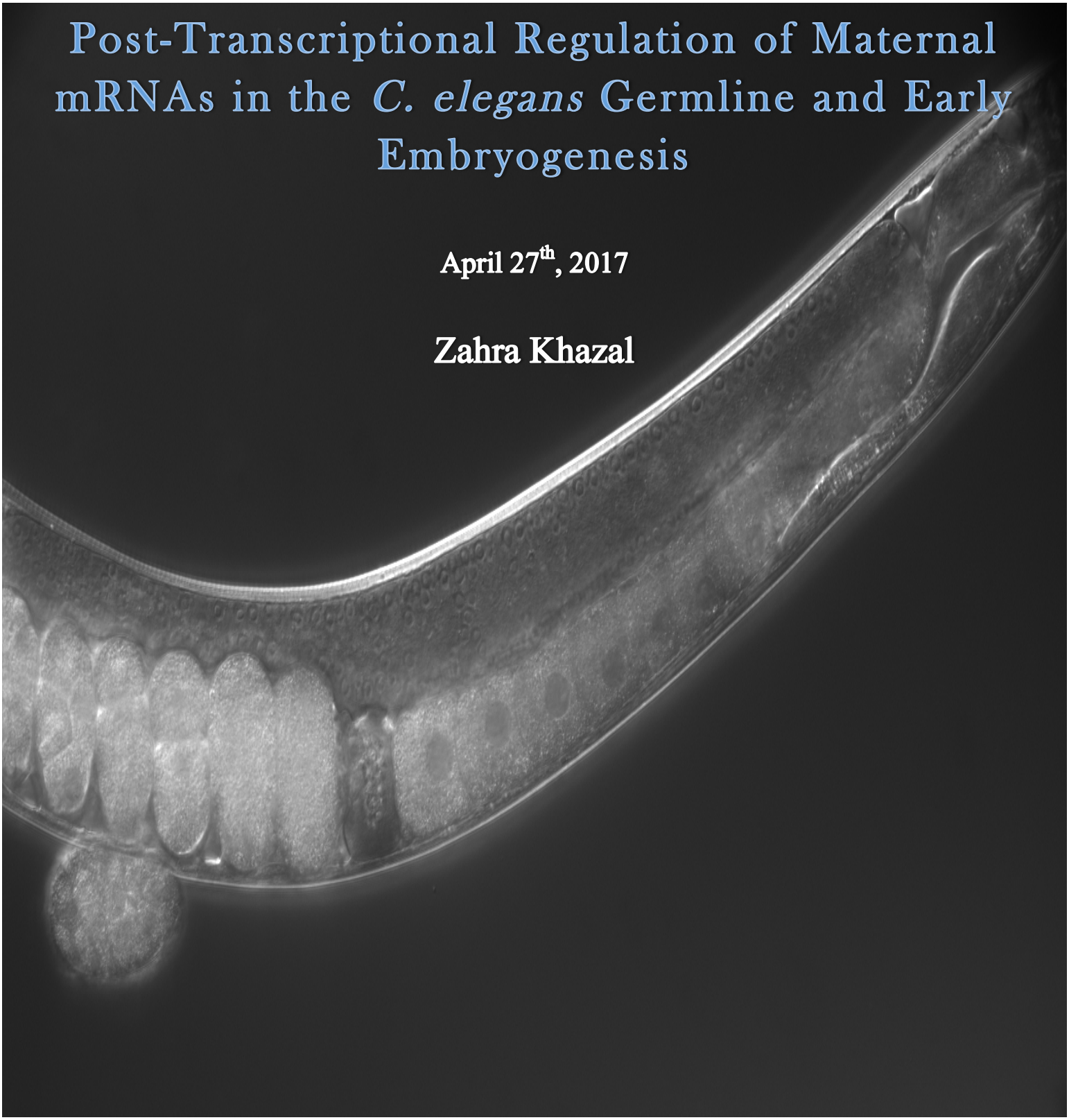
Post-Transcriptional Regulation of Maternal mRNAs in the *C. elegans* Germline and Early Embryogenesis



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Post Transcriptional Regulation of Maternal mRNAs in the C. elegans Germline and Early Embryogenesis

A Major Qualifying Project

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By

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Abstract

Maternal RNAs are transferred through the oocyte cytoplasm to the fertilized progeny. In metazoans, these transcripts play a critical role in patterning embryonic development prior to the maternal-to-zygotic transition. The 3'UTR appears to be the primary determinant controlling the pattern of maternal RNA expression in the germline and in the embryo. The 3'UTR also directs robust translation in male gametes, suggesting a possible role for mRNA inheritance through the male germline in *C. elegans*. Specifically, the *set-2* 3'UTR drives strong translation of a GFP reporter in sperm. This study aims on identifying *cis* acting elements in this UTR that confer strong sperm-specific translation, and screening for *trans* acting RNA binding proteins that influence sperm translation efficiency.

Acknowledgments

Special thanks to Professor Jagan Srinivasan for advising this project, Professor Sean Ryder for sponsoring this research and for his guidance through the duration of the project, my colleagues at the Ryder Lab for their assistance, and my parents for their continuous support.

Introduction

By the end of the 19th century, comparative embryology was still a new, developing field. Scientists were studying the steps that lead to the development of complex multicellular organism from a single, fertilized cell. Specifically, embryologists wanted to find key connection ideas that could unit observations made between species in an effort to recombine their discoveries with Darwin's published principle of natural selection. Modern embryologists are still focusing on the same questions 130 years later using modern techniques of molecular and genetic biology and tools that allow them to study the genome-wide analysis of gene expression patterns. Similar observations made in the embryogenesis molecular development suggest a common ancestor between metazoan species but lack explaining the morphological differences between them.

One of the main concepts that arises from embryogenesis studies is the post-transcriptional regulation of maternal mRNAs, which is essential to the development of the zygote. During the development of the oocytes, the egg chromosomal content becomes locked in meiosis until fertilization happens, which prevents the transcription of the mRNAs that the new organism inherits. In the earlier stages of oogenesis, maternal transcripts are formed and reversibly silenced. Additionally, DNA of most animal embryos does not get transcribed until the zygote has divided one or more times. Generally, zygotic transcription begins after multiple cell divisions and a several patterning and cell fate specification events. Therefore, initiation of maternal transcripts by maternal regulatory elements offers the initial step for formation of the body

organization (Farley and Ryder, 2008).

C. elegans as a Model Organism

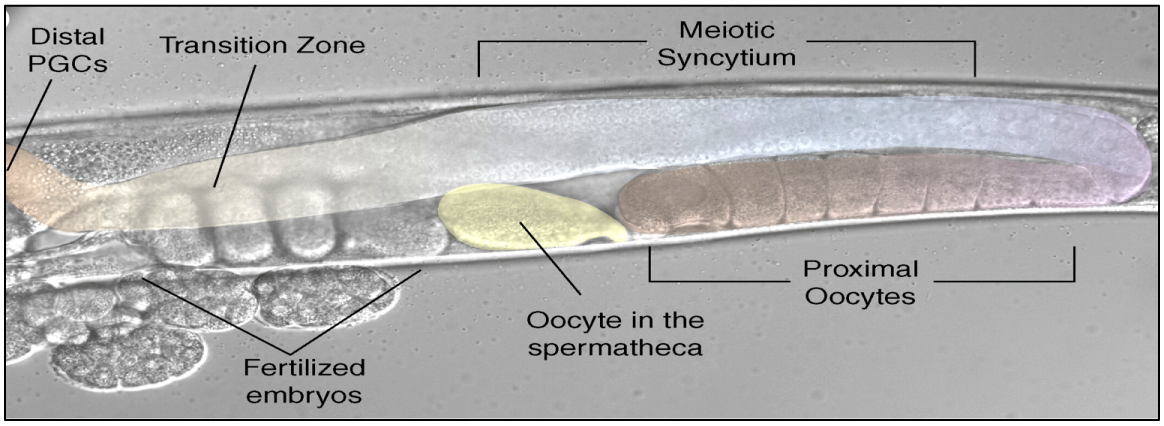
Caenorhabditis elegans (nematode worm) is one of the common model organisms used in laboratories along with *Drosophila melanogaster* (fruit fly), *Xeno- pus laevis* (frog), *Danio rerio* (zebrafish), and *Mus musculus* (mouse) (Farley and Ryder, 2008). In this study, *C. elegans* offered a great system to study the process of oogenesis. This free-living soil nematode is found worldwide and has two genders. With males arising at a frequency of <0.2%, this nematode exists primarily as self-fertilizing hermaphrodites allowing the complete process of early embryogenesis to be studied in one organism (Kaymak and Ryder, 2013). One of the their important features is their transparency, which allows to microsciablly visualize different parts of the worm, specfically the germline as a visible gradient of development using flourescnet reporter protein. Additionally, *C. elegans* is very easy to maintian and grow, using *Escherichia coli* as their diet. They also produce rapidly and have a short life span, taking only 3 days to develop from an egg to an adult. Furthermore, *C. elegans* have a well-annotated genome. For example, at least 38% of the *C. elegans* protein-coding genes have predicted orthologs in the human genome and 60-80% of human genes have an ortholog in the *C. elegans* genome. Thus, this makes findings of experiments using *C. elegans* as a model organims easily applicable to humans in the molecular level (Kaletta and Hengartner, 2006).

C. elegans Germline

Oogenisis begins at the distal tip of the gonad where a population of primordial germ cells mitotically divide as seen in **Figure 1**. The distal PGCs then transition into meiosis and their plasma membrane disappears, forming a syncytium of meiotically arrested nuclei. The nuclei recellularize, which create immature oocytes that remain arrested in meiosis I until they approach the spermatheca, an area that contains spermatocytes. The oocytes will then complete both meiotic divisions and enter the spermatheca, where fertilization occurs (Farley and Ryder, 2008).

Figure 1: False-color image of C. elegans germline (Farley and Ryder, 2008)

Once fertilized, the oocyte completes meiosis and quickly creates an anterior-posterior body axis, with the point of sperm entry determining the posterior pole of the embryo. Embryonic cell division happens along this axis, creating a larger anterior blastomere (embryo cell), and a smaller posterior blastomere. The larger anterior blastomere is the first of six founder cells that form during the main cell divisions **Figure 2**. The founder cells will commit themselves to differentiate into a limited subset of tissue types. In opposition, the smaller



posterior daughter cell is the responsible precursor of the entire germ line, also known as the P lineage. The process of asymmetric division is repeated three more times until the germline blastomere undergoes one final symmetric division to produce two primordial germ cells. These cells are transcriptionally inactive until larval development (Farley and Ryder, 2008).

