# THE ROLE OF mRNA TRANSLATION MECHANISMS IN GERM CELL AND EMBRYONIC DEVELOPMENT IN C. ELEGANS.

by

Hannah B. Umphlett

A Signature Honors Project Presented to the

Honors College

East Carolina University

In Partial Fulfillment of the

Requirements for

Graduation with Honors

by

Hannah B. Umphlett

Greenville, NC

April 2023

Approved by: Brett D. Keiper, PhD

Brody School of Medicine at East Carolina University

Department of Biochemistry and Molecular Biology

# The role of mRNA translation mechanisms in germ cell and embryonic development in *C. elegans*

Hannah B. Umphlett

Department of Biochemistry and Molecular Biology, Brody School of Medicine (ECU)

**Abstract**

Reproductive cells and embryos use mRNA regulation as a primary means of gene expression. mRNA translational mechanisms rely on the eIF4 translation factors. These include eIF4E, which binds mRNA 7-methylguanosine caps, and eIF4G, which binds eIF4E and guides the mRNA to the ribosome for translation. Germ cells and embryos have the unusual capacity to use both cap-dependent and cap-independent translation mechanisms. Both use eIF4G (called "IFG-1" in *C. elegans*), but only cap-dependent uses eIF4G. Previous research in the model organism *C. elegans* has indicated that these mechanisms function at different times and for different mRNAs during germ cell development into sperm and oocytes. Germ cell development is also highly temperature dependent. Each of these aspects in turn impact the fertility of the nematode.

Genetic engineering by means of the CRISPR-Cas9 system allowed us to introduce N-terminal tags into the *ifg-1* gene. We noticed early that some of these tags had deleterious effects on fertility, specifically on embryo hatching, while others were fully tolerated. This study addresses the viability of IFG-1 tagged with short peptides including V5, 3xMyc, and a short internal deletion of V5, along with the longer fluorophore mCherry. Embryonic lethality was compensated by balancer chromosomes in strains where tags were not tolerated. Indeed, addition of 3xMyc and the full mCherry protein (3xMyc::mCherry) fused to IFG-1 was viable. However, replacement of the 3xMyc tag with the short peptide V5 caused full embryonic lethality at all

temperatures tested (15C, 20C, 25C). To determine if the V5 *per se* was toxic, we used CRISPR-Cas9 to make an internal deletion in V5. Remarkably, deletion of just 3 amino acids within V5 (dV5) restored nearly wild type levels of fertility (egg laying) at all temperatures. Remarkably, the viability of those embryos was observed only at 25C. To exclude the possibility of a recombination event in the *dV5::ifg-1* gene that restored a functionally wild type *ifg-1* gene, we conducted genomic PCR in the strains, even those mothers used in the egg laying/hatching experiment at 25C. Correct predicted genotypes were observed for all strains at the *ifg-1* locus by PCR.

Our results indicate that the N-terminus of the IFG-1 is very sensitive to structural or sequence changes that affect the protein synthesis required for embryo hatching. This portion of the protein is upstream of the portion that binds to eIF4E—the cap binding protein. Cap-dependent mRNA translation is known to be critical for cells in growing or differentiating stages. We are attempting to evaluate the importance of the balance between cap-dependent and -independent mechanisms in the developmental processes associated with embryo hatching.

**Acknowledgements**

It is difficult to describe how truly grateful I am for the people who made this research project possible. This includes but is not limited to the members of the Keiper lab (Dr. Brett Keiper, Eun Suk Kim, Gita Gajjar, Molly Lasure), Dr. Myon Hee Lee (BSOM Internal Medicine Department), and Dr. Dustin Updike. I want to thank all for their extensive technological assistance, knowledge, and support during this project. More specifically, I would like to express my gratitude to Dr. Keiper for his exemplary mentorship, his compassion, and his patience over the last year and a half.

This work was supported by the National Science Foundation grants MCB-2119959 and MCB-1714264 to Dr. Keiper.

## Table of Contents

## List of Figures

## Introduction

*Caenorhabditis elegans* is an excellent model organism to model fertility due to the hermaphroditic nature of the worm, the fast-paced temperature-dependent life cycle, and the ease of genetic engineering by the CRISPR-cas9 system. As the worms are self-fertile, self-fertilized eggs will accumulate in the gonad until they are laid by the mother worm (Schafer 2005). The timing of when the eggs are laid and whether they are laid at all depends on both temperature and homeostatic factors. The typical temperature range of study is 15C to 25C, with 20C being the standard incubation temperature. Lower temperatures will extend the lifespan, while higher temperatures accelerate growth. At 20C, the average length of time to adulthood is 3.5 days (Corsi, et al, 2015) (Fig 1). Moreso, the worms are small and thousands are capable of growing on an agar plate seeded with an E. coli food source. Larval stage 1 worms are typically 0.25 millimeters and fully grown adults average to 1.0 millimeters. If the plate is unseeded or without the E. coli food source, nematode development is arrested. The worms are capable of surviving starvation for at least one month at higher temperatures and up to six months at lower incubation temperatures (Corsi, et al, 2015).

