THE APPLICATION OF RNA INTERFERENCE TO KNOCK OUT GROUCHO EXPRESSION IN *DROSOPHILA* S2 CELLS

By

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ABSTRACT

The development of a complex nervous system requires the actions of intricate genetic mechanisms that influence and maintain the differentiation of common nervous system progenitor cells into neurons and glial cells. In *Drosophila melanogaster*, the gene *glial cells missing* (*gcm*) encodes the transcription factor Gcm that, when active, causes early nervous system cells to preferentially differentiate into glial cells by activating the *reversed polarity* (*repo*) gene, as well as others. However, the Gcm protein also plays a role in the differentiation of both macrophages and tendon cells. This suggests that there are other transcription factors or cofactors that interact with Gcm, leading to its different functions under different contexts. The possible role of Groucho as a collaborator with Gcm can be examined by performing RNA interference in Drosophila S2 cells to remove the Groucho protein, which was attempted in this experiment. While the results were inconclusive, some important ideas for the reproduction of the experiment were obtained.

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LIST OF ABBREVIATIONS

cDNA	complementary DNA
CNS	central nervous system
DEPC	diethylpyrocarbonate
dsRNA	double-stranded RNA
FBS	fetal bovine serum
GB	glioblast
gcm	*glial cells missing* gene
Gcm	glial cells missing protein
GMC	ganglion mother cell
gro	*groucho* gene
Gro	groucho protein
mRNA	messenger RNA
NB	neuroblasts
NGB	neuroglioblast
NGS	normal goat serum
PBS	phosphate-buffered saling
PCR	polymerase chain reaction
Pen/Strep	penicillin/streptomycin
PNS	peripheral nervous system
PNT	PBS/NGS/Triton
pntp1	*pointed p1* gene
Pntp 1	pointed p1 protein
repo	*reversed polarity* gene
Repo	reversed polarity protein

ttk69	*tramtrack isoform 69* gene
RNAi	RNA interference
siRNA	small interfering RNA
ssRNA	single-stranded RNA
TTK69	tramtrack isoform 69 protein

CHAPTER 1

BACKGROUND AND INTRODUCTION

1.1: Drosophila Nervous System Development and Genetic Mechanisms

Complex nervous systems include two types of cells: neurons and glia. Neurons transmit information, while glial cells exist to support and maintain neurons and their environment. In *Drosophila*, most glial cells originate from neural stem cells in the ventral neurogenic ectoderm and peripheral ectoderm on either side of the midline; midline glia, whose development and specifications diverge from most glia, are the exception (Lee and Jones, 2005; Jones, 2001). Therefore, the rest of the information presented in this thesis regards the lateral glia of the peripheral nervous system (PNS) and central nervous system (CNS). In the PNS, neural progenitors known as sensory organ precursors delaminate, or migrate, from the ectoderm. They then undergo a series of cell divisions to ultimately form neurons, glia, and other support cells (Jones, 2004). In the CNS, there are three types of neural progenitors. Neuroblasts (NBs) give rise only to neurons. Glioblasts (GBs) will give rise only to glia. Lastly, neuroglioblasts (NGBs) give rise to mixed glial/neuronal lineages and can be subdivided into two types. Type 1 NGBs produce both a GB and a NB after one asymmetric division. Type 2 NGBs, on the other hand, give rise to a series of ganglion mother cells (GMCs) that subsequently divide to give rise to either two sibling neurons or a neuron/glia pair (Jones, 2004).

In the nervous system of *Drosophila*, one gene, *glial cells missing* (*gcm*), is primarily responsible for the determination of neuroprogenitor cells into glial cells. In *gcm* loss-of-function Drosophila embryos, nearly all glial cells are lacking, and most presumptive glial cells mature as neurons instead. Alternatively, in *gcm* gain-of-function embryos, ectopic expression of Gcm causes presumptive neurons to differentiate into glial cells instead (Jones, 2004). This shows that *gcm* is both necessary and sufficient for glial cell development. *gcm* encodes a transcription factor, Gcm, that is transiently expressed in all prospective glia, aside from midline glia. Three genes that have been identified as targets of Gcm are the transcription factors *reversed polarity* (*repo*), *pointed p1* (*pntp1*), and *tramtrack isoform 69* (*ttk69*). Repo and Pntp1 act together to regulate expression of the *loco* gene, whose encoded protein is necessary for the morphogenesis of glial cells (Lee and Jones, 2005; Jones, 2004). TTK69, a repressor, acts to suppress neuronal development (Jones, 2004). Thus, it seems that expression of Gcm initiates glial cell formation both by promoting glial cell differentiation through transcription factors Repo and Pointed, while also suppressing neuronal development through the activation of repressor TTK69. While *repo* is expressed exclusively in all lateral glial cells (Jones, 2004), *gcm* also plays a role in the development of tendon cells and immature hemocytes into phagocytic macrophages (Johnson et al., 2012; Jones, 2004). This suggests that there are different cofactors interacting with Gcm that lead to different affects and controls under different developmental contexts.