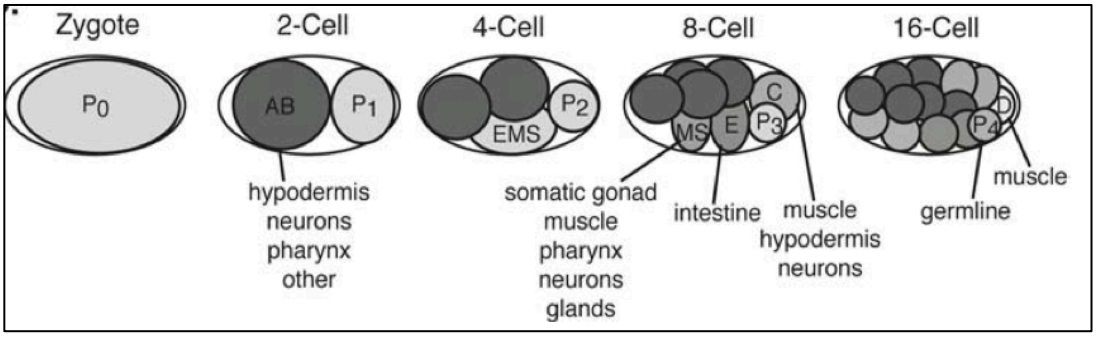


Figure 2: Founder cells fate of the early C. elegans embryo (Farley and Ryder, 2008)

Post-transcriptional Regulation of Maternal mRNA in Oogenesis

In *C. elegans*, post-transcriptional regulation of maternal mRNAs controls gene regulation during oogenesis and early embryogenesis (Kaymak and Ryder, 2013). Hermaphrodites produce both sperm and eggs from the same place of primordial germ cells. The orders of making eggs and sperms and the timing of the transition from sperm making to egg production are both closely regulated. Spermatocytes are made during larval stages of development, while oocytes are produced in adults. Changing from spermatogenesis to oogenesis depends mainly on the post-transcriptional regulation of a few key transcripts in the germline (Farley and Ryder, 2008).

Post-transcriptional regulation of mRNA is mainly facilitated through *cis*-

regulatory elements existing in the un-translated regions (UTRs) of the transcript. A study of germline-expressed genes in the nematode *C. elegans* validates that 3′ UTRs are ample to drive patterned gene expression in the germline. Furthermore, most transcripts do not contain a unique 5′ UTR, as 5′-end formation in nematodes is facilitated primarily by *trans*-splicing of one of two key sequences (Merritt et al., 2008). Thus, the absence of a unique 5' UTR between genes makes studying the RNA binding proteins easier since the binding region is contained in one region of the gene.

RNA binding proteins are a specific class of protiens that act to regulate gene expression by binding to specific mRNA transcripts resulting in increased or decreased translation (Kaymak and Ryder, 2013). Using the idea that the 3' UTR is the main regulatory element and understanding that affinity does not necessarily correlate to regulation, we have developed a hypothesis on the regulation of gene expression by these RNA binding proteins. The developed cluster hypothesis states that if there's a cluster of binding sites in the 3' UTR then that gene is regulated primarily by those RNA binding proteins.

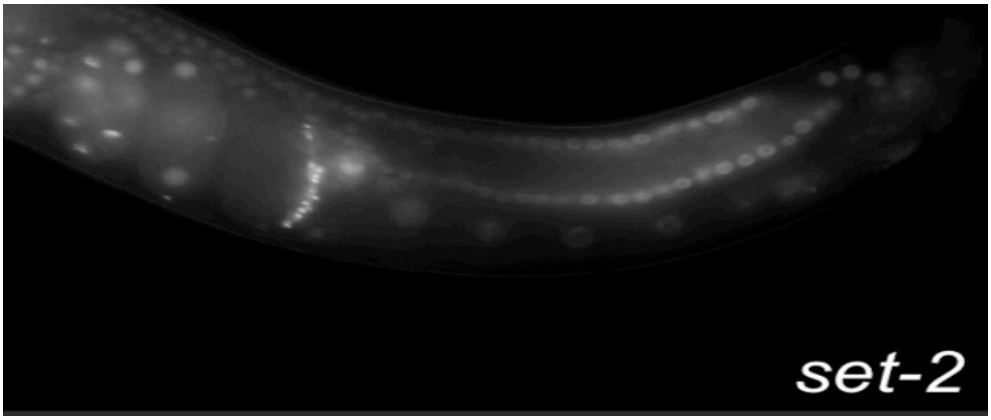
Set-2 Reporter Construct

Set-2 is a reporter construct that was integrated using standard reporter MosSC in the Ryder Lab. Set-2 3' UTR showed faded GFP expression in the distal end followed by an increased expression in the syncytial region, which then decreased around the recellularization region and oocytes. The GFP expression remained strong in providing an example of a 3'UTR that can direct expression of a reporter in male gametes (Kaymak

et al., 2016). Set-2 encodes a histone H3K4 methyltransferase and regulates its levels. It plays a role in germline development, postembryonic development, and RNA interference (Erdelyi, Wang, Suleski, & Wicky, 2016). As shown in **Figure 3**, there is a GFP expression in the spermatheca which is not normally seen in other reporter constructs.

Figure 3: Set-2 GFP expression (Kaymak et al., 2016).

RNAi and RNA-Binding Proteins



RNAi interference technique was used identify RNA-binding proteins that directly or indirectly control the expression pattern of the set 3'UTR reporter strain. RNAi is a way to "silence" genes by preventing the creation of the proteins that they code for without changing the DNA of the organism (Liou, 2010). The genes we are interested in inhibiting are puf-5, Daz-1, Alg-3, and Csr-1. Each one of these genes was

selected due to the proteins that they code for which we believe will cooperate with Set-2 and cause a noticeable modification in the mutant lacking that specific gene. Daz-1 is an RNA-binding protein required for oogenesis. Therefore, silencing Daz-1 will to the nonappearance of oocytes and sterility. Puf-5 is required for embryonic viability and oocyte maturation (Kaymak, Wee, & Ryder, 2010). On the other hand, Alg-3 is required in process of spermiogenesis in which spermatids become mobile ameboid sperm. Finally, Csr-1 is required for chromosome segregation, and embryonic viability. Embryos of Csr-1(RNAi) are mainly inviable, with disordered metaphase chromosomes and anaphase DNA bridges (Wormbase, 2017).

Materials and Methods

Maintaining *C. elegans*

Worms are manintained by being kept on agar plates and using 50µL of *a E. coli* HT115(DE3) wild-type culture to feed the worms. The agar plates are made by mixing 20g agar, 2.5g peptone, 3g NaCl, and 1 liter of purified water. After mixing the components together, the solution was then autoclaved and was left to cool on a stirrer to avoid solidifying. By using 25ml pipette, 25ml of 1M KPO_4, 1mL of 1M $CaCl_2$, 1mL of 1M $MgSO_4$, and 1mL of mg/ml cholesterol were added. Generally, 10mL of the media was needed to be poured for the 6cm plates.

To continue the propagation of worm cultures, the worms are “chunked” when the plates become overgrown, usually after 2-3 days. To chunk the plates, a metal spatula is

heated and cleaned using ethanol, then a square cut is made on the old, overgrown plate. This cut is then placed on the edge of the *E. coli* food in the new plate face-down to allow the worms to easily move in the new plate.

The "picking" process was also used when singling-out one worm from the progeny was needed. This was done by using a flattened end metal tool, which is sterilized using ethanol and heat before picking the worms.

Seeding RNA-Binding Proteins

To perform RNA interference, plates containing RNAi "food" for the worms were created. The food contained of HT115(DE3) *E. coli* bacteria including the desired plasmid needed to introduce the interfering RNA. Frozen stocks from the -80 C° of each desired RNAi food were streaked on LB plates that contained ampicillin and tetracyclin, and were then incubated overnight to grow colonies. Genes silenced using RNAi included Daz-1, Puf-5, Alg-3, and Csr-1 in addition to the HT115(DE3) *E. coli* that acted as a control by not silencing any genes.

To prepare bacterial culture for the RNA-binding proteins colonies, 2mL of LB broth, 100µg/mL of ampicillin, and 12.5µg/mL of tetracyclin were sued. A colony of the anticipated RNA-binding protein was picked from a plate and added to the culture. The culture was shaken at 37 C° overnight. 500µL of this culture was then added to an Erlenmeyer flask containing 50mL of LB broth, 50µL of ampicillin and 250µL of tetracyclin. The cultures were left in the shaker at 37 C° until the OD595 of the samples

reached 0.4. IPTG was then added to the cultures to 0.4mM, and the cultures were again shaken for four hours. An additional 100µg/mL of ampicillin and 12.5µg/mL of tetracyclin were added.

50µL of the culture was seeded onto a worm plate. These plates were made by combining 20g agar, 3g NaCl, 2.5g peptone, and 1 liter of purified water. This solution was mixed then autoclaved, and 1 mL of 1M $MgSO_4$, 25 mL of 1 M KPO_4, and 1 mL of 5 mg/mL cholesterol were added. 4mL of the mixture was poured into each 3.5cm plate and left to dry. After the plates dried, the RNAi food was seeded onto each plate, and left to dry overnight before use.

Bleaching

Before seeding worms on a plate containing RNAi food, the worm plates were bleached to kill off all adult worms and plate only eggs on the new plates. The bleach only dissolves the worm body, leaving their eggs undamaged. Plates were chunked and allowed to grow for approximately 72 hours, or until a significant number of eggs had been laid on the plate. A mixture of 1mL bleach and 250µL of 5M NaCl was added to a 1.5mL Eppendorf tube and vortexed. 3mL of filtered, pure water were added to the plates to loosen the eggs and worms from the plates, and the water containing the worms and eggs was added to a 15mL falcon tube. The bleach mixture was added to the falcon tube containing the worms and eggs, and the tube was vortexed and incubated at room temperature for 7 minutes. The tube was then centrifuged at 1100xg for 1 minute at 4°C, and all the supernatant was vacuumed off leaving on 1mL. Filtered, pure water was

added to the tube up to 6mL, and the tube was vortexed and spun again using the same settings as the first spin. The supernatant was vacuumed off as much as possible without disturbing the pellet, and the resulting mixture was pipetted several times to mix. 20-30 eggs were deposited onto each plate containing the desired RNAi food and the plates were incubated at 25°C for approximately 48 hours to allow the worms to grow and develop a germline.