As discussed previously, *C. elegans* is hermaphroditic (XX) and capable of self fertilization, but a male sex is also present (XO). The worms are diploidy with 12 total chromosomes with 5 autosomes and 1 sex chromosome. A large volume of eggs are produced in healthy wildtype hermaphroditic mothers--300, on average. The eggs are incredibly resilient and tough, allowing for embryonic development outside of the mother's body. Typically the eggs will reside within the mother until they reach 24 cells, at which point, they are laid (Corsi, et al, 2015). The eggs will then develop and mature through four larval stages before reaching adulthood.

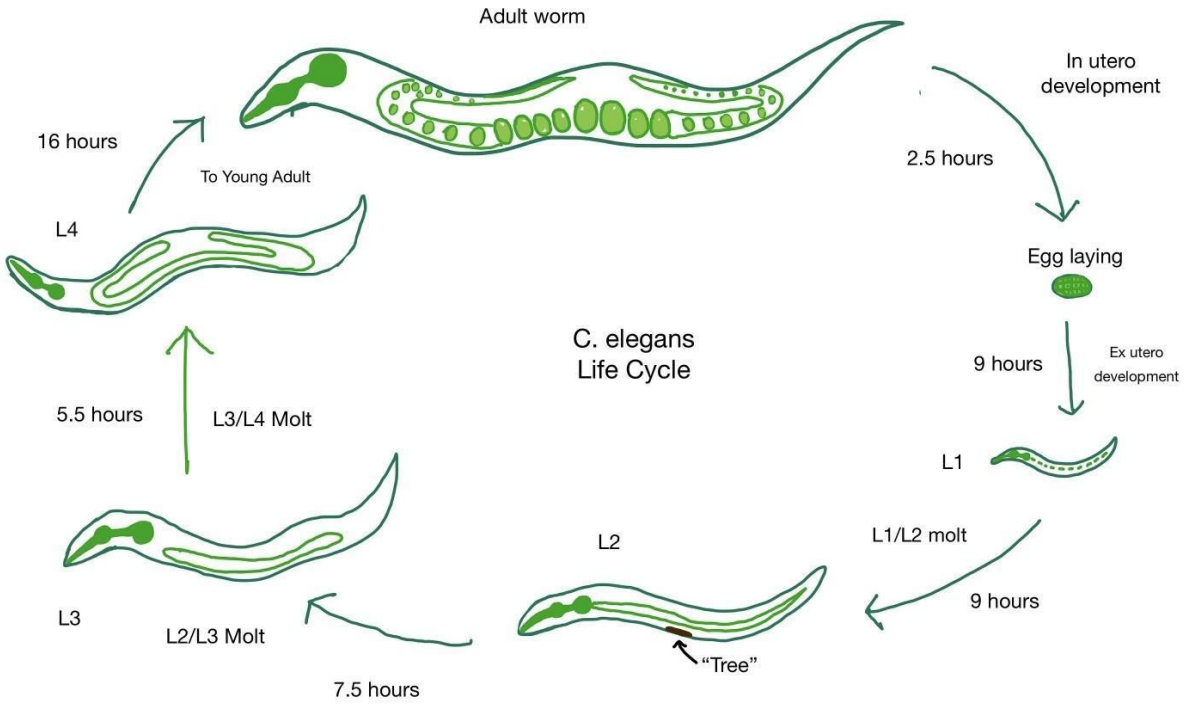


**Figure 1.** ***Caenorhabditis elegans life cycle from egg laying to adulthood.*** *The average life cycle of C. elegans is 3.5 days at 20C; it begins with the laying of an egg and development through four larval stages. The focus of this research is sperm-egg fertilization, in utero development, and the laying and hatching of the eggs.*

Cap-independent and cap-dependent mRNA translation mechanisms have been shown to have roles in germ cell and embryonic development in the organism (Keiper, 2019). Caps, or 7-methyldiguanonsyl-triphosphates (m7GpppG), serve as post-transcriptional modifications to protect the mRNA from degradation. This allows for the spliceosome to recognize the introns and "splice" them out. The mRNA is then ushered out of the nucleus, also via cap-binding. In the cytoplasm, translation factors in the eIF4 group recognize the m7G cap to assemble the initiation complex with ribosomal subunits. This is the mechanism for canonical cap-dependent translation (Figure 2). Cap-independent mRNA translation refers to a non-canonical mechanism in which

the ribosome binds on the mRNA by means of an IRES (internal ribosome entry site). The process does not utilize recognition of the 5' cap, but does utilize the other initiation proteins, including eIF4G (here, IFG-1) in order to bind to the sequence. Previous research has indicated these mechanisms function at different times within the gonad of *C. elegans*, which in turn, impacts the fertility of the nematode (Contreras, et al., 2008; Contreras, et al., 2011; Morrison, et al., 2014).