Potential Gcm cofactors were addressed by in a previous dissertation presented at the University of Mississippi (Nipper, 2014). Nipper performed a double interaction screen, a variation of a one-hybrid yeast screen for identifying interactions

between protein and DNA. A one-hybrid yeast screen is performed using tandem repeats of target or "bait" DNA that are cloned into a pAbAi vector that is then integrated into the yeast genome upstream of a reporter gene through homologous recombination. The pAbAi vector carries the reporter gene *Aureobasidin A resistance*, which confers the ability to grow in the presence of the Aureobasidin A antibiotic (Nipper, 2014). When a fusion protein binds to the "bait" sequence, it causes expression of the *AbAi* reporter gene, providing the ability to grow on selective media containing Aureobasidin A (Nipper, 2014).

A yeast expression vector carrying the *gcm* gene was introduced by transforming it into the Y1HGold/AbAi yeast reporter strain, creating a second dimension in the screen (Nipper, 2014). Now, proteins that interact with Gcm as co-factors or other binding partners should interact with the reporter system, in addition to those capable of binding the "bait" DNA. These double interaction screens were performed simultaneously with an original one-yeast hybrid screen. One of these screens produced yeast colonies in the presence of Aureobasidin A that contained both *gcm* and *groucho* cDNA. Although the *groucho* fusion protein induced resistance on its own, suggesting that it may interact with endogenous factors, the resistance was notably increased when both *gcm* and *groucho* were present, suggesting a synergistic interaction between *gcm* and *groucho* in the yeast cells (Nipper, 2014).

The Groucho family of proteins consists of corepressors characterized by a conserved N-terminal glutamine-rich region (Q domain), which has been implicated in several protein-protein interactions and is, most importantly, required for the homotetramerization of Groucho (Song et al., 2004). They also possess a highly

conserved C-terminal WD-repeat domain necessary in the binding of many proteins involved in repression (Song et al., 2004). Gro, the *Drosophila* member of the Groucho family, is present almost ubiquitously within cells and is required for nearly every aspect of embryonic development, including sex determination, neurogenesis, segmentation, dorsoventral patterning, terminal patterning, eye development, and wing development (Song et al., 2004). Gro, like other members of the Groucho family, is a corepressor, and most evidence thus far suggests that Gro mediates long-range repression (Song et al., 2004). This means that the corepressor is able to silence a particular locus regardless of where the corepressor is positioned relative to the activators or the core promoter, contrasting some other corepressors that can only repress transcription when bound within 100 base pairs of activators or the core promoter.

1.2: RNA Interference in *Drosophila* S2 Cells

Theoretically, this possible interaction can be examined by performing RNA interference (RNAi) to knock out the presence of Gro in *Drosophila* S2 cells in hopes of measuring any potential effects on Repo reporter expression. RNAi, sometimes referred to as RNA silencing, is a naturally occurring mechanism thought to be used for protection against viruses and transposons (Agrawal et al., 2003), but in recent years, this mechanism has been applied in laboratory settings in order to create a loss-of-function phenotype in multiple organisms, including *Drosophila* flies. RNAi has been shown to be a two-step process. The first step occurs when an RNA nuclease binds to the large double-stranded RNA and cleaves it into smaller RNA fragments

called small interference RNAs (siRNAs) that range from twenty-one to twenty-five nucleotides in size (Agrawal et al., 2003). RNase III- type endonucleases are responsible for this degradation into siRNAs and do so by creating staggered cuts along both strands of the dsRNA, leaving a 3' overhang of two nucleotides (Agrawal et al., 2003). An RNase III discovered in *Drosophila* termed Dicer is thought to be involved in this initiation step of RNAi (Fig. 1). Dicer has four domains: an amino-terminal helicase domain, two RNase III motifs, a dsRNA binding domain, and a PAZ domain (Agrawal et al., 2003). This enzyme is also found to have an ATP-binding motif, suggesting that the process of RNAi requires the expense of energy. The second step of RNAi occurs when the siRNAs join with the multinuclease RNA-induced silencing complex (RISC), which then degrades the homologous single-stranded mRNAs (Fig. 1). This complex is believed to activate in the presence of ATP, exposing the antisense portions of the siRNAs. The antisense siRNAs then bind with the homologous mRNAs, and the activated complex cleaves the mRNA in approximately the middle of the duplex region (Agrawal et al., 2003).