Worm Imaging

After 48 hours, worms that had reached the young adult stage were imaged. 10-15 worms were picked off the plates using the picking technique and placed onto a slide containing 2% agar and 10 µL levamisole to immobilize the worms without killing them. The germlines of the worms were imaged at 40X in order to investigate differences between the RNAi strains.

Gateway Cloning

This technique was used to generate MosSCI compatible reporter constructs, and generate transgenic worms using moderate-throughput injection-based method. Gateway cloning is a recombinational cloning technology that uses protein expression and cloning of PCR products by using site-specific recombination enzymes. This technique makes use of a master clone having a particular gene that can be rapidly transferred to desired destination vectors. The BP reaction is a site specific recombination reaction between the B and P sites leading to generation of the master clone. While the LR reaction, which is

the main reaction pathway of the Gateway system, consists of a recombination reaction between a master clone and a destination vector (Thermo Fisher Scientific, 2016). For the full, detailed protocol, see **Appendix I**.

Results and Discussion

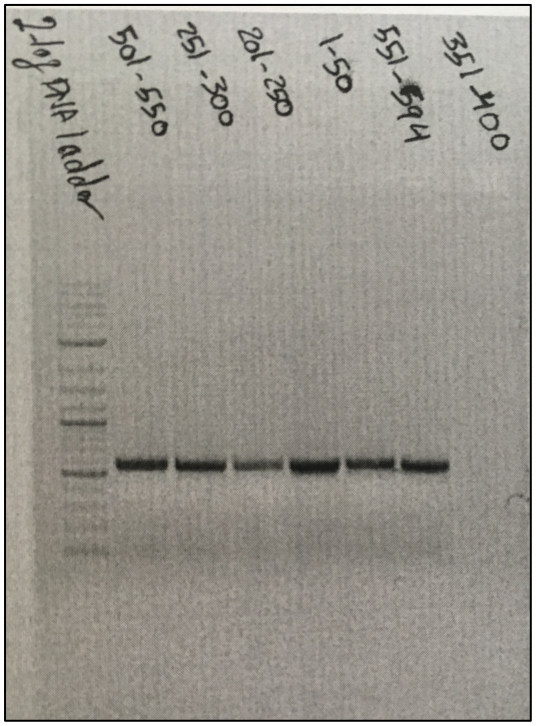
The aim of this project was to identify the necessary and sufficient elements to mediate sperm-specific expression using a strain with a Set-2 3'UTR.

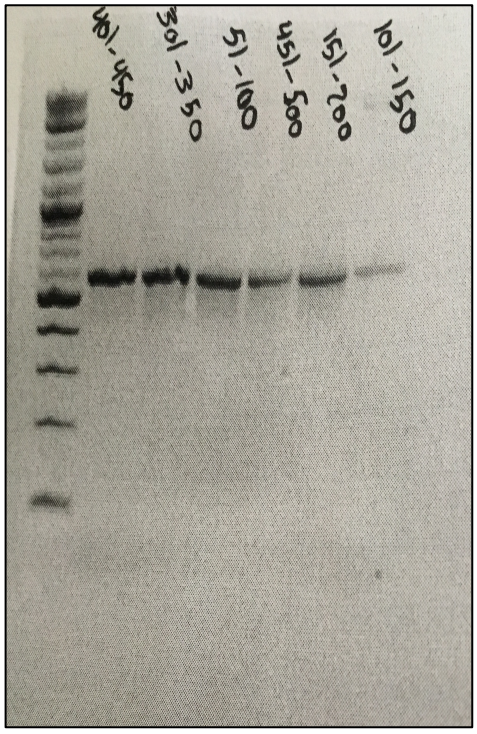
By using the Genome Browser, it was determined that the C. elegans 3'UTR is 594 base pairs long. Then, 12 mutant plasmids were generated; each plasmid was 544 base pairs. This was done by making 50 nucleotide deletions starting with the first 50 b.p. then to the next 50 b.p. and so on. The complete 12 plasmid deletions are shown in **Appendix II.** Then by following the Gateway Cloning reporter construct protocol, forward and reverse primers were made for the C. elegans 3'UTR. There were 4 primers generated, 2 forward (one for the wild-type plasmid and one for the mutant plasmid) and 2 reverse primers (one for the wild-type plasmid and one for the mutant plasmid), shown in **Appendix III**.

After receiving both the 12 plasmid mutants and their primers, polymerase chain reaction (PCR) was run to verify the size of the 12 plasmid mutants to confirm if the primers are binding correctly. **Figure 4** below shows the results of the PCR trials that were run:

Figure 4: Polymerase chain reaction results for the 12 mutant plasmids

As seen in the figure above, all 12 mutant plasmids had the same expected size of ~544 nucleotide. The next step in the process was to run the BP reaction for all of the 12 mutant plasmids. Samples from the BP reactions were sent for sequencing to check for any mismatches, deletions or insertions. The results of all 12 plasmids were confirmed and there were no issues observed. The following step was to perform the LR reaction. Unfortunately, the LR reaction was not successful. Therefore, it was important to trouble shoot and investigate each component that went into the reaction and find the factors that lead to the unsuccess of this step. Possible reasons that were already investigated were the use of old components and/or possible contamination. After confirming that was no contamination and using freshly made parts, we are now trying to use new super competent DH5-α cells instead of regular competent cells. The trouble-





shooting process for the constant failure of the LR reaction is still going, and the Gateway Cloning technique will be resumed once a solution is found for the LR reaction.

RNAi

Another issue that was encountered in this project was the transgene silencing of the germline in the Set-2 strain. As seen in **Figure 5**, on the heterozygous, there might be only one copy of the integration on just one allele, which prevents fluorescence under GFP.

Homozygous	Heterozygous
GFP $^{+/+}$ + GFP$^{+/+}$	GFP $^{+/-}$ + GFP$^{+/-}$
100% GFP $^{+/+}$	75% GFP^{+} / 25% GFP^{-}
Green	(1)GFP $^{+/+}$: (2)GFP $^{+/-}$: (1)GFP $^{-/-}$
	Green Faint Black

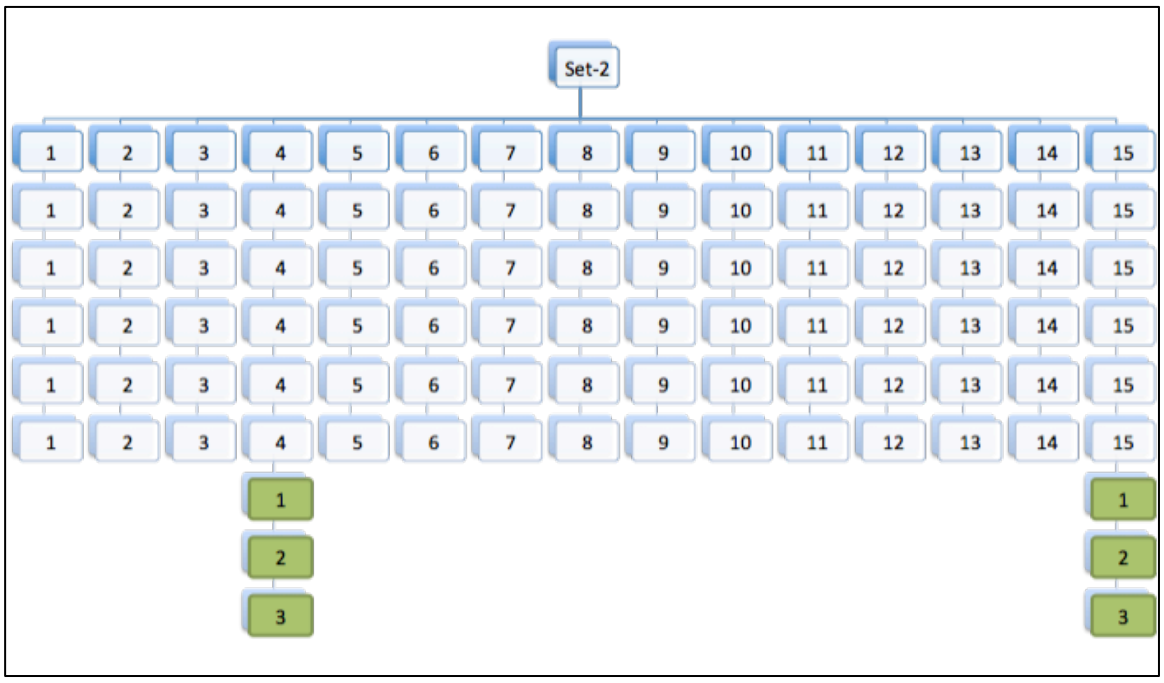
Figure 5: Transgene silencing

To overcome this issue, we have singled out 15 worms from a new set-2 strain and kept singling out worms from each progeny until the 6th generation **(Figure 6)**.

Figure 6: Process of singling out worms and making new progenies to find flouresing worms

There were about 8/10 worms from plates #4 and #15 that showed fluorescence in the oocytes and spermatheca. Then, worms were singled out from those two progenies until nicely fluorescing worms in the 3rd generation were observed.

The princible behind this step is to increase the chance of getting a homozygous worms that will self-fertilize and have strong transgenes which will allow for the flourescing under the GFP and eventually being able to continue the RNAi food screeining.