The aforementioned proteins include the eIF4G translation initiation factor, also known as IFG-1 in *C. elegans* nomenclature. Translation factor eIF4G associates with eIF4E and guides the mRNA to the ribosome for translation. Cleavage of the translation initiation factors-specifically the eIF4G protein at a hinge region-induces cap-independent synthesis of apoptotic proteins, therefore triggering apoptosis (Contreras, et al 2008). Physiological apoptosis does occur naturally in order to remove germline cells within the gonad that are not selected to further develop into oocytes and later eggs.

The purpose of this study was to look at the effects of tagging the IFG-1 protein in vivo using Crispr gene engineering. The tags were added by inserting DNA sequences to cause in-frame fusion proteins with N-terminal additions to IFG-1; specifically the long p170 isoform of the protein (Contreras, 2008). Tags included both a fluorescent protein [mCherry (red, ~230 amino acids) and short peptide sequences (11-31 amino acids) that serve as antibody affinity tags (antigens) for affinity purification and immunostaining. In one case, the viral V5 tag (14 amino acids) was internally deleted of just 3 amino acids to create a shorter 11 amino acid tag that could still be recognized. Forms of IFG-1 fused to all three tags were studied through the course of this research--3xMyc, V5, and dV5. Figure 2 depicts the tags added to the IFG-1 gene through CRISPR-cas9--specifically 3xMyc, V5, and dV5.

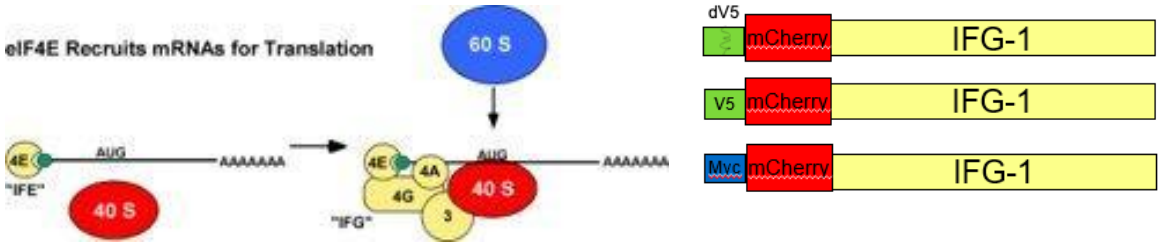


**Figure 2.** ***Cap-dependent translation mechanisms, specifically the eIF4G or "IFG-1" protein.***


**Figure 3**. ***ifg-1 is the gene of focus of this study, specifically exon 1.*** *Two forms of mRNA are made, as indicated by the two arrows, encoding p130 and p170 to catalyze cap-dependent and cap-independent translation.*

## Methods

*Creating fusion protein strains*

Genetic engineering by the CRISPR-Cas9 system allowed for the study of the *ifg-1* gene of the organism. Within the CRISPR-Cas9 system, a target sequence is identified to which a guide RNA binds with a complementary DNA sequence. The nuclease enzyme cas9 uses the "PAM," or protospacer adjacent motif sequence, to bind to the correct spot at the guide RNA's position. The target sequence is then cut and a mutation can be inserted into the genome. The PAM is typically found 3-4 nucleotides downstream from the cut. This system of genetic engineering is accomplished in C elegans to produce stably engineered new strains of worms by means of microinjecting the gonads of the parent worms with a solution containing the Cas9 enzyme, a guide RNA, and a homologous recombination repair DNA template containing the

sequences to be inserted. Successful mutations are witnessed in the F1 generation of the injected mothers. N-terminal tags were inserted into the *ifg-1* gene on chromosome II of the mother worms by microinjection. Successful gene engineering was scored in the F1 and F2 offspring generations by following the "rolling" phenotype (caused by the co-injection of a dominant *rol-6* gene on a plasmid) and by genomic PCR as depicted in Figure 9. It was noticed early that some of these tags had deleterious effects on fertility, specifically on embryo hatching, while others were fully tolerated. These markedly differing effects of tags on fertility and embryo viability became the focus of the subsequent study.

*Balancing unviable mutant alleles with the fluorescent-marked chromosome II strain*

In the cases where an engineered strain was infertile, it could not be stabilized and propagated in the homozygous state. Therefore, the heterozygous F2 worms were crossed with another strain containing a GFP-pharynx marker (mIn1) also on chromosome II. The resulting mutant/mIn1 strain could be isolated and stably propagated as a heterozygous strain (Figure 4). Homozygous engineered worms were selected from parent plates by their lack of pharyngeal green fluorescence.

