserved sequences encode the eIF3/eIF4A (MIF4G) domain suggesting that cbIFG-1 participates in ribosome recruitment and translation. The second *C. briggsae* gene, *cbifg-2*, was discovered that generates a transcript containing only five exons. Interestingly, the last two exons are the only sequences shared with *C. elegans ifg-1* p170 and p130 as well as *C. briggsae ifg-1*. In addition, these sequences also encode the MIF4G domain suggesting that it associates with ribosomes. The 5' end of *cbifg-2* contains divergent sequence that is unrelated to eIF4G from any species (Figure C.1A). These results indicate that at least two protein isoforms may exist that differ in their N-termini.

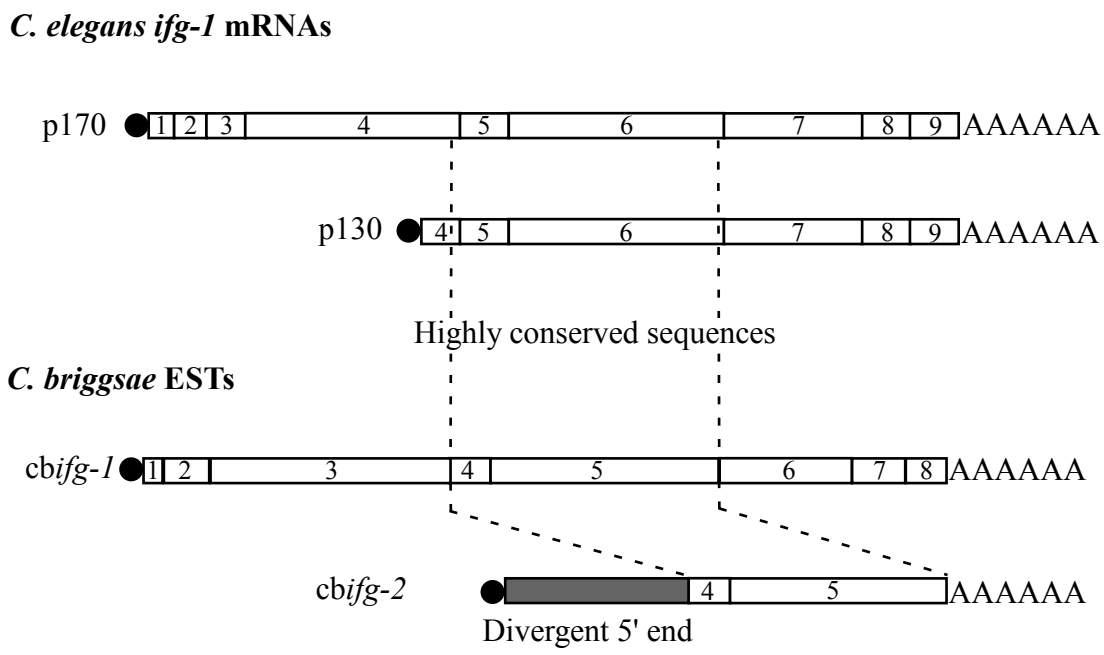
In order to determine whether multiple protein isoforms exist in *C. briggsae*, western blot analysis was performed from total worm lysates of *C. elegans* and *C. briggsae*. The sequences encoding the MIF4G domain was originally used to generate the epitope recognized by the central domain antibody (Contreras 2005). Therefore the central domain antibody which detects both IFG-1 p170 and p130 in *C. elegans* was also used to detect IFG-1 related proteins in *C. briggsae*. Two high molecular proteins were detected at 180 and 170 kDa which closely co-migrated with ceIFG-1 p170 (Figure C.1B). In addition, a third major protein was discovered that migrated at 130 kDa almost identically with ceIFG-1 p130. These observations indicate that long and short eIF4G isoforms are also produced in *C. briggsae*. Additional protein analysis using the ceIFG-1 N-terminal antibody will have to be conducted in order to determine if these isoforms differ in the N-terminus.

## *Conclusion*

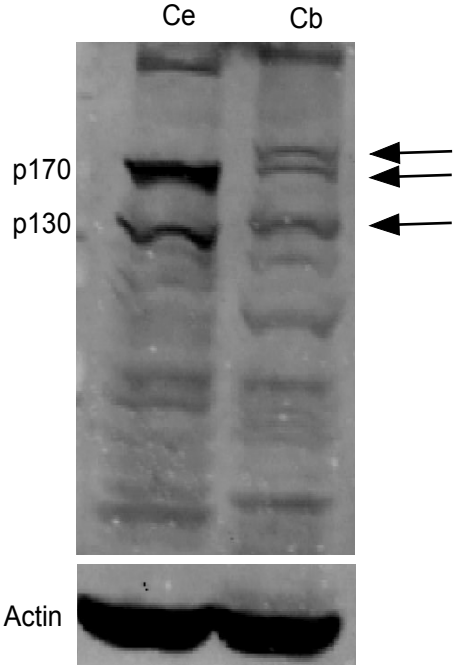
**FIGURE C.1 Comparison of *C. elegans* and *C. briggsae* IFG isoforms.** (A) Diagram comparing IFG mRNAs in two *Caenorhabditis* species. *ifg-1* p170 and p130 mRNAs are derived (see Chapter 2) from a single gene. Analysis of *C. briggsae* ESTs show that multiple RNAs exist derived from separate genes. *ifg-1* and *ifg-2* differ significantly in their 5' sequences but share high homology in exon 4 and 5. These exons are highly conserved between species and are very similar to exon 5 and 6 in *C. elegans*. Coincidentally these sequences encode the core ribosome recruitment domain shared among all eIF4G proteins. (B) Western blot shows multiple protein isoforms of *C. briggsae* eIF4G. Compared to *C. elegans* IFG-1 p170 and p130, there appear to also be p170 and p130 analogous proteins along with a p180 that migrates higher. All three are marked by arrows.

# Figure C.1

A




B




Western Blot
(ceIFG-1 central antibody)

Approximately 100 million years of evolution separate *C. elegans* and *C. briggsae (*Stein, Bao et al. 2003). Yet both species share an identical distribution of long and short eIF4G protein isoforms. Unlike *C. elegans* IFG-1, *C. briggsae* contains at least three major protein isoforms encoded by two different genes, *cbifg-1* and *cbifg-2*. In addition the fact that the only eIF4G sequences retained in *cbifg-2* encode the MIF4G domain suggest a highly conserved selection factor required for the minimal region necessary to recruit ribosomal complexes. It is likely that the separation of gene products in *C briggsae* occurred as a function of physical isolation or selective pressures from the environment (temperature, nutrient availability, etc). The synthesis of IFG-1 isoforms is tethered to a single gene in *C. elegans* which could make this species "unfit" under certain conditions where now both gene products are susceptible to loss of function. In order to preserve the subfunctions of eIF4G isoforms during speciation, duplication may have been a necessary solution that prevented genetic drift (disappearance of gene variants) and the loss of compensatory activities (protein synthesis modalities). Further interpretation of the evolutionary significance of eIF4G isoforms and the effects on translation mechanism are discussed in Chapter 5. The results described here indicate that *C. briggsae* and perhaps other *Caenorhabditis* species may be used to further address orthologous (interspecies) and paralogous (intraspecies) translation mechanisms and developmental pathways that require long and short eIF4G proteins.