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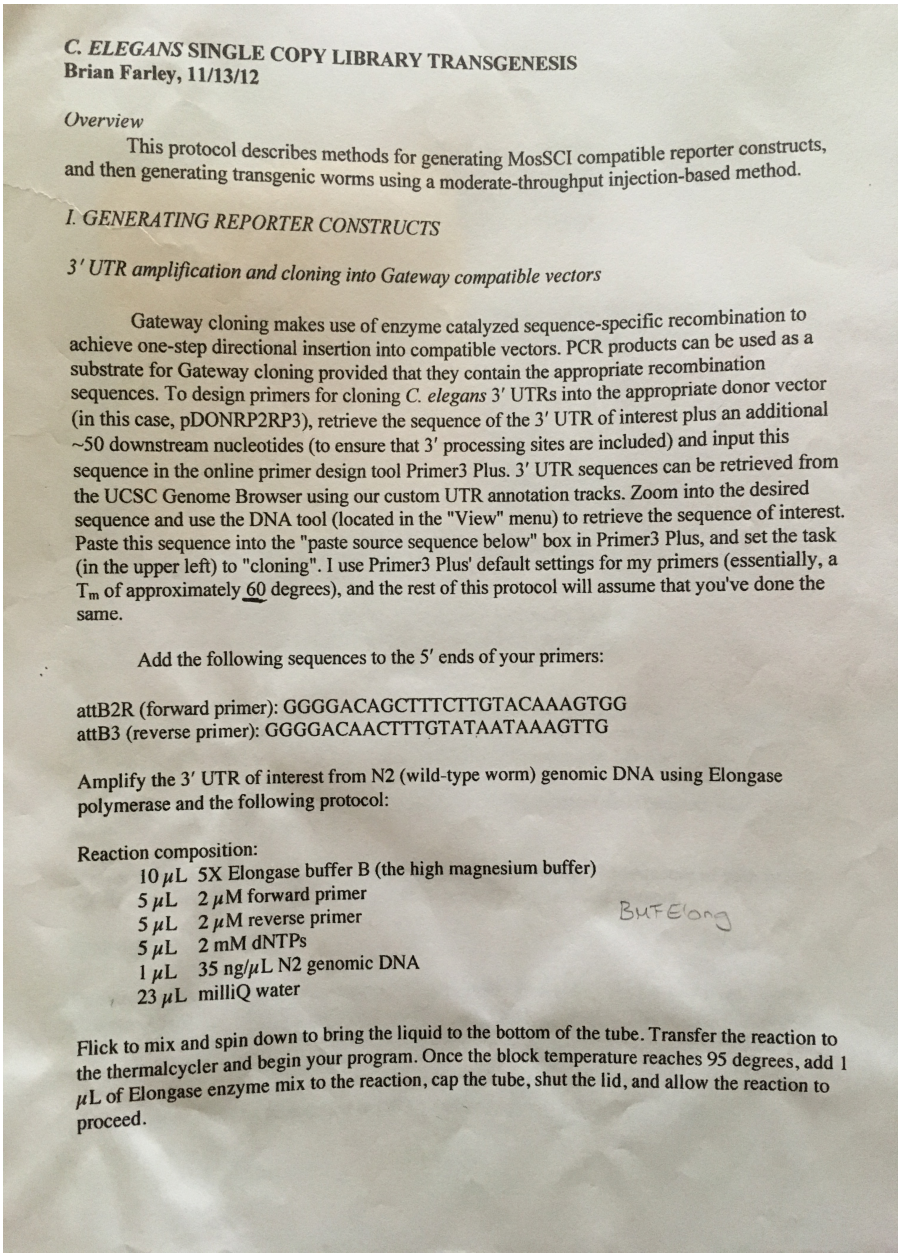

C. *ELEGANS* SINGLE COPY LIBRARY TRANSGENESIS
Brian Farley, 11/13/12

Overview

This protocol describes methods for generating MosSCI compatible reporter constructs, and then generating transgenic worms using a moderate-throughput injection-based method.

I. GENERATING REPORTER CONSTRUCTS

3′ UTR amplification and cloning into Gateway compatible vectors

Gateway cloning makes use of enzyme catalyzed sequence-specific recombination to achieve one-step directional insertion into compatible vectors. PCR products can be used as a substrate for Gateway cloning provided that they contain the appropriate recombination sequences. To design primers for cloning *C. elegans* 3′ UTRs into the appropriate donor vector (in this case, pDONRP2RP3), retrieve the sequence of the 3′ UTR of interest plus an additional ~50 downstream nucleotides (to ensure that 3′ processing sites are included) and input this sequence in the online primer design tool Primer3 Plus. 3′ UTR sequences can be retrieved from the UCSC Genome Browser using our custom UTR annotation tracks. Zoom into the desired sequence and use the DNA tool (located in the "View" menu) to retrieve the sequence of interest. Paste this sequence into the "paste source sequence below" box in Primer3 Plus, and set the task (in the upper left) to "cloning". I use Primer3 Plus' default settings for my primers (essentially, a T_m of approximately 60 degrees), and the rest of this protocol will assume that you've done the same.

Add the following sequences to the 5′ ends of your primers:

attB2R (forward primer): GGGGACAGCTTTCTTGTACAAAGTGG
attB3 (reverse primer): GGGGACAACTTTGTATAATAAAGTTG

Amplify the 3′ UTR of interest from N2 (wild-type worm) genomic DNA using Elongase polymerase and the following protocol:

Reaction composition:

10 μL	5X Elongase buffer B (the high magnesium buffer)
5 μL	2 μM forward primer
5 μL	2 μM reverse primer
5 μL	2 mM dNTPs
1 μL	35 ng/μL N2 genomic DNA
23 μL	milliQ water

BufElong

Flick to mix and spin down to bring the liquid to the bottom of the tube. Transfer the reaction to the thermalcycler and begin your program. Once the block temperature reaches 95 degrees, add 1 μL of Elongase enzyme mix to the reaction, cap the tube, shut the lid, and allow the reaction to proceed.

Appendix I: Gateway Cloning Protocol

Thermalcycler protocol:

Pre-amplification denaturation:		95 degrees, 2 minutes
Cycling	Denaturation:	95 degrees, 30 seconds
	Annealing:	55 degrees, 30 seconds
	Extension:	68 degrees, 2 minutes

Repeat the three cycling steps a total of 35 times.

Run 10 μL of the reaction on a 1X TAE, 1% agarose gel to confirm that it worked, and then use a Qiagen PCR clean-up kit and determine the DNA concentration using the Nanodrop to prepare the PCR product for the BP Gateway reaction.

BP reaction

Set up the following reaction:

- X μL PCR product (aim for ~100 ng; the Invitrogen protocol recommends 20-50 fmol of PCR product, and a 1 kb dsDNA product is ~0.65 ng/fmol)
- 1 μL 150 ng/μL pDONRP2RP3
- → 1X TE (10 mM Tris pH 8.0, 1 mM EDTA) to 8 μL
- 2 μL BP Clonase II

8 µL BP pDONR insert } X
8 - X
↓
TE

Incubate at room temperature (or 25 degrees, but this isn't really necessary) for one hour, and then add 1 μL of the Proteinase K supplied with the BP Clonase II. Incubate at 37 degrees for 15 minutes.

Transform 5 μL of this reaction into 100 μL of our homemade $RbCl_2$ competent DH5alpha using the standard transformation protocol (30 minutes on ice, 2 minute heatshock at 37 degrees, 1 hour outgrowth in LB at 37 degrees), and plate the entire reaction on LB-Kan plates.

NOTE: pDONRP2RP3 alone must be propagated in ccdB-resistant cells (such as DB3.1), and should be grown in LB+Kan+Chlor

The next day, pick a few colonies for mini-preps and sequencing. The sequence of your insert can be determined using M13 Forward and M13 Reverse sequencing primers.

LR Reaction

To assemble the full transgenic construct, I use a multi-site gateway reaction catalyzed by LR Clonase II Plus. For MosSCI insertions on chromosome two, use pCFJ150 as your destination vector. Destination vectors for other insertion sites can be found on the Jorgensen Lab MosSCI website and obtained from Addgene.

pCFJ150 alone must be propagated in DB3.1 cells and grown in LB+Amp+Chlor.

Each transgenic construct must contain three parts: a promoter (in pDONRP4P1R), an open reading frame (in pDONR221 or pDONR201) and a 3′ UTR (in pDONRP2RP3). All of my transgenic constructs use the *mex-5* promoter (pCM1.111), and the mouse ornithine decarboxylase::GFP::histone 2B (*his-58*) open reading frame. Alternate promoters can be obtained from the promoterome (which the Walhout lab owns), and alternate promoters and ORFs can be cloned into their respective donor vectors using a similar protocol to that described for UTRs (promoters must use attB4 (forward) and attB1R (reverse) sites, while open reading frames must use attB1 (forward) and attB2 (reverse) sites).

Once all of the desired parts are collected, assemble the following reaction:

20 fmol	pCFJ150 (about 110 ng) 100 ≤
10 fmol	pCM1.111 (about 25 ng) 30
10 fmol	pBMF2.7 (about 25 ng) 30
10 fmol	your 3′ UTR in pDONRP2RP3 (probably between 20-30 ng)
	1X TE to 8 μL
2 μL	LR Clonase II Plus

Incubate overnight (>16 hours) at room temperature or 25 degrees, and then add 1 μL of the Proteinase K provided with the enzyme. Incubate at 37 degrees for 15 minutes, and then transform 5 μL of the reaction into highly competent (~10^9 colony forming units/μg DNA) DH5alpha or equivalent (we've successfully used NEB 5-alpha before). → use 1B-AMP Plat

Insertion can be confirmed via PCR using the following primers (sequence listed 5′ to 3′):

BMF69 (in GFP): CCTTTCGAAAGATCCCAACG
BMF479 (in the "right" homology arm): acgcccaggagaacacgttag

Successful clones should give a single PCR product of approximately 1.2 kb (depending on the length of your 3′ UTR) using Taq.

To sequence putative inserts, use the following primers:

Forward: pCFJ150_unc119_SEQ_F TTCGCTGTCCTGTCACACTC
Reverse: M13 Forward (not a typo!): GTAAAACGACGGCCAGT

Quickchange (I refuse to spell this the way Stratagene does) mutagenesis

Quickchange reactions can be performed in either the entry vector (your sequence cloned into pDONRP2RP3) or the assembled transgene. Assembled transgenes are 9-10 kb, so it may be easier to perform quickchanges in the entry vector (only ~4 kb).

I use PrimerX to design Quickchange primers. The default settings will likely fail (because of how GC-poor 3′ UTRs tend to be), so you can make the following modifications: minimum GC% 20%; maximum total length 60 nt; maximum flanking lengths 30 nt; minimum T_m 70

degrees. Use the primer pair with the highest T_m. If these settings still fail to produce a useable primer pair, you can uncheck the "force terminal G or C" option, but only do this as a last resort.

Reaction composition:

25 ng	plasmid template
5 μL	2 μM forward primer
5 μL	2 μM reverse primer
5 μL	**1 mM dNTPs (THIS IS CRUCIAL)**
5 μL	10X Pfu buffer
	Water to 49 μL
1 μL	Pfu Turbo

Thermalcycler protocol

Preamplification denaturation		95 degrees, 30 seconds
Cycling	Denaturation	95 degrees, 30 seconds
	Annealing	55 degrees, 1 minute (primer T_m is irrelevant)
	Extension	68 degrees, 2 minutes/kb of plasmid

Repeat the cycling steps for a total of 20 cycles.