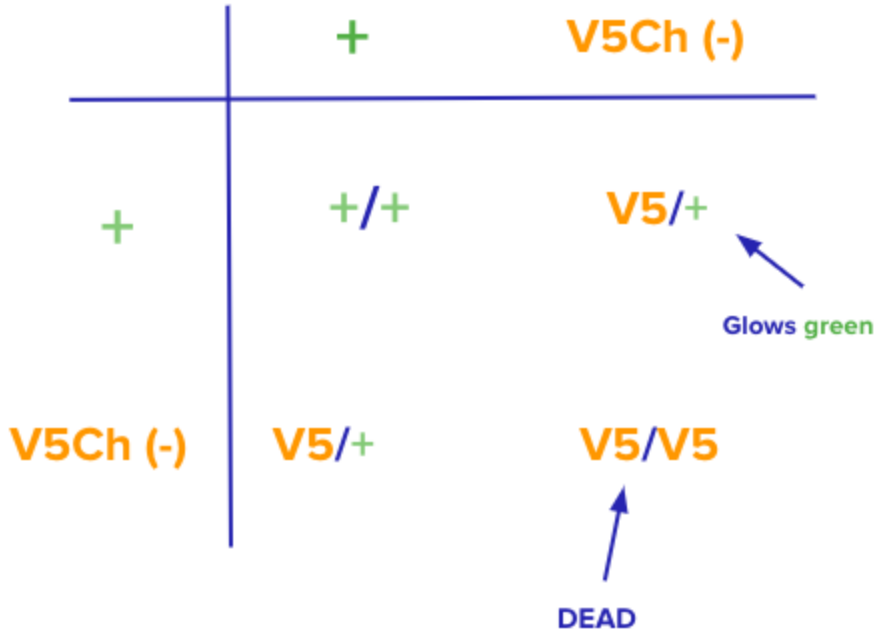


**Figure 4.** ***A Punnett square depicting the genotypes possible as a result of the genetic balancing.*** *The common chromosome II balancer mIn1 carries a myo-2::gfp fusion that is easily recognized in live worms under fluorescence. This can be used, and selected against, to isolate homozygous engineered worm (here "V5Ch") that could otherwise not be propagated as a strain.*

Fluorescent tags, specifically pharynx-GFP and gonadal-mCherry, were used to identify dV5/mIn1 progeny. Worms that exhibit both green GFP and red gonads indicated a successful cross and balancing of the strain. Embryonic lethality assays were then performed on the homozygous F1 worms derived from the balanced strains at the three temperatures again. Individual homozygous mothers (V5/V5, dV5/dV5) were then grown on plates at the three different temperatures of incubation. A fertility/embryonic lethality assay was performed on their F2 offspring in which the numbers of hatchlings and eggs laid were monitored over the course of several days until complete hatching could be expected from the wild type control strain. During the process of an embryonic lethality assay, two sets of plates--A and B plates--were utilized.

Mother worms are transferred over from the A to the B plates, and both were assessed in the counting assay. Mothers were eventually picked off into a 2uL solution of SWL:PrK in a PCR tube to prepare for genomic PCR to verify their genotype. Two different sets of triple primer combinations were used. The primers were designed using the Sequence Builder Pro "DNAstar" computer program. In each assay a triple primer set was used that would detect both the wild type *ifg-1* gene (e.g. from the balanced worms) and the Crispr-engineered *ifg-1* gene (see Figure 9). The first set contained V5S1, -136s, and mCh104a primers to target the Crispr-engineered *V5::mCherry::ifg-1* allele. The second set contained -136s, 223a, and mCh104a to target the wild type and *3xMyc::mCherry::ifg-1* allele (see schematic in Figure 9). A 2.0% agarose gel was utilized in order to visualize smaller fragments of DNA and their differences to confirm the genotype of the worms. Originally, the Phusion58 polymerase was used for the PCR, but Amplitaq DNA polymerase was instead utilized for the final confirmatory gel. In this case the Amplitaq enzyme showed a greater efficiency, likely due to the primer complexity

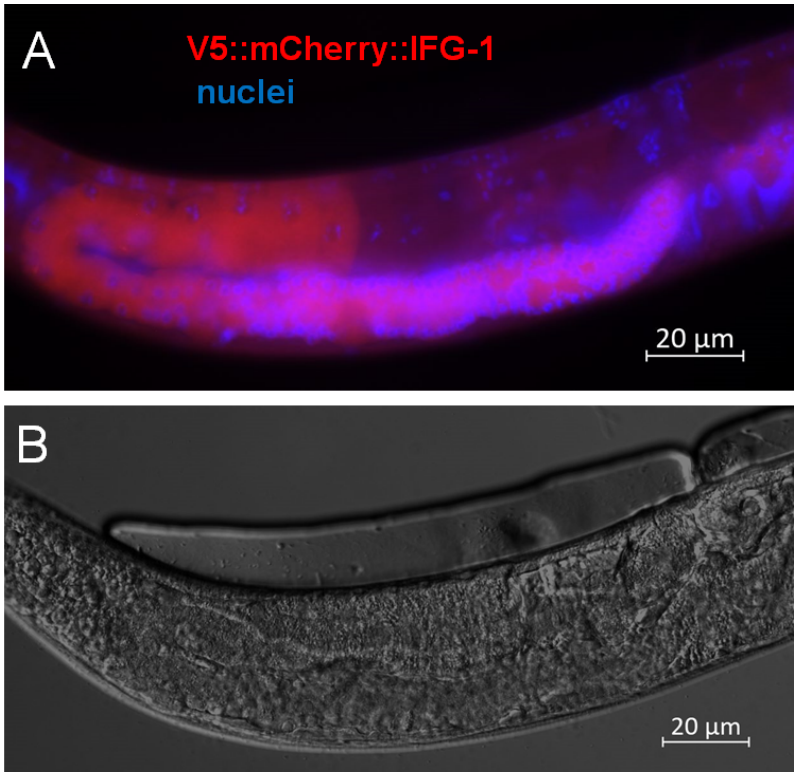


**Figure 5.** ***Fluorescent microscopy of C. elegans nematode.*** *Image A is illuminated by DAPI staining on cell nuclei and the fluorescent tag mCherry. Image B is the same worm, but without fluorescence.*