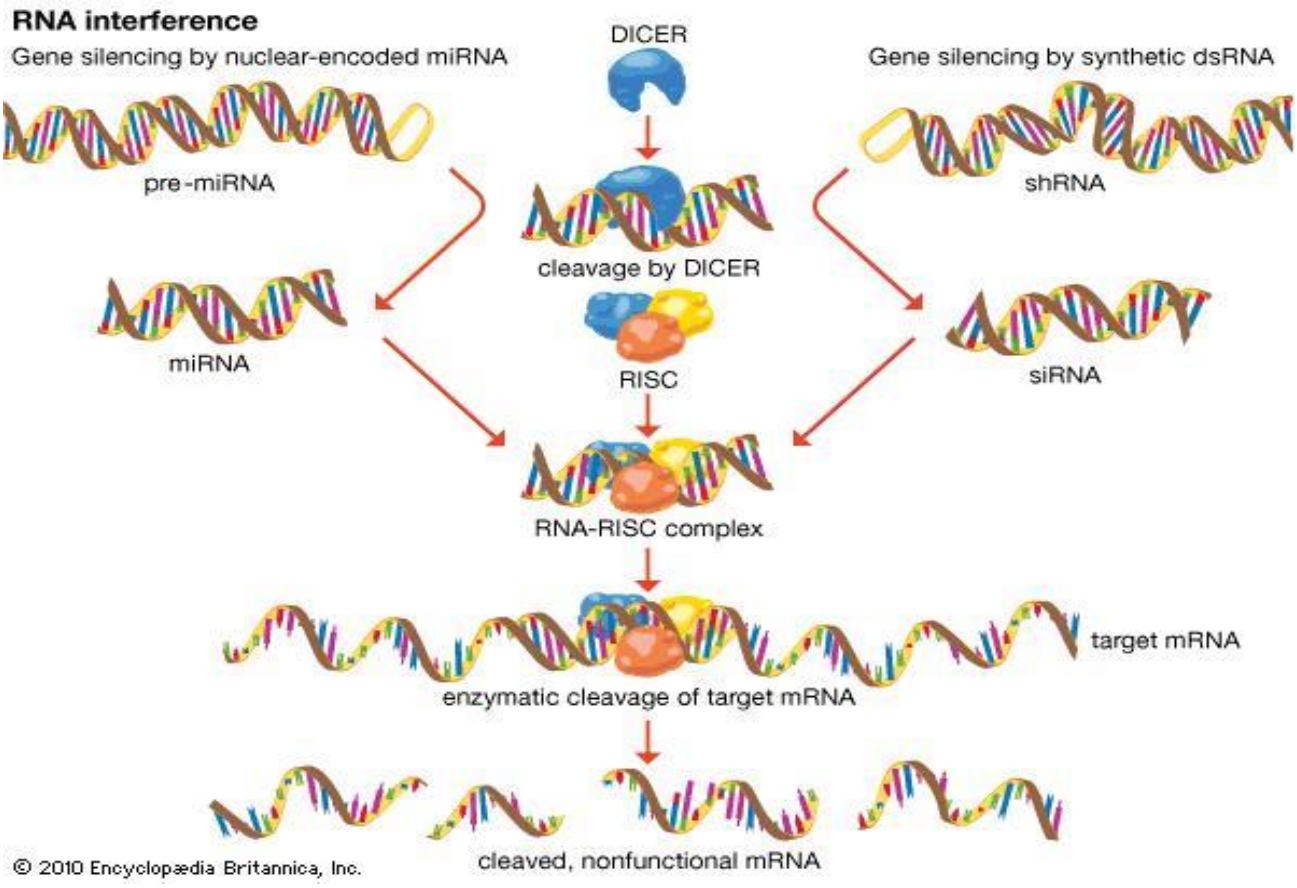

Figure 1. RNAi process. RNAi can occur using endogenous dsRNA (left side of figure) or synthetic dsRNA (right side of figure). Dicer cleaves the synthetic dsRNA into small siRNAs. These siRNAs join with the RISC complex, which then acts to cleave the endogenous mRNA at the points complementary to the siRNAs.

RNAi is commonly used to knock out protein expression in *Drosophila* S2 cells, a cell line isolated from late-stage (20-24 hours old) *Drosophila* embryos by Imogene Schneider in 1972 that is easily maintained as a semi-adherent monolayer at room temperature without CO_2 (Yang and Reth, 2012). In order to use RNAi to create a *groucho* loss-of-function phenotype in *Drosophila* S2 cells, one must design and synthesize an appropriate dsRNA molecule to knock out the gene of interest. The first step in this process is the preparation of a PCR template. Generally, the primer

begins with the T7 RNA polymerase sequence (5' – TAATACGACTCACTATAGG – 3'), which is followed by a sequence specific to the gene of interest (Rogers and Rogers, 2008). The PCR template should be of a length between 300 and 1000 base pairs and have a melting point that is between 52 and 58 degrees Celsius (Rogers and Rogers, 2008). It is very important to make sure the sequence chosen is as specific as possible for the gene of interest, in order to prevent unintentional mRNA degradation of other proteins. If possible, cDNA corresponding to the *groucho* sequence should be used for PCR amplification (Worby et al., 2001). Once the PCR product is obtained and isolated, in vitro transcription reactions are performed in order to obtain the dsRNA (Rogers and Rogers, 2008).