Add one μL of DpnI and incubate at 37 degrees for one hour. Transform 5 μL into our $RbCl_2$ DH5alpha cells using the standard heatshock + outgrowth protocol. Plate the entire reaction and confirm mutation by sequencing (I sequence at least three colonies per reaction).

Library assembly

To combine multiple reporter constructs for library transgenesis, mix an equal mass of each construct together (this assumes that each construct is similar in size; adjust accordingly if this isn't true) and determine the concentration of the pool using the Nanodrop. Treat this library as if it is a single plasmid when making injection master mixes.

II. GENERATING TRANSGENIC WORMS

Overview

MosSCI makes use of a strain carrying a single (well, technically two) copy of the Mos1 transposon in the genome. By injecting DNA encoding a transposase capable of excising that transposon, a genomic double stranded break is formed. Using a transgenic construct flanked by sequences homologous to that DSB, your transgene of interest will occasionally be inserted into the genome in a single copy at that defined location via DSB repair.

The initial injection strain is uncoordinated, and the transgenic construct contains a gene that rescues this phenotype located between the homologous repair regions (adjacent to your

transgene) of the vector backbone. Thus, rescue of the uncoordinated phenotype is a marker for the presence of your transgene.

C. elegans can also form extrachromosomal arrays from endogenously supplied DNA. These are undesirable for two reasons: 1) these arrays typically contain hundreds of copies of your gene of interest, and expression may be variable from worm to worm, and 2) extrachromosomal arrays are efficiently silenced in the germline. To select against extrachromosomal arrays, MosSCI makes use of two visual screening markers (that each express a fluorescent protein in somatic tissue and thus indicate the presence of extrachromosomal arrays) as well as a heat shock-inducible selection that selects against arrays.

Uncoordinated worms are injected, and their progeny are screened for wild-type rescue. Plates with rescued worms are then heat shocked to induce the negative selectable marker, and surviving wild-type worms are likely to have an integrated copy of your transgene.

Master mix assembly

I use the following plasmids at the following concentrations for MosSCI injections:

Name	Description	Final concentration
pCFJ601	*Peft-3::Mos1* transposase	50 ng/μL
pMA122	*Phsp::peel-1* (heat shock neg. selection)	10 ng/μL
pCFJ90	*Pmyo-2::mCherry* (pharynx marker)	2.5 ng/μL
pCFJ104	*Pmyo-3::mCherry* (body wall marker)	5 ng/μL
Targeting vector/Library of targeting vectors		25 ng/μL

I intentionally keep the total concentration of DNA low to prevent stable extrachromosomal transgenic array formation. Stable arrays will likely lead to transgene silencing (especially when using our constructs – essential proteins seem to avoid silencing if they're tagged on the C-terminus according to the Mello Lab) that can only be reversed with multiple crosses. Avoid silencing at all costs!!

Prior to mixing all of the plasmids in the injection mix together, spin them for ten minutes at max speed in a microcentrifuge. I usually make 20-50 μL of master mix at a time, which is enough for 12 – 30 needles.

Preparing worms for injection

The SINGLE MOST IMPORTANT criterion for a successful integration event is injecting worms of an appropriate age. The injection strain we use (EG6699) is uncoordinated and develops an increasingly severe egg-laying phenotype with age. Thus, it is critical that worms are injected during early adulthood. I typically try to inject worms that have about ten or so embryos in their uterus, as younger worms will likely not survive the injection and older worms generate far fewer rescued progeny. This is a very narrow window, so significant effort must be made to ensure worms of the correct age are available.

At least one week before you plan to inject, pick individual L4 injection strain (EG6699) worms to 35 mm plates seeded with Comamonas (DA1877). I pick ten, and keep 5 at 15 degrees and 5 at room temperature. This will generate semi-synchronous populations of worms that are ready to inject 6-7 and 3-4 days later, respectively. If you single worms out for multiple consecutive days, you ensure that worms will almost always be available for injection.

Alternatively, if you only have starved out plates, you can wash the larvae from those plates with water, spin the worms down in a microcentrifuge at 1100xg, remove most of the supernatant, and plate the remaining larvae. When I use this approach, I seed three 60 mm plates and distribute them between 15 degrees, room temperature, and 25 degrees. The former method is much more likely to give you a steady supply of injectable worms, but this method allows you to start large semi-synchronous cultures rapidly.

Preparing needles for injection

At least an hour before you want to inject (but preferably not more than four), start pulling and loading needles for injection. I use Kwik-Fil™ capillary tubes (World Precision Instruments, part number 1B120F-4), which contain an internal filament that speeds loading. Please wear gloves while handling these tubes, as particulate matter entering the tubes will likely cause clogged needles later on.

To pull needles for microinjection, use the Sutter P-97 micropipette puller in the lab. To start the instrument, turn it on using the power switch on the lower left side. You'll be asked for a program number to use; choose program 50, which has been optimized for worm needles.

For reference, these are the settings I use. Don't change them unless you absolutely have to, as reoptimizing puller settings will take the better part of an afternoon.

Program 50, for *C. elegans* microinjection needles
For a 2.5 mm box filament, using borosilicate glass

Pressure = 500
Heat = 575 (RAMP + 5)*
Pull = 45
Velocity = 80
Delay = 120

*The RAMP value is dependent on the filament and glass that you're using. If either of these ever change, you'll need to perform a ramp test by mounting a capillary tube and selecting RAMP from the special functions menu (accessible by pressing CLR, and then 0 – this step is important, as pressing 1 will delete your program, followed by 1).

To load a capillary tube for pulling, slide it into the groove on the pipette holder on your "dominant" side using your dominant hand. Gently slide the tube towards the filament, being careful not to make contact with the filament. I hold the capillary with both hands during this step. Slide the tube until the capillary is approximately 5 mm away from the groove in the pipette

holder. At this point, the tube should be in the filament chamber. Turn the clamp until it is finger tight; you can let go of the pipette at this step. Press the metal tab above the pipette holder to which you've clamped your tube, and slide the holder towards the filament chamber. Release the tab, and using your dominant thumb, hold the holder in place via the small handle near the clamp. With your off hand, press the metal tab above the other pipette holder, and slide it towards the filament using the index finger of your dominant hand. While holding both holders in place, tighten the clamp on the loose holder with your off hand.

Once the pipette is secured, press the green pull button. The filament will heat up, and your capillary will be pulled into two symmetric needles. After the pull is complete, the puller will display the amount of time that heat was applied during the pull. This should be between 9.5 and 11 seconds; chronic deviations from this number suggest that the filament will probably need to be replaced.

Remove the needles from the holders by loosening the clamps, and place them in the plastic pipette storage box. I pull three capillary tubes (for a total of six needles) for each injection mix I'm using; this dramatically increases your chances of having a good needle and some spares.

While pulling the needles, spin down your master mix at max speed in a microcentrifuge for 10 minutes. This prevents particulate matter from being loaded in your needles.

Once the needles are pulled and the master mix has been spun, you can load the needles. I backfill my needles using an aspirator and a hand drawn needle. To make a hand drawn needle, hold a capillary tube using both hands at both ends, and place the center in the flame of an ethanol burner. When the glass is fairly soft (you can easily deform it, but it isn't collapsing on itself) pull the tube up out of the flame and out using both hands. Snap the capillary in half and use one half to load your needles. Hand drawing needles takes some practice!

Mount your hand drawn needle in the white port of your aspirator and draw some of your master mix into the needle using suction. Insert the tip of the hand drawn needle into the back of an injection needle, at least a few millimeters deep. Expel a couple of microliters of injection mix into the injection needle, and then place the needle back in the pipette storage box. Tilt the tip slightly down, and when all of your needles are loaded, close the lid.

It will take between 15 minutes and an hour for the injection mix to completely flow to the tip of the needle. Monitor the needles' progress every fifteen minutes or so; if they aren't completely loaded within thirty minutes, seal the back of the needle by stretching some parafilm over the opening to prevent evaporation.

I've tried loading needles the day before I want to use them and storing them at 4 degrees overnight, but I think the vibration of the deli case compressor dulled the needles. This doesn't mean that pre-loading won't work, just that I've never gotten it to produce satisfactory needles.

If the needles look loaded, check the tips using the highest magnification on the dissection scope. If you see any air bubbles trapped in the narrowest parts of the needle, discard it. These bubbles

are unlikely to migrate out of the tip and will absorb most of the force used to expel liquid by the microinjector, which will completely prevent flow.

Mounting and breaking filled needles

Once a suitable needle is found, mount it in the microinjector. Remove the brass needle holder from the micromanipulator, and loosen (but don't remove!) the nut farthest away from the tubing. Remove the leftover needle that's likely in place and discard it, and insert your needle into the nut until you feel resistance. Hand-tighten the nut and re-mount the brass holder in the micro manipulator.

Turn the light on on the microinjector, and use the micromanipulator to position the tip of your needle in the path of the light. This is most easily done if you're looking directly at the needle. The top-most control will move the needle in the x-axis, the middle control the y-axis, and the bottom controls will both move the needle in the z-axis (the topmost of these two is the coarse control, while the bottommost is fine control).

Once the needle is in the light path, prepare an agar-pad bearing coverslip (I'll describe how to make these pads later in the protocol). Place the coverslip agar side up on a 35 mm plate lid under the dissection scope. Using your pick, place a drop of halocarbon oil on the coverslip but away from the pad. Place your oil-bearing coverslip on the injection scope stage with the oil side towards the needle. Position the short edge of the coverslip so that it is in the center of the field of view using the 5X objective. Focus on the edge of the coverslip, and then (using the micromanipulator) move the tip of the needle to the center of the field of view.

Switch to 20X and repeat, unfocus the objective, switch to 40X, and refocus (THIS IS REQUIRED WHENEVER YOU SWITCH TO OR FROM THE 40X OBJECTIVE!!!). Make the slight adjustments required to center the tip of the needle, and make sure that the edge of the coverslip is still visible in your field of view. Once the needle is centered, don't touch the x- or y-axis controls unless you have to. Using the glide stage, GENTLY touch the coverslip to the tip of the needle. If the coverslip is perpendicular to the needle, you'll likely produce a flat edge, while any other configuration of needle and coverslip will likely (but not always) produce a beveled tip. I prefer the flat edge, but you should figure out what works for you. The needle should bend slightly, and when you pull the needle away, the tip should be slightly broken back. Any contact between the needle and coverslip is sufficient to break the needle.