**Results**

*The microscopy observation of the strains with red fluorescent tagged IFG-1 p170 and various peptide tags.*

Strain KX214 originally featured a deletion of the V5 gene--dV5--that was deleterious to the fertility rates of the nematode at three temperatures of growth. The fertility progressively worsened, culminating in no hatchlings. The strain was then genetically balanced with wildtype *C. elegans*. Genetic balancers are chromosomal rearrangements that allow for lethal mutations to be maintained in heterozygous worms; this prevents recombination and brings back fertility without heavily altering the genome of the strain.

The fluorescent tags employed—mCherry and GFP--were inserted to be able to differentiate genotypes by the phenotypes present. Gonadal mCherry with no pharynx GFP presentation was the goal phenotype *(*V5:mCherry-ifg-1(-/-)) as it would indicate a homozygous genotype. Due to the initial genetic balancing and crossing, heterozygous genotypes were undesirable, fluorescing green and having a "dumpy" phenotype. Worms that fluoresced both mCherry and GFP were also avoided. Homozygous worms were successfully picked and grown for the embryonic lethality assays.

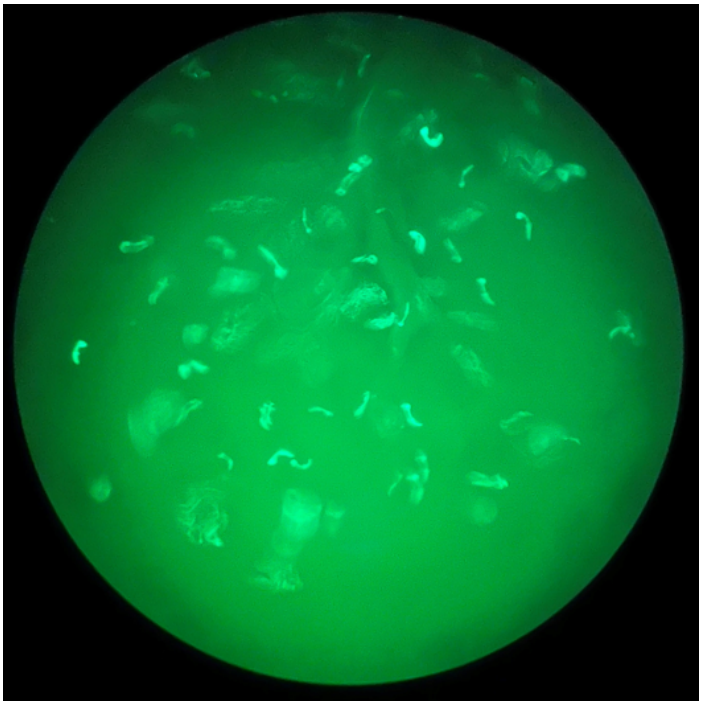

**Figure 6.** ***Microscope view of worms bearing mutation balanced by a GFP-pharynx-tagged chromosome II****. Heterozygous worms were photographed on a fluorescent microscope through the lens with a cell phone camera.*

*Assessing changes in fecundity (fertility) caused by the type of tag.*

Fertility of the worm was measured in two aspects: egg laying and successful hatching. Wildtype worms, on average, laid 200 to 300 eggs at the three test temperatures. All wildtype worms would hatch, with the exception of one egg occasionally failing to brood. The

V5::mCherry fusion tag, however, was severely stunted in both egg laying and brooding. At the typical incubation temperature of 20C, wildtype worms laid about 298 eggs. On the other hand, V5 tagged mother worms laid 40 eggs. The dV5::mCherry tag was drastically different; it presented nearly normal levels of egg laying at 15C and 20C. In fact, dV5 laid more eggs than the wildtype N2 worms at both 15C and 20C. 3xMyc::mCherry presented lower egg laying levels than dV5 and wildtype, but far better than the V5 tag. Egg laying is directly related to fertilization within the hermaphroditic mothers; therefore the defects lie in sperm or oocyte formation.