The medium is then removed from the S2 cells and replaced with new medium containing diluted dsRNA, a method known as soaking, in order to degrade native *gro* RNA within the cells (Rogers and Rogers, 2008). The S2 cells are derived from a macrophage-like lineage, meaning they are phagocytic in nature. The loss of the protein may then be directly measured using a Western blot analysis, which requires an antibody specific for the protein or by staining the cells to observe loss of protein (Worby et al., 2001). Once a loss-of-function phenotype is obtained, its effect on the expression of *Repo* reporters can be analyzed. The following experiment attempts the performance of RNAi of Gro in *Drosophila* S2 cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 Selection of Primers for Polymerase Chain Reaction

The primers and desired DNA sequence were selected using the gro-RJ cDNA within a pUAST plasmid by trying to find sections that were specific to *gro* and contained an approximately 50% G/C content. The forward primer chosen was located between base pairs 1915 – 1934 on the cDNA, and the reverse primer chosen was located between base pairs 2639 – 2658 of the cDNA. A basic representation can be seen in Fig. 2. The length of the entire selected DNA sequence was thus 744 base pairs of a single exon. The specificity of the entire sequence was tested by running a BLAST search of the sequence on FlyBase to compare its similarity to other gene sequences. The primers were ordered from Integrated DNA Technologies with the T7 promoter sequence (5' TAATACGACTCACTATAG 3') attached to each of them.

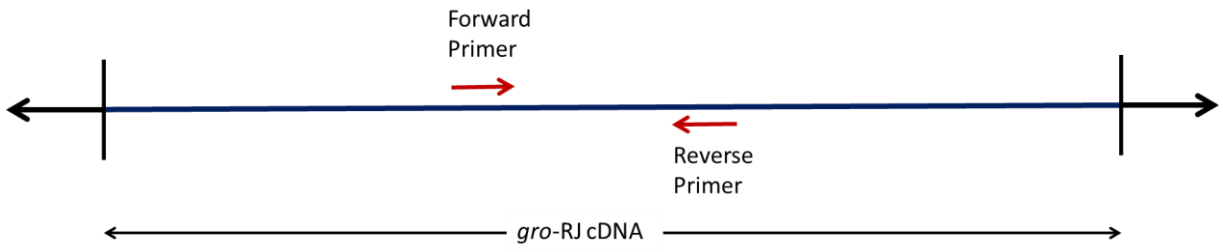

Figure 2. Primers within plasmid. The forward and reverse primers are chosen from approximately the middle of the gro-RJ Cdna, which is within the pUAST plasmid. The length of the chosen sequence is 744 base pairs, and begins at location 1915 and ends at position 2658 of the cDNA.

2.2 Preparation of Template DNA

The pUAST – *gro* plasmid was transformed into E. coli and grown on a streak plate. A culture was selected, and the DNA was isolated using a standard mini-prep and digested with the restriction enzymes Xho1 and EcoR1 to ensure the DNA obtained from the culture was the *gro* cDNA. After a positive result, the pUAST *gro* DNA was digested with Xba1 and diluted 1:100 in DEPC treated water to be used in the polymerase chain reaction.

2.3 Performance of Polymerase Chain Reaction (PCR)

A PCR reaction was run with a total volume of 100 µL by combining the following in a PCR tube: 10 µL of forward primer (10 µM), 10 µL reverse primer (µM), 10 µL 10x PCR buffer, 10 µL dNTPs (2mM), 4 µL DNA template (diluted 1:100 in DEPC treated water), 2 µL AmpliTaq polymerase, and 54 µL of DEPC treated water. Once the reactants were combined, they were run using the 3-Step protocol in the MJ PTC-100 thermal cycler.

1. 94 ºC 2:00 min.
2. 94 ºC 0:30 min.
3. 60 ºC 0:30 min.
4. 72 ºC 1:30 min.
5. Repeat to step 2 – 29 times (is this correct? Is it possibly repeat step 2-4 rather 1&1 as this wording implies)
6. 72 ºC 7:00 min.
7. 4 ºC Hold

A 1 µL sample of the PCR product was then run on a 0.8% agarose gel to confirm that the reaction had given the correctly sized product.

The PCR product was subjected to a phenol:chloroform extraction in order to purify the product. An equal volume (100 µL) of phenol:chloroform was added to the PCR product. The two were mixed by vortexing and then centrifuged at 12000 rpm for 15 min. at 4 ºC. The supernatant (aqueous portion) was transferred to a new 1.5 mL Eppendorf tube.