Using the micromanipulator, raise the needle up, and drop it in the halocarbon drop on your coverslip. This prevents your injection mix from drying at the tip of the needle and forming clogs. Set the regulator above the injection scope to ~30 psi and check for flow by using the foot pedal. You should see a steady, but slow flow from the needle.

If the needle is ready, remove it from the oil using the micromanipulator, and remove the coverslip from the stage.

Microinjecting worms

Transfer the coverslip from the injection scope stage and place it on the dissection scope (I use a 35 mm plate lid to lift the coverslip off of the dissection scope stage whenever I'm using that microscope). Using your pick, spread a thin, even layer of oil on the agar pad large enough to accommodate four or more worms. The agar will dry worms out VERY fast, and the oil slows this process down. Without flaming the oil off of the pick (a little oil on your pick is a great adhesive!), pick a single young adult worm (~5-10 embryos in the uterus; you'll need to use the highest magnification to find these), and transfer it to the thin layer of oil on the coverslip. Try to place the worm in a straight line at ~45 degree angle (with the worm pointing up and to the left). It's more important to avoid rolling the worm on the pad than it is to position it perfectly, so once any part of the worm is stuck to the pad, DON'T MOVE IT! Press very gently along the length of the worm using your pick to make sure it's stuck to the pad. You can transfer more than one worm to the pad if you're comfortable with injecting; if you choose to do so, make sure that you move them one at a time and that the worms have at least a worm-length between them.

Once your worms are mounted, transfer the coverslip to the injection scope, and find your worm(s) using the 20X objective. You want the vulva of each worm you're injecting to face away from the needle, so make a mental note of which worms are facing which way. Only inject worms with their vulva in this orientation; flip the coverslip around later to catch the rest.

Still at 20X, focus on the worm you'd like to inject and bring the needle down into the plane of focus using the micromanipulator. You're looking for a clear spot near the center of the worm; this is the gonad. Typically, only one will be visible, and the other will be obscured by the intestine. Using the glide stage, move this spot adjacent to, but not touching your needle and switch to 40X (make sure to defocus first!).

Focus on the rachis of the visible gonad arm. The correct focal plane is identifiable by the appearance of two parallel tracks of nuclei in the gonad. Once this is in focus, use the micromanipulator to bring just the tip of your needle into focus – most of the needle should be out of focus! Using the glide stage, move the worm into the needle until the cuticle and a small portion of the gonad is deformed. If your needle is exceptionally sharp, it may slide right into the worm, but this is a rarity! Tap GENTLY on the stage, and your needle should enter the gonad. If the needle is making contact with the intestine, center the needle tip in the rachis before injecting. Once the needle appears to be in the worm, press the foot pedal and check for flow. If you see liquid flowing in both directions away from your needle, and the clear spot of the gonad expands, congratulations! You just injected a worm! Your flow rate should be low enough so that it takes a second or two to completely fill the gonad; any faster than this and you are likely to irreparably injure the worm. The gonad is completely full once the flow makes it around the bend and to the oocytes. If you see individual nuclei moving away from your needle, you're probably in the wrong focal plane and have not placed the needle in the gonad, but are instead injecting into the body cavity. If multiple embryos exit through the vulva, STOP FILLING THE WORM, as the intestine will likely follow the embryos shortly.

Withdraw the needle from the worm, and press the foot pedal to clean the needle.

I only inject one gonad arm, as typically only one arm is visible. The second arm is much more challenging to find, and you'll likely over-fill the worm (leading to worm rupture and death) even if you find it.

Once all of the worms on the coverslip are injected, raise the needle using the micromanipulator and transfer the coverslip to the dissecting scope. Use your aspirator and a hand drawn needle to place a drop of M9 on top of the worms and your oil spot. The oil will float to the surface, and your worms will swim as well as they can (which isn't very well) if they're still alive. Using your aspirator, transfer each worm to an individual 35mm plate.

I usually try to inject between 20 and 40 worms (I average one integrant per 10 injections on a good day), and it takes about an hour or two.

Let the worms recover at room temperature for an hour, and then transfer the plates to the 25 degree incubator.

If your needle gets clogged (or your flow slows), raise the pressure at the regulator and try starting flow again. If this doesn't work, you can touch the needle to the edge of the coverslip again, making sure to place the needle back into the oil each time you want to check your flow. If this fails, you can apply pressure using the foot pedal while contacting the edge of the coverslip, but this will likely break your needle wide open, so only do it as a last resort. If none of these approaches produce a satisfactory needle, swap it out for one of your spares and start over.

Following injected worms

Allow the plates containing your injected worms to starve completely – this should take about seven days at 25. Any plates that aren't starved after seven days probably had an injected worm with a low (or no) brood size, and aren't likely to produce integrants. Screen the plates for worms that move wild-type; these are plates where you had a transgenic rescue and these are the most likely to produce integrants. At the low DNA concentrations I inject, I frequently get wild-type movement without seeing red fluorescence (the marker for extrachromosomal array formation and propagation), suggesting that my injection conditions don't form arrays efficiently, and thus likely escape germline transgene silencing.

Identify the plates with rescued progeny and mark them. I heat shock ALL of my plates, but I prioritize the rescued plates for downstream processing and only look at the plates without rescued progeny if my rescued plates give me no integrants.

Clean out the immersion heater water bath in the far fume hood in 970X1 if it needs it (and it probably will), and then turn it on and let it heat to 34 degrees. Put some aluminum blocks from one of dry heat baths into the bath too to bring them to 34. While this is happening, tightly wrap your plates in parafilm (they're going into the waterbath, so make the wraps as water-tight as

possible). I make equally sized stacks of plates (adding empties if necessary) to make it easier to weigh the plates down. Place your stacks of plates in the bath and put an aluminum block on top to keep them submerged. You may need a third hand to make this possible.

Heat shock the plates for at least an hour but no more than two, and then remove them from the water bath, remove the parafilm, and leave the plates at RT for at least four hours. One hour at 34 is sufficient to induce expression of the negative selection gene, but it takes four hours for the protein it encodes to kill worms.

After four hours have elapsed, cut each of the plates that had rescued worms in half using a sterile spatula. Transfer each half to a 60mm plate (make sure to keep the two plates with chunks from the same 35mm plate together!), oriented so that worms must crawl out of the chunk to find food. This will make it MUCH easier to find wild-type worms later.

Place the plates at 25, and the following day, screen for larval (L1, L2, or dauer) wild type worms. Most of the time, plates with successful integrations will have tens of wild type worms (suggesting an integration event in the injected worm producing an F1 integrant that gives rise to all of the F2s you see), but I'll occasionally get integrants from plates with only one or two larval, wild-type worms. Set aside your putative integrants; if you have plates with an "early" integration event, you can proceed to PCR screening for integration that day. Plates with fewer wild-type worms should be allowed to grow for a few more days.

From each pair of plates with integrants, single out five or so worms and check for homozygosity. Once you have your insert homozygosed, you can screen for GFP expression as well as confirm insertion via PCR.

PCR verification of insertion

I use single-worm PCR using a primer that anneals in GFP and another that anneals to a region of the genome downstream of the insertion site to confirm integration.

Primers:
BMF69 (forward): CCTTTCGAAAGATCCCAACG
BMF480 (reverse): atcgggaggcgaacctaactg

Place 5 µL of 30 mM Tris pH 8.8 + 1 mg/mL NEB Proteinase K in a lid of an overturned PCR tube (use strip caps) for each worm you'd like to use as a PCR template.

Pick one worm into each drop; place the caps on PCR tubes and spin down using the PCR tube rotor mini-fuge. Place the tubes on dry ice for 10 minutes (this plus the thawing helps lyse the worms). Transfer the tubes to the thermalcycler and run the protocol NUKEWORM (65 degrees, 1 hour; 95 degrees, 15 minutes. The first step promotes Proteinase K-catalyzed proteolysis, while the second inactivates Proteinase K).

While the worms are being nuked, prepare a master mix of the following PCR reaction:

5 µL 10X Pfu buffer
5 µL 2 µM BMF69
5 µL 2 µM BMF480
5 µL 2 mM dNTPs
24 µL H_20

Once the worm lysis is complete, add 44 µL of the master mix to each lysed worm, and place the tubes in the thermalcycler (at room temp). Begin the protocol listed below, and once the block temperature reaches 95 degrees, add 1 µL of Pfu Turbo to each reaction. Seal the tubes and close the lid.

Thermalcycler protocol		
Preamplification denaturation		95 degrees, 2 minutes
Cycling	Denaturation	95 degrees, 30 seconds
	Annealing	55 degrees, 30 seconds
	Extension	68 degrees, 4 minutes
Post amplification extension		68 degrees, 10 minutes

Repeat the cycling steps a total of 35 times.

Run the products on a 1X TAE, 1% agarose gel; successful integrations should give a single product of ~3 kb. Use the Qiagen PCR cleanup kit to prepare the PCR products for sequencing.

I have two forward sequencing primers (one in GFP and one in H2B), and one reverse sequencing primer (in the homology arm). The reverse primer sequences a lot of useless sequence in the arm, so I'm working on developing a better primer.