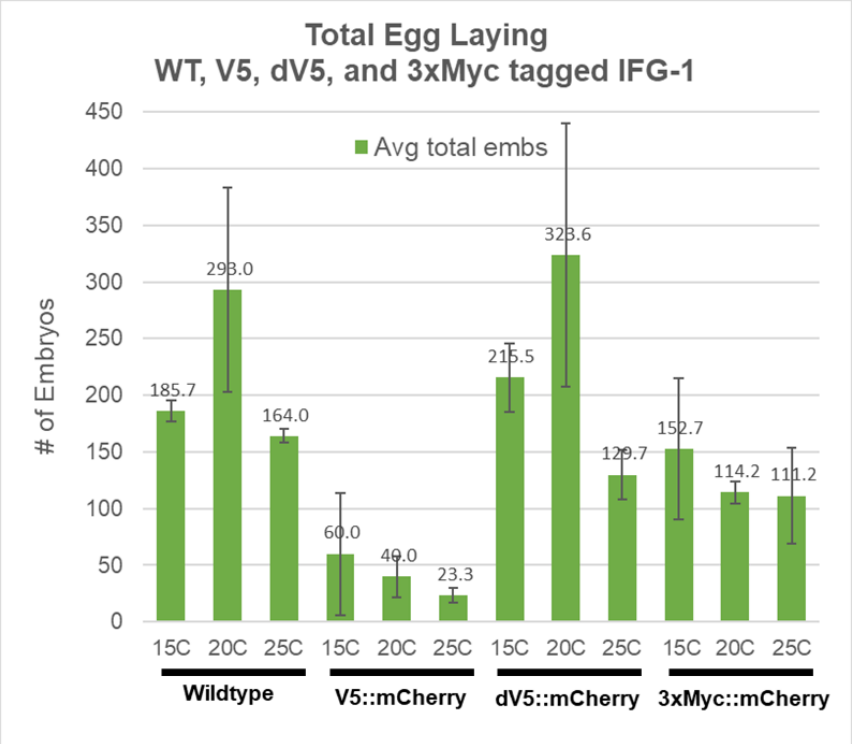


**Figure 7.** ***Eggs laid by IFG-1 tagged mothers—WT, dV5, V5, and 3xMyc.*** *V5 stunted egg laying substantially, whereas dV5 had nearly equal rates of egg-laying to wildtype worms, despite extensive lethality. Myc egg laying was dampened, but viability was better than dV5.*

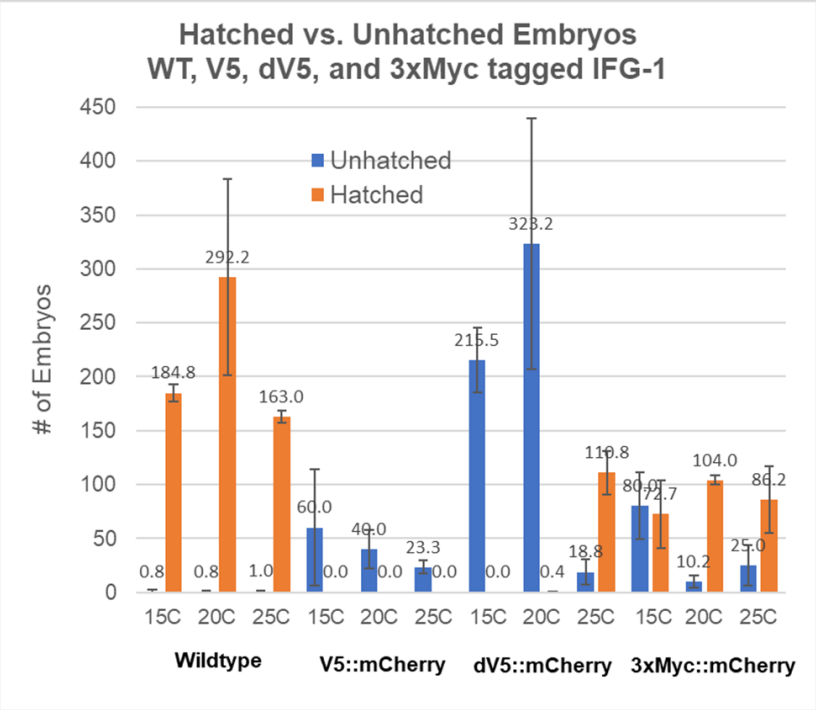


**Figure 9.** ***Embryonic lethality of various IFG-1 tags: WT, dV5, V5, Myc****. The V5 tag added to mCherry causes severe embryonic lethality compared to Myc tag. Internal deletion within the tag (dV5) alleviates lethality at 25C only.*

Embryonic lethality--that is, whether or not the eggs hatch into L1s--differs widely amongst the fusion tags within ifg-1. No eggs hatched with the V5::mCherry tag--indicating full embryonic lethality. dV5 eggs did not hatch at 15C or 20C despite the high levels of laying at the respective temperatures. However, 85-90% of the dV5 eggs laid at 25C hatched. The 3xMyc::mCherry tag fared well with adequate levels of hatching at all tested temperatures.