The PCR product was then precipitated according to Green's and Sambrook's manual *Molecular Cloning*. First, 1/10 volume (10 µL) of 3 M sodium acetate (pH 5.2) and 2 volumes (200 µL) of 100% ethanol were added to the PCR product, and this mixture was incubated at -20 ºC for 5 minutes. The mixture was then centrifuged at 12000 rpm for 10 minutes at 4 ºC before the supernatant was removed. The pellet was rinsed with 100 µL of 70% ethanol and centrifuged at the same speed and temperature for another 2 minutes. The ethanol was removed, and the pellet was dried using a speed vacuum. The pellet was resuspended in 50 µL of nuclease-free water, and the concentration of the PCR product DNA was then measured using a spectrophotometer.

2.4 Transcription and Annealing of Double-Stranded RNA

The transcription reaction was performed according to the Thermo Scientific TranscriptAid T7 High Yield Transcription Kit. The following were combined at room temperature in the order given to a total volume of 20 µL: 3.3 µL DEPC treated water, 4 µL 5x TranscriptAid reaction buffer, 8 µL rNTPs mix, 2.7 µL DNA template (~ 1 µg), and 2 µL TranscriptAid enzyme mix. The contents were mixed thoroughly by vortexing and centrifuged briefly, then incubated at 37 ºC for 2 hours. 2 µL of RNase-free DNase were added to the reaction mix, and the mixture was incubated at 37 ºC for an additional 30 minutes in order to remove the template DNA.

Before precipitation, the RNA transcript was subjected to a phenol:chloroform extraction, as described in the previous section. In order to precipitate the RNA transcript, the following steps were performed similar to the precipitation of the PCR DNA, but with slight alterations. 1/10 the volume (2.2 µL) of 3 M sodium acetate (pH 5.2) and 2.5 volumes (55 µL) of 100% ethanol were added to the transcription product. The solution was mixed by vortexing and then incubated at -20 ºC for 30 minutes. The precipitated RNA was collected by centrifuging at 12000 rpm for 30 minutes at 4 ºC. The supernatant was removed, and the pellet was washed with 1 mL of 70% ethanol to remove residual salt and centrifuged at 12000 rpm for 5 minutes. As much of the 70% ethanol as possible was removed, and the pellet was dried using a speed vacuum. The RNA pellet was then dissolved in 50 µL of DEPC treated water.

1 µL of the resuspended RNA transcript was then run on a 1% agarose gel, along with a 2 µL sample of the RiboRuler high range ready-to-use RNA ladder provided in the kit, and another 1 µL was run with a 1 kb DNA ladder. The concentration of the RNA transcript was measured using a spectrophotometer. A test annealing reaction was then performed by combining 0.5 µL of the RNA transcript and 1 µL of 10x annealing buffer (1 M potassium acetate, 300 mM HEPES – potassium hydroxide, 20 mM magnesium acetate, and DEPC treated water to 100 mL). This product was run on a gel to ensure the annealing reaction was successful. A large-scale reaction was then performed to produce a dsRNA solution at 0.5 µM by combining 100 pmol (6.3 µL) of the ssRNAs, 10 µL of 10x annealing buffer, and 83.7 µL of DEPC water. The dsRNA was precipitated as described in section 2.3, and resuspended in 37.8 µL of DEPC water to yield a dsRNA solution at 1 µg/ µL.

2.5 Maintenance of *Drosophila* S2 Cell Cultures

In order to begin the cell cultures, 5 mL of S2 complete media (450 mL Schneider's S2 media combined with 50 mL of heat-inactivated FBS and 5 mL of penicillin/streptomycin) were added to a 15 mL conical tube. A 1 mL aliquot of liquid nitrogen frozen cells from a previously maintained culture was thawed and added to the conical tube. The cells were then centrifuged at 1800 rpm for 5 minutes. As much media as possible was removed from the pellet, and 10 mL of fresh S2 complete media was added. The cells were resuspended by pipetting, transferred to a T-25 tissue culture flask, and incubated at 25 ºC for 48 hours.

For regular cell maintenance, the flasks were removed from the incubator every 48 hours, and the cells were washed off the bottom of the flask by pipetting the cell solution up and down several time while holding the flask at an approximately 45-degree angle. The cells were then transferred to a 15 mL conical tube and centrifuged at 1200 rpm for 5 minutes. The supernatant of old media was removed, with approximately 2 mL of the old media left in the tube. A volume of 8 mL of fresh S2 complete media was then added to bring the volume to 10 mL, and the cells were resuspended by pipetting. A 5 mL sample of this cell solution were then added to 10 mL of S2 complete media in a T-75 flask and incubated at 25 ºC for another 48 hours.

After two transfers, the cell culture was split to form a backup culture in case one of the cultures became contaminated. Both cultures were maintained as described above simultaneously. All work done with the S2 cells was done in a sterile tissue culture hood,

while wearing gloves and a lab coat. The hood was wiped down with 70% ethanol before and after each use.