Primers:
BMF69 (forward): CCTTTCGAAAGATCCCAACG
LR_H2B_SEQ_F (forward): ACCGTGTCCTCAAGCAAGTT

BMF479 (reverse): acgcccaggagaacacgttag

~fin~

Appendix II: 12 Plasmid Mutants

The original DNA sequence:

>ce6_ct_UTRs_9640 range=chrIII:4922371-4922963 5'pad=0
3'pad=30 strand=- repeatMasking=none
TATGATTCGTCTAGTTTTTAAAATTTATATTCCATTCGATCTCTTCTAGTT
TTTTAATATTTTTTTATTTATTTAAATTTTCCTATCCAATTCCCAATTCGC
TGGTTCGAAGTTGGAAATTGCTATCTGATAAGAATAGGCCGGTTCCTACT
TTTTTTTTAAATATTGAAAACTGTATCATTGTTATTCATTCTCCTTATGG
TGAGCTGAGAAATCCTTGTATTATTAGTGGAATTCGTTATTGTTGAACAA
TTGTTTGAGTGTATGTTATACGATACAAGCTCCCCTTTTCAACTAGATAC
TTCTACATTTTATCAAATCTCTTGTTTCTCTATAGGTTTACGAATTGTTT
TCTTGGAGGATTTCCTCTACAGTCTCTTGTTTATCTCTCTATACTTGAAC
ATCTCTGAAATCATTCATTTTCCCTTGAACTCTGAACAATTGGTCTTTCC
AAAACAACCTCCCATTCAAGTCTTTATTCATTCGTGTTTAAAGTTAATTT
CAGCTAGTCTGAATAGTTTTATGTTTTAAAAAGTGTTTTATTATGTTCTT
TAAATGAAATAATTTTAAAAGTCATGGTTTTCGTATTTTAAAA

The Mutant DNA Sequences:

Mutated set-2 3UTR (Del: nt 1-50)
TTTTTTAATATTTTTTATTTATTTAAATTTTCCTATCCAATTCCCAATTCGCTGGTTCG
AAGTTGGAAATTGCTATCTGATAAGAATAGGCCGGTTCCTACTTTTTTTTTTAAATATTG
AAAACTGTATCATTGTTATTCATTCTCCTTATGGTGAGCTGAGAAATCCTTGTATTATT
AGTGGAATTCGTTATTGTTGAACAATTGTTTGAGTGTATGTTATACGATACAAGCTCCC
CTTTTCAACTAGATACTTCTACATTTTATCAAATCTCTTGTTTCTCTATAGGTTTACGA
ATTGTTTTCTTGGAGGATTTCCTCTACAGTCTCTTGTTTATCTCTCTATACTTGAACAT
CTCTGAAATCATTCATTTTCCCTTGAACTCTGAACAATTGGTCTTTCCAAAACAACCTC
CCATTCAAGTCTTTATTCATTCGTGTTTAAAGTTAATTTCAGCTAGTCTGAATAGTTTT
ATGTTTTAAAAAGTGTTTTATTATGTTCTTTAAATGAAATAATTTTAAAAGTCATGGTT
TTCGTATTTTAAA

Mutated set-2 3UTR (Del: nt 51-100)
TATGATTCGTCTAGTTTTTAAAATTTATATTCCATTCGATCTCTTCTAGGCTGGTTCGA
AGTTGGAAATTGCTATCTGATAAGAATAGGCCGGTTCCTACTTTTTTTTTTAAATATTGA
AAACTGTATCATTGTTATTCATTCTCCTTATGGTGAGCTGAGAAATCCTTGTATTATTA
GTGGAATTCGTTATTGTTGAACAATTGTTTGAGTGTATGTTATACGATACAAGCTCCCC
TTTTCAACTAGATACTTCTACATTTTATCAAATCTCTTGTTTCTCTATAGGTTTACGAA
TTGTTTTCTTGGAGGATTTCCTCTACAGTCTCTTGTTTATCTCTCTATACTTGAACATC
TCTGAAATCATTCATTTTCCCTTGAACTCTGAACAATTGGTCTTTCCAAAACAACCTCC
CATTCAAGTCTTTATTCATTCGTGTTTAAAGTTAATTTCAGCTAGTCTGAATAGTTTTA
TGTTTTAAAAAGTGTTTTATTATGTTCTTTAAATGAAATAATTTTAAAAGTCATGGTTT
TCGTATTTTAAA

Mutated set-2 3UTR (Del: nt 101-150)
TATGATTCGTCTAGTTTTTAAAATTTATATTCCATTCGATCTCTTCTAGTTTTTTAATA
TTTTTTATTTATTTAAATTTTCCTATCCAATTCCCAATTCCTTTTTTTTTTAAATATTGA
AAACTGTATCATTGTTATTCATTCTCCTTATGGTGAGCTGAGAAATCCTTGTATTATTA
GTGGAATTCGTTATTGTTGAACAATTGTTTGAGTGTATGTTATACGATACAAGCTCCCC
TTTTCAACTAGATACTTCTACATTTTATCAAATCTCTTGTTTCTCTATAGGTTTACGAA
TTGTTTTCTTGGAGGATTTCCTCTACAGTCTCTTGTTTATCTCTCTATACTTGAACATC
TCTGAAATCATTCATTTTCCCTTGAACTCTGAACAATTGGTCTTTCCAAAACAACCTCC
CATTCAAGTCTTTATTCATTCGTGTTTAAAGTTAATTTCAGCTAGTCTGAATAGTTTTA
TGTTTTAAAAAGTGTTTTATTATGTTCTTTAAATGAAATAATTTTAAAAGTCATGGTTT
TCGTATTTTAAA

Mutated set-2 3UTR (Del: nt 151-200)
TATGATTCGTCTAGTTTTTAAAATTTATATTCCATTCGATCTCTTCTAGTTTTTTAATA
TTTTTTATTTATTTAAATTTTCCTATCCAATTCCCAATTCGCTGGTTCGAAGTTGGAAA
TTGCTATCTGATAAGAATAGGCCGGTTCCTAGGTGAGCTGAGAAATCCTTGTATTATTA
GTGGAATTCGTTATTGTTGAACAATTGTTTGAGTGTATGTTATACGATACAAGCTCCCC
TTTTCAACTAGATACTTCTACATTTTATCAAATCTCTTGTTTCTCTATAGGTTTACGAA
TTGTTTTCTTGGAGGATTTCCTCTACAGTCTCTTGTTTATCTCTCTATACTTGAACATC
TCTGAAATCATTCATTTTCCCTTGAACTCTGAACAATTGGTCTTTCCAAAACAACCTCC
CATTCAAGTCTTTATTCATTCGTGTTTAAAGTTAATTTCAGCTAGTCTGAATAGTTTTA
TGTTTTAAAAAGTGTTTTATTATGTTCTTTAAATGAAATAATTTTAAAAGTCATGGTTT
TCGTATTTTAAA

Mutated set-2 3UTR (Del: nt 201-250)
TATGATTCGTCTAGTTTTTAAAATTTATATTCCATTCGATCTCTTCTAGTTTTTTAATA
TTTTTTATTTATTTAAATTTTCCTATCCAATTCCCAATTCGCTGGTTCGAAGTTGGAAA
TTGCTATCTGATAAGAATAGGCCGGTTCCTACTTTTTTTTTTAAATATTGAAAACTGTAT
CATTGTTATTCATTCTCCTTATAATTGTTTGAGTGTATGTTATACGATACAAGCTCCCC
TTTTCAACTAGATACTTCTACATTTTATCAAATCTCTTGTTTCTCTATAGGTTTACGAA
TTGTTTTCTTGGAGGATTTCCTCTACAGTCTCTTGTTTATCTCTCTATACTTGAACATC
TCTGAAATCATTCATTTTCCCTTGAACTCTGAACAATTGGTCTTTCCAAAACAACCTCC
CATTCAAGTCTTTATTCATTCGTGTTTAAAGTTAATTTCAGCTAGTCTGAATAGTTTTA
TGTTTTAAAAAGTGTTTTATTATGTTCTTTAAATGAAATAATTTTAAAAGTCATGGTTT
TCGTATTTTAAA

Mutated set-2 3UTR (Del: nt 251-300)
TATGATTCGTCTAGTTTTTAAAATTTATATTCCATTCGATCTCTTCTAGTTTTTTAATA
TTTTTTATTTATTTAAATTTTCCTATCCAATTCCCAATTCGCTGGTTCGAAGTTGGAAA
TTGCTATCTGATAAGAATAGGCCGGTTCCTACTTTTTTTTTTAAATATTGAAAACTGTAT
CATTGTTATTCATTCTCCTTATGGTGAGCTGAGAAATCCTTGTATTATTAGTGGAATTC
GTTATTGTTGAACACTTCTACATTTTATCAAATCTCTTGTTTCTCTATAGGTTTACGAA
TTGTTTTCTTGGAGGATTTCCTCTACAGTCTCTTGTTTATCTCTCTATACTTGAACATC
TCTGAAATCATTCATTTTCCCTTGAACTCTGAACAATTGGTCTTTCCAAAACAACCTCC

CATTCAAGTCTTTATTCATTCGTGTTTAAAGTTAATTTCAGCTAGTCTGAATAGTTTTA
TGTTTTAAAAAGTGTTTTATTATGTTCTTTAAATGAAATAATTTTAAAAGTCATGGTTT
TCGTATTTTAAA

Mutated set-2 3UTR (Del: nt 301-350)
TATGATTCGTCTAGTTTTTAAAATTTATATTCCATTCGATCTCTTCTAGTTTTTTAATA
TTTTTTATTTATTTAAATTTTCCTATCCAATTCCCAATTCGCTGGTTCGAAGTTGGAAA
TTGCTATCTGATAAGAATAGGCCGGTTCCTACTTTTTTTTTTAAATATTGAAAACTGTAT
CATTGTTATTCATTCTCCTTATGGTGAGCTGAGAAATCCTTGTATTATTAGTGGAATTC
GTTATTGTTGAACAATTGTTTGAGTGTATGTTATACGATACAAGCTCCCCTTTTCAACT
AGATTTTCTTGGAGGATTTCCTCTACAGTCTCTTGTTTATCTCTCTATACTTGAACATC
TCTGAAATCATTCATTTTCCCTTGAACTCTGAACAATTGGTCTTTCCAAAACAACCTCC
CATTCAAGTCTTTATTCATTCGTGTTTAAAGTTAATTTCAGCTAGTCTGAATAGTTTTA
TGTTTTAAAAAGTGTTTTATTATGTTCTTTAAATGAAATAATTTTAAAAGTCATGGTTT
TCGTATTTTAAA

Mutated set-2 3UTR (Del: nt 351-400)
TATGATTCGTCTAGTTTTTAAAATTTATATTCCATTCGATCTCTTCTAGTTTTTTAATA
TTTTTTATTTATTTAAATTTTCCTATCCAATTCCCAATTCGCTGGTTCGAAGTTGGAAA
TTGCTATCTGATAAGAATAGGCCGGTTCCTACTTTTTTTTTTAAATATTGAAAACTGTAT
CATTGTTATTCATTCTCCTTATGGTGAGCTGAGAAATCCTTGTATTATTAGTGGAATTC
GTTATTGTTGAACAATTGTTTGAGTGTATGTTATACGATACAAGCTCCCCTTTTCAACT
AGATACTTCTACATTTTATCAAATCTCTTGTTTCTCTATAGGTTTACGAATTGTACATC
TCTGAAATCATTCATTTTCCCTTGAACTCTGAACAATTGGTCTTTCCAAAACAACCTCC
CATTCAAGTCTTTATTCATTCGTGTTTAAAGTTAATTTCAGCTAGTCTGAATAGTTTTA
TGTTTTAAAAAGTGTTTTATTATGTTCTTTAAATGAAATAATTTTAAAAGTCATGGTTT
TCGTATTTTAAA