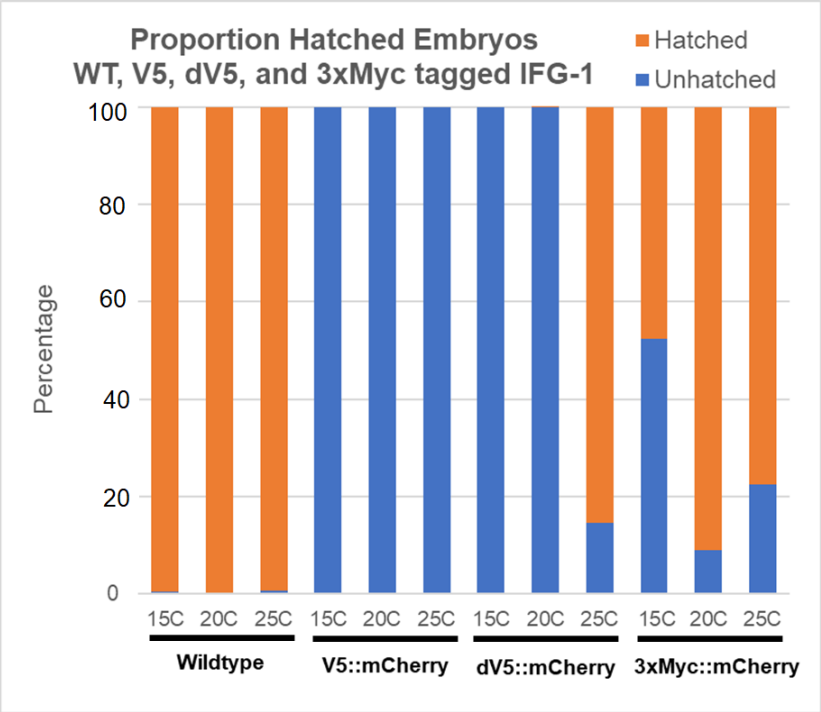


**Figure 8.** ***Percentage of embryonic lethality of IFG-1 tags.*** *All forms of V5 were substantially more toxic than 3xMyc when added to mCherry::IFG-1. Myc-tagged were nearly like WT at 20C.*

*Assessing changes in embryo viability*

All tags tested allowed for the F1 generation of worms to become mothers and lay eggs, heavily suggesting that some functions of the IFG-1 protein are supported by the tags V5, dV5, and 3xMyc. Previous research has indicated that null mutations in the ifg-1 gene did not cause arrest at either egg laying or L1 hatching (Contreras, et al 2008) but rather as L2 larvae. Null mutations refers to a complete lack of IFG-1 production within the worm. The role of IFG-1 in the physical maturation of the worm and its reproduction are potentially distinct in the developmental stage. Furthermore, because several of the tested strains laid normal or above average egg numbers which failed to hatch, the potential of a later defect within the embryonic division stage is very possible.

A genomic PCR was performed in order to confirm the results and the genotypes of the tagged worms (Fig 10). No recombination event occurred to restore the wild type ifg-1 gene, as the small base pair differences between V5 and dV5 were visible and drastically differed from the wildtype banding.

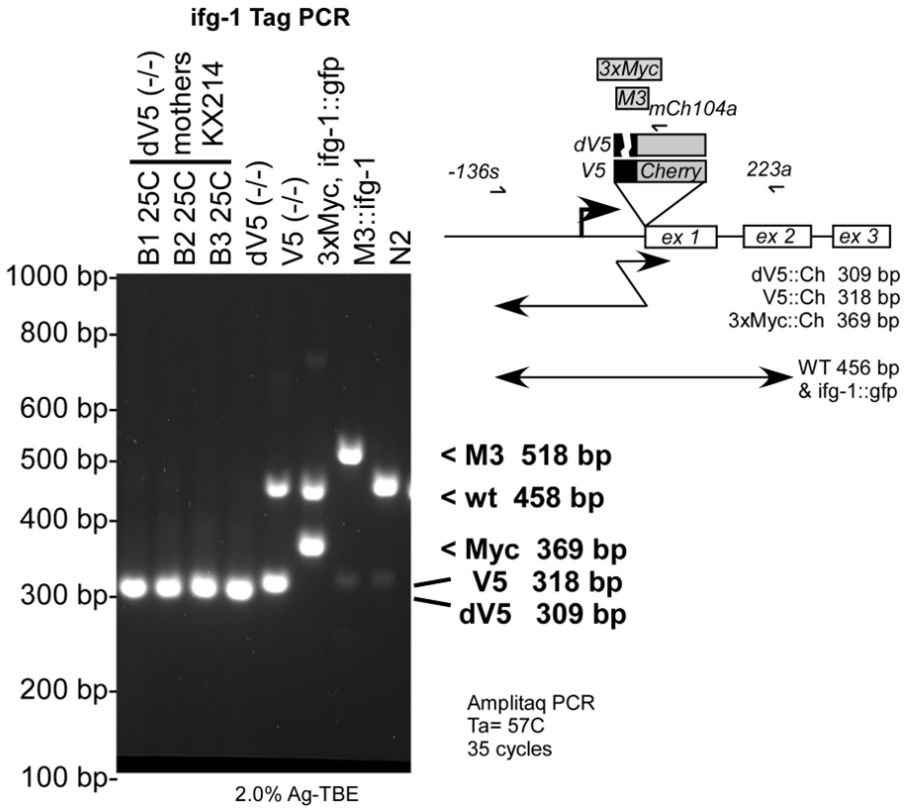


**Figure 10.** ***Genomic PCR of whole adult worms from each strain.*** *PCR reactions and subsequent agarose gels (Fig. 2) confirmed the genotype and the size of ifg-1 gene insertions, suggesting no recombination event occurred that might restore the wild type ifg-1 gene.*