2.6 Performance of RNAi on S2 Cells and Staining of Cells

The S2 cells were counted using a hemocytometer by combining 10 µL of resuspended cells, 490 µL of 1X PBS, and 500 µL of 0.4% Trypan blue in a 1.5 mL Eppendorf tube and adding 10 µL of this mixture onto the hemocytometer. Trypan blue stains only dead cells, so these cells were not counted. The number of cells counted in the four quadrants was then divided by 4, multiplied by 2 (Trypan dilution), multiplied by 50 (original dilution), and multiplied by 10,000 (hemocytometer dilution). This yielded a cell count of 22.5×10^6 cells/mL, which was then diluted to 1×10^6 cells/mL with Schneider's *Drosophila* medium without FBS or Pen/Step.

RNAi was then performed on the cells using a soaking technique. A 400 µL sample of the diluted cells were added to each well in a 12-well cell culture plate and incubated at 25 °C for 24 hours. After 24 hours, 6 µL of the 1 µg/ µL dsRNA solution were added to six of the wells and mixed by gently moving the plate back and forth in straight lines. Nuclease-free water was added to the other six wells as a control. The cells were then incubated for 30 min. at 25 °C. Next, 800 µL of Schneider's *Drosophila* medium with FBS and pen/strep were added to each well, and the cultures were incubated for 2 days at 25 °C.

The cells were stained on a 12-well slide that had been treated with a 1 mg/mL solution of Poly-L-lysine, meant to help the cells adhere to the slide. The slide was placed in a vertical Coplin jar and completely covered with Poly-L-lysine for 10 minutes.

The slide was then washed with distilled water several times over the course of 10 min and dried in a glass slide rack. After 48 hours, the cells were removed from the incubator and resuspended by gently pipetting them up and down. A 75 µL sample of the cells from each well of the tissue culture dish was added to the complementary well on the slide, with wells 1-6 being RNAi positive and 7-12 being RNAi negative. The cells were allowed to settle for 45 minutes in a humid chamber at room temp before being gently tipped to remove excess media. While the slide was held flat, 2 mL of 3.7% formaldehyde fix were added to the top of the slide and allowed to flow into the wells. The slide was incubated in the humid chamber for another 15 minutes before excess solution was gently poured off. The slide was then washed three times with 1X PBS. While the slide was held flat, 2 mL of 1x PBS were pipetted at the top of the slide, and the excess was gently poured off. This was repeated twice. The slide was then carefully dried between the wells with a Qtip, making sure never to allow the Qtip to touch inside the wells.

The primary antibody used was anti-Gro-s and was first diluted 1:5 in PNT (0.1% Triton, 1% NGS, and 1X PBS). A 15 µL sample of the primary antibody was added to each well, and the cells were incubated in the humid chamber for one hour. The slide was then washed with PBS and dried with the Qtip as previously described. A 15 µL sample of the secondary antibody (CY3-goat anti-mouse), diluted 1:1000 in PNT, was added to each of the wells on the slide, and this was also incubated for 1 hour in the humid chamber at room temp. The slide was then washed and dried, as previously described. Several small drops of Vectamount + DAPI were added to slide between the

wells, and two half coverslips were gently placed on the slide, causing the Vectamount to spread into the wells. The slide was then examined under a fluorescent scope.

CHAPTER 3

RESULTS

The primers selected for PCR had an approximately 50% G/C content, and the entire sequence to be transcribed into RNA was 744 bp. The entire sequence of the primers as ordered can be seen in figure 3.

Forward Primer	5′ GGATCCTAATACGACTCACTATAGGCAGTTAGCCAGCTGGATTGT 3′
Reverse Primer	5′ GGATCCTAATACGACTCACTATAGGCTTCGTAGACAGTAGCCTTC 3′

Figure 3. Sequences of primers. The above sequences represent the primers used in a polymerase chain reaction to amplify a 744 bp sequence of a groucho exon. The blue portion (3'end) is the actual primer selected from the gro-RJ cDNA. The red (5'end) represents the T7 promoter sequence added to the primers.

When run on a gel, the PCR product (Fig. 4A) revealed a single band that is approximately the size of the target DNA sequence (744 bp) as it is located between the 500 bp ladder fragment and 1000 bp fragment, but closer to the 1000 bp. The concentration of the DNA obtained from the PCR was found to be 0.3687 µg/µL. The product of the transcription reaction was found to have a concentration of approximately 6 µg/µL and was run with both a DNA ladder (Fig. 4B) and an RNA ladder (Fig. 4C). The RNA ladder consists of single-stranded (ss) molecules, while the DNA ladder consists of double-stranded (ds) molecules. ssRNAs should move through the gel faster than dsRNAs. Thus, the shift seen in the transcript products relative to the ladder

indicates that the bottom band (white star) is most likely the ssRNAs formed in the transcription reaction, and the band above it is dsRNA that annealed on its own during the reaction. The second to last RNA band (black star) seen with the DNA ladder (Fig. 4B) is in approximately the same position relative to the ladder as the double-stranded DNA (Fig. 4A). Because residual DNA should have been degraded by the DNase reaction described previously, this suggests that this band is likely the desired dsRNA complementary to the *gro* target sequence.

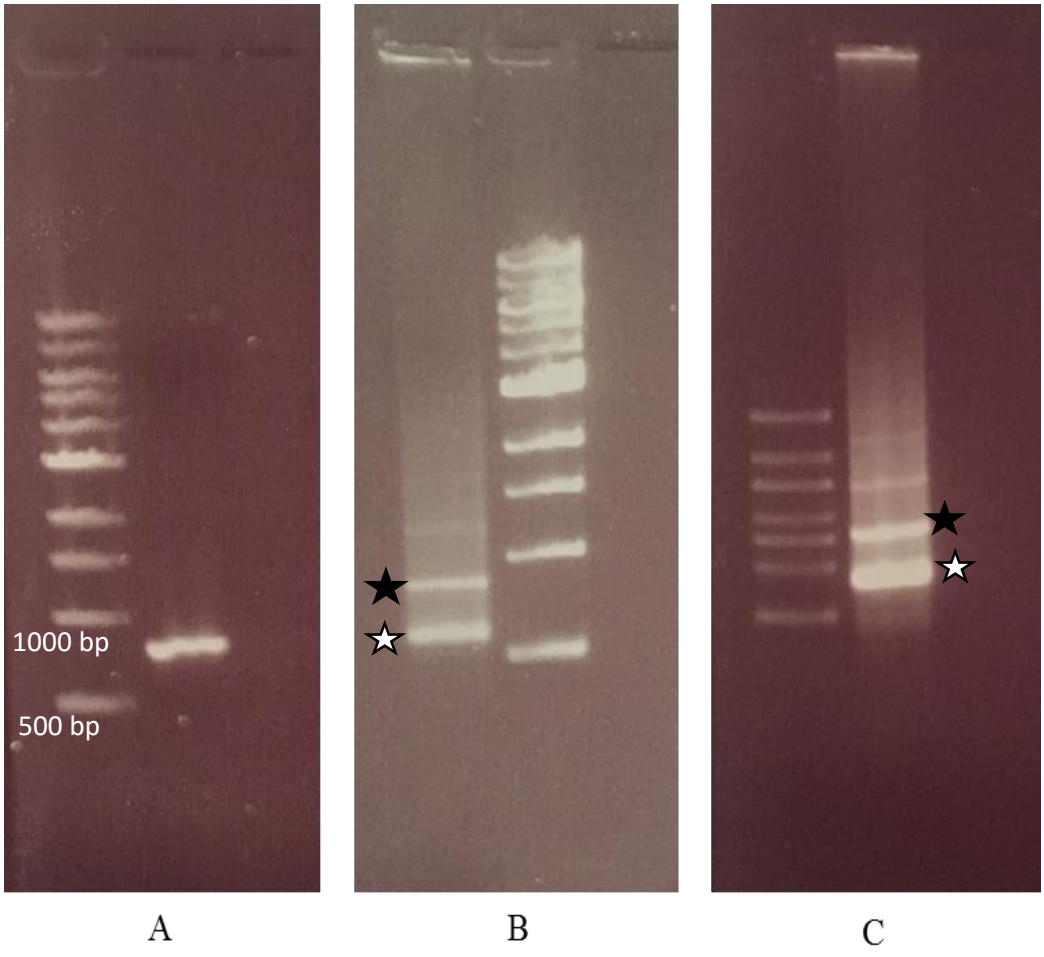

Figure 4. Products of transcription reaction. Part A shows the product of the PCR reaction next to a DNA ladder. Part B shows the products of the transcription reaction next to a double-stranded DNA ladder. Part C shows the products of the same transcription reaction next to a single-stranded RNA ladder. All samples were run on a 1% agarose gel.

The annealing reaction, when run on a 1% agarose gel (Fig. 5), revealed that all of the single-stranded RNA did not anneal. However, the relative brightness of the top and bottom bands changed compared to their brightness before the annealing reaction (Fig. 5). The bottom band (ssRNA) was brighter than the top band (dsRNA) before the annealing reaction (Fig. 5, right-most lane), but the top band appears brighter than the bottom band after the annealing reaction (Fig. 5, center lane). This suggests that, although the annealing reaction was not completely effective, some of the previously ssRNA did anneal to form dsRNA, which combined with the already dsRNA that likely self-annealed during the transcription reaction.

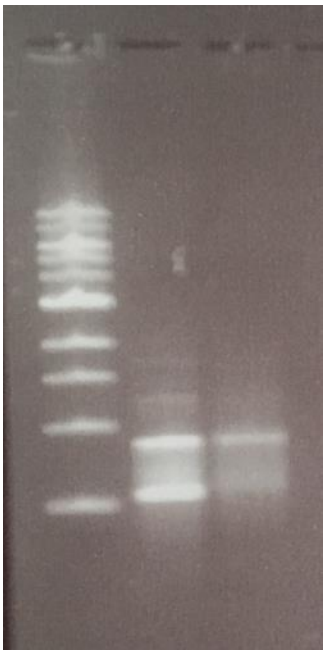

Figure 5. Products of annealing reaction. This shows the results of running on a 1% agarose gel the products of the annealing reaction performed on the RNA obtained from transcription. The products of the annealing reaction (very right lane) are being compared to the products of the transcription reaction prior to annealing (middle lane). The products of the annealing reaction run on the gel were more dilute than the transcript products, which explains the decreased brightness.

After the performance of RNAi on the *Drosophila* S2 cells, they were examined under the fluorescent scope. While cells could be seen under normal light, no cells could be seen with fluorescence that had picked up the DAPI or the Gro stain. All of the cells'

nuclei should have stained with DAPI, which was a control, and the RNAi negative cells should have stained for the Gro protein. The cells, upon closer examination, did not look the same as S2 cells stained in past experiments. The results of the experiment are thus difficult to ascertain and considered inconclusive because it seems the experiment was not actually performed on *Drosophila* S2 cells.

CHAPTER 4

DISCUSSION

Because Gcm plays a role in the development of glial cells in the nervous system, tendon cells, and immature macrophages, it can be concluded that there are other transcription factors or cofactors interacting with Gcm in different contexts to lead to developmental lineages. Identifying these potential collaborators has proven to be a difficult task, but there is evidence to suggest that Gcm and Gro may interact with one another based on their simultaneous presence in a double interaction yeast screen (Nipper, 2014). One approach to addressing this potential interaction is to knock out *groucho* expression, which could potentially be done using RNAi.

The synthesis of this dsRNA is a multi-step process that involves selection of the primers and sequence to be reproduced, performance of PCR to amplify the selected sequence, transcription of ssRNAs complementary to the selected *gro* sequence, and annealing of these ssRNAs to form appropriate dsRNAs. The polymerase chain reaction yielded 0.3687 µg/µL of an approximately 744 bp strand of DNA, which matched the length of the DNA sequence selected from an exon within the *gro* cDNA present in the pUAST plasmid. The transcription reaction, performed with a Thermo Scientific high

yield transcription kit, yielded a large amount of product (~ 6 µg/µL), but the product showed multiple bands upon gel electrophoresis.

Examining the bands against an RNA ladder, which is single-stranded, and a DNA ladder, which is double-stranded, helped in determining what the bands most likely represented. The bottom-most band was at a position of approximately 744 bases with the ssRNA ladder (Fig. 2B), but appeared much shorter against the dsRNA ladder (Fig. 2C). This suggests that the band contains ssRNA from the transcription reaction because it represents the target size on the single-stranded ladder. The second to last band, on the other hand, appears larger than 744 bp against the single-stranded ladder (Fig. 2B) and at 744 bp against the double-stranded ladder (Fig. 2C). This suggests that the band contains a 744 bp long segment of dsRNA, which would have appeared longer compared to a single-stranded ladder because double-stranded molecules are larger and take longer to move through the agarose gel. The addition of RNase-free DNase prior to gel electrophoresis of the product should have ensured that the band is not residual DNA template. The other bands visible above these two aforementioned bands could have resulted from some of the ssRNA forming secondary structures within itself, causing it to become bulky and move slowly through the gel. Another measure that could be performed to ensure the band is composed of dsRNA and the bottom band of ssRNA would be to treat a sample of the transcript product with RNase A, which degrades ssRNA, but not dsRNA.

When attempting to anneal the remaining ssRNA so that the entire sample would be double-stranded, the reaction did not appear to be completely effective. There was still a band representing ssRNA. However, the ssRNA band became less bright than the

dsRNA band, whereas the ssRNA band was brighter than the dsRNA band prior to performing the annealing reaction (Fig. 3B). This suggests that the annealing reaction could increase the efficacy of the RNAi. The annealing reaction performed may not have been completely effective because our transcript product was actually a combination of ssRNA, dsRNA, and possibly some other RNA configurations. The protocol from *Molecular Cloning* called for 50 pmol of each primer, but it was not possible to distinguish what percentage of the total 6 µg/µL concentration was just single-stranded. Even without the annealing reaction, a useful amount of dsRNA appears to have been produced. Because the S2 cells are derived from a macrophage-like lineage and exhibit a similar phagocytic nature (Schneider, 1972), they should have taken up the dsRNA molecules during soaking, which could then take part in the RISC complex.

After staining the cells to examine the results of the RNA interference, viewing them under the fluorescent scope yielded inconclusive results. While there were many cells visible under normal light conditions, it appeared that no cells picked up any stain, either the DAPI or the Gro stain. All cells' nuclei should have stained with the DAPI, and the RNAi negative cells should have stained with the Gro stain. Some previously stained S2 cells were examined to see what the results should resemble, and this examination revealed that the cells on the slide were much