Mutated set-2 3UTR (Del: nt 401-450)
TATGATTCGTCTAGTTTTTAAAATTTATATTCCATTCGATCTCTTCTAGTTTTTTAATA
TTTTTTATTTATTTAAATTTTCCTATCCAATTCCCAATTCGCTGGTTCGAAGTTGGAAA
TTGCTATCTGATAAGAATAGGCCGGTTCCTACTTTTTTTTTTAAATATTGAAAACTGTAT
CATTGTTATTCATTCTCCTTATGGTGAGCTGAGAAATCCTTGTATTATTAGTGGAATTC
GTTATTGTTGAACAATTGTTTGAGTGTATGTTATACGATACAAGCTCCCCTTTTCAACT
AGATACTTCTACATTTTATCAAATCTCTTGTTTCTCTATAGGTTTACGAATTGTTTTCT
TGGAGGATTTCCTCTACAGTCTCTTGTTTATCTCTCTATACTTGACCAAAACAACCTCC
CATTCAAGTCTTTATTCATTCGTGTTTAAAGTTAATTTCAGCTAGTCTGAATAGTTTTA
TGTTTTAAAAAGTGTTTTATTATGTTCTTTAAATGAAATAATTTTAAAAGTCATGGTTT
TCGTATTTTAAA

Mutated set-2 3UTR (Del: nt 451-500)
TATGATTCGTCTAGTTTTTAAAATTTATATTCCATTCGATCTCTTCTAGTTTTTTAATA
TTTTTTATTTATTTAAATTTTCCTATCCAATTCCCAATTCGCTGGTTCGAAGTTGGAAA
TTGCTATCTGATAAGAATAGGCCGGTTCCTACTTTTTTTTTTAAATATTGAAAACTGTAT
CATTGTTATTCATTCTCCTTATGGTGAGCTGAGAAATCCTTGTATTATTAGTGGAATTC

GTTATTGTTGAACAATTGTTTGAGTGTATGTTATACGATACAAGCTCCCCTTTTCAACT
AGATACTTCTACATTTTATCAAATCTCTTGTTTCTCTATAGGTTTACGAATTGTTTTCT
TGGAGGATTTCCTCTACAGTCTCTTGTTTATCTCTCTATACTTGAACATCTCTGAAATC
ATTCATTTTCCCTTGAACTCTGAACAATTGGTCTTTTTCAGCTAGTCTGAATAGTTTTA
TGTTTTAAAAAGTGTTTTATTATGTTCTTTAAATGAAATAATTTTAAAAGTCATGGTTT
TCGTATTTTAAA

Mutated set-2 3UTR (Del: nt 501-550)
TATGATTCGTCTAGTTTTTAAAATTTATATTCCATTCGATCTCTTCTAGTTTTTTAATA
TTTTTTATTTATTTAAATTTTCCTATCCAATTCCCAATTCGCTGGTTCGAAGTTGGAAA
TTGCTATCTGATAAGAATAGGCCGGTTCCTACTTTTTTTTTTAAATATTGAAAACTGTAT
CATTGTTATTCATTCTCCTTATGGTGAGCTGAGAAATCCTTGTATTATTAGTGGAATTC
GTTATTGTTGAACAATTGTTTGAGTGTATGTTATACGATACAAGCTCCCCTTTTCAACT
AGATACTTCTACATTTTATCAAATCTCTTGTTTCTCTATAGGTTTACGAATTGTTTTCT
TGGAGGATTTCCTCTACAGTCTCTTGTTTATCTCTCTATACTTGAACATCTCTGAAATC
ATTCATTTTCCCTTGAACTCTGAACAATTGGTCTTTCCAAAACAACCTCCCATTCAAGT
CTTTATTCATTCGTGTTTAAAGTTAATTTTAAATGAAATAATTTTAAAAGTCATGGTTT
TCGTATTTTAAA

Mutated set-2 3UTR (Del: nt 551-594)
TATGATTCGTCTAGTTTTTAAAATTTATATTCCATTCGATCTCTTCTAGTTTTTTAATA
TTTTTTATTTATTTAAATTTTCCTATCCAATTCCCAATTCGCTGGTTCGAAGTTGGAAA
TTGCTATCTGATAAGAATAGGCCGGTTCCTACTTTTTTTTTTAAATATTGAAAACTGTAT
CATTGTTATTCATTCTCCTTATGGTGAGCTGAGAAATCCTTGTATTATTAGTGGAATTC
GTTATTGTTGAACAATTGTTTGAGTGTATGTTATACGATACAAGCTCCCCTTTTCAACT
AGATACTTCTACATTTTATCAAATCTCTTGTTTCTCTATAGGTTTACGAATTGTTTTCT
TGGAGGATTTCCTCTACAGTCTCTTGTTTATCTCTCTATACTTGAACATCTCTGAAATC
ATTCATTTTCCCTTGAACTCTGAACAATTGGTCTTTCCAAAACAACCTCCCATTCAAGT
CTTTATTCATTCGTGTTTAAAGTTAATTTCAGCTAGTCTGAATAGTTTTATGTTTTAAA
AAGTGTTTTATTATGTTC

Appendix III: Primers

5’**TAT**GAT**TCG**TCT**AGT**TTT**TAA**AAT**TTA**TAT**TC**/CATTCGATCTCTTCTAGTT
TTTTAATATTTTTTATTTATTTAAATTTTCCTATCCAATTCCCAATTCGC
TGGTTCGAAGTTGGAAATTGCTATCTGATAAGAATAGGCCGGTTCCTACT
TTTTTTTTAAATATTGAAAACTGTATCATTGTTATTCATTCTCCTTATGG
TGAGCTGAGAAATCCTTGTATTATTAGTGGAATTCGTTATTGTTGAACAA
TTGTTTGAGTGTATGTTATACGATACAAGCTCCCCTTTTCAACTAGATAC
TTCTACATTTTATCAAATCTCTTGTTTCTCTATAGGTTTACGAATTGTTT
TCTTGGAGGATTTCCTCTACAGTCTCTTGTTTATCTCTCTATACTTGAAC
ATCTCTGAAATCATTCATTTTCCCTTGAACTCTGAACAATTGGTCTTTCC
AAAACAACCTCCCATTCAAGTCTTTATTCATTCGTGTTTAAAGTTAATTT

CAGCTAGTCTGAATAGTTTTATGTTTTAAAAAGTGTTTTATTATGTTCTT
TAAATGAAATAATTTTAAAAGT/CATGGTTTTCGTATTTTAAAA 3’

Forward Primer:
5’GGGGACAGCTTTCTTGTACAAAGTGGGAATATAAATTTTAAAAACTAGACGAATCAT
A 3’

Reverse Primer:
5’GGGGACAACTTTGTATAATAAAGTTGTTTTAAAATACGAAAACCATG 3’

Mutated set-2 3UTR (Del: nt 1-50)
TTTTTTAATATTTTTTATTTATTTAAATTTT/CCTATCCAATTCCCAATTCGCTGGTTC
GAAGTTGGAAATTGCTATCTGATAAGAATAGGCCGGTTCCTACTTTTTTTTTTAAATATT
GAAAACTGTATCATTGTTATTCATTCTCCTTATGGTGAGCTGAGAAATCCTTGTATTAT
TAGTGGAATTCGTTATTGTTGAACAATTGTTTGAGTGTATGTTATACGATACAAGCTCC
CCTTTTCAACTAGATACTTCTACATTTTATCAAATCTCTTGTTTCTCTATAGGTTTACG
AATTGTTTTCTTGGAGGATTTCCTCTACAGTCTCTTGTTTATCTCTCTATACTTGAACA
TCTCTGAAATCATTCATTTTCCCTTGAACTCTGAACAATTGGTCTTTCCAAAACAACCT
CCCATTCAAGTCTTTATTCATTCGTGTTTAAAGTTAATTTCAGCTAGTCTGAATAGTTT
TATGTTTTAAAAAGTGTTTTATTATGTTCTTTAAATGAAATAATTTTAAAAGTCATGGT
TTTCGTATTTTAAA

Forward Primer:
5’GGGGACAGCTTTCTTGTACAAAGTGGAAAATTTAAATAAATAAAAAATATTAAAAAA
3’

Reverse Primer:
5’GGGGACAACTTTGTATAATAAAGTTGTTTTAAAATACGAAAACCATG 3’

Mutated set-2 3UTR (Del: nt 551-594)
TATGATTCGTCTAGTTTTTAAAATTTATATTCCATTCGATCTCTTCTAGTTTTTTAATA
TTTTTTATTTATTTAAATTTTCCTATCCAATTCCCAATTCGCTGGTTCGAAGTTGGAAA
TTGCTATCTGATAAGAATAGGCCGGTTCCTACTTTTTTTTTTAAATATTGAAAACTGTAT
CATTGTTATTCATTCTCCTTATGGTGAGCTGAGAAATCCTTGTATTATTAGTGGAATTC
GTTATTGTTGAACAATTGTTTGAGTGTATGTTATACGATACAAGCTCCCCTTTTCAACT
AGATACTTCTACATTTTATCAAATCTCTTGTTTCTCTATAGGTTTACGAATTGTTTTCT

TGGAGGATTTCCTCTACAGTCTCTTGTTTATCTCTCTATACTTGAACATCTCTGAAATC
ATTCATTTTCCCTTGAACTCTGAACAATTGGTCTTTCCAAAACAACCTCCCATTCAAGT
CTTTATTCATTCGTGTTTAAAGTTAATTTCAGCTAGTCTGAATAGTTTTAT/GTTTTAA
AAAGTGTTTTATTATGTTC

Forward Primer:
5’GGGGACAGCTTTCTTGTACAAAGTGGGAATATAAATTTTAAAAACTAGACGAATCAT
A 3’

Reverse Primer:
5’GGGGACAACTTTGTATAATAAAGTTGGAACATAATAAAACACTTTTTAAAAC
3’