**Discussion**

Reproductive cells and embryos utilize mRNA translation mechanisms to regulate gene expression. Cap-dependent mRNA translation mechanisms are catalyzed l by the ifg-1 gene, specifically within exon 1. The mRNA comes in two forms of varying lengths: p130 and p170 (Figure 3). As seen by the results, the role of cap-dependent translation cannot be understated. Peptide tags fused to the large fluorophore mCherry directly impacted fertility irregardless of their size. Rather, the sequence nature was far more significant. For example, the 3xMyc::mCherry construct was fully viable with minimal fertility impact at a much larger size, yet the smaller V5 tag fused to the fluorophore caused full embryonic lethality at *all* three temperatures tested. The V5 tag induced complete embryonic lethality with a low number of eggs and no hatchlings. dV5, on the other hand, has an 85-90% hatching rate at 25C. dV5 is just 9 base pairs smaller than V5. Despite dV5 being 3 amino acids smaller, dV5 damages the ifg-1 gene *less*. 3xMyc, despite being larger than V5, also damages the ifg-1 gene less.

Fertility, which was measured by the number of eggs laid due to sperm and oocyte potency, varied across the four strains of worm tested. Embryonic lethality refers to cell cycle development and whether the eggs matured into L1 larvae. Wildtype worms are highly fertile, producing 200-300 eggs typically across the temperature ranges. V5 worms produced a maximum of 60 eggs at 15C, a number which dwindled as the temperature rose. dV5 worms were more “fertile” at 15C and 20C, yet the eggs failed to hatch at these respective temperatures. Thus, they were embryonically lethal at cooler temperatures. Intriguingly, the higher incubation temperature of 25C restored fertility and reproductive function within the worms. 3xMyc produced an adequate number of eggs with nearly complete levels of hatching at lower temperatures. Interestingly, the levels of egg laying between all three temperatures of 3xMyc and

25C dV5 are very similar (Fig 7.)

Null mutations of the IFG-1 protein typically cause arrest and apoptosis at the L2 larval stage, rather than egg laying or L1 molting. Wildtype worms avoided arrest nearly 100% of the time, with the exception of a lone egg failing to mature. V5 worms completely arrested prior to the L1 stage at all tested temperatures. 3xMyc was only slightly affected, with the majority of the small number of eggs maturing. dV5 experienced arrest at both 15C and 20C, but not 25C. Again, the role of IFG-1 in the maturation and life cycle of the worm and its reproductive capabilities are highly nuanced in development.

The future of this research lies in the human application of the eIF4e/g translation factors. Specifically how misregulation and overexpression of these factors are linked to human prostate and breast cancers (Hiller et al 2009, Silvera et al 2009). Potential cancer therapies exist in the form of modulating levels of eIF4G to decrease the incidences of these cancers. However, it becomes clear that fertility and reproduction are directly impacted by the modulation of these translation factors. Thus, care must be taken to avoid adverse effects on human reproduction.

## Works Cited


Contreras, V., Friday, A.J., Morrison, J.K., Hao, E. and Keiper, B.D. (2011) Cap-Independent translation promotes *C. elegans* germ cell apoptosis through Apaf-1/CED-4 in a caspase-dependent mechanism. PLoS ONE, 6, e24,444.

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

Corsi, AK, Wightman, B, and Chalfie, M (2015). A Transparent window into biology: A primer on Caenorhabditis elegans (June 18, 2015), WormBook, ed. The C. elegans Research Community, WormBook, doi/10.1895/wormbook.1.177.1, http://www.wormbook.org.

Kim, H., Ishidate, T., Ghanta, K.S., Seth, M., Conte, D., Jr., Shirayama, M. and Mello, C.C. (2014) A Co-CRISPR Strategy for Efficient Genome Editing in Caenorhabditis elegans. Genetics, 197, 1069-1080.

Huggins, H.P. and Keiper, B.D. (2020) Regulation of Germ Cell mRNPs by eIF4E:4EIP Complexes: Multiple Mechanisms, One Goal. Front Cell Dev Biol, 8, 562.

Keiper, B. (2019) Cap-Independent mRNA Translation in Germ Cells. Int J Mol Sci., 20, 173.

Morrison, J.K., Friday, A.J., Henderson, M.A., Hao, E. and Keiper, B.D. (2014) Induction of cap-independent BiP (hsp-3) and Bcl-2 (ced-9) translation in response to eIF4G (IFG-1) depletion in *C. elegans*. Translation, 2, e28935.

Schafer WR. Egg-laying. 2005 Dec 14. In: WormBook: The Online Review of C. elegans Biology [Internet]. Pasadena (CA): WormBook; 2005-2018. Available from: https://www.ncbi.nlm.nih.gov/books/NBK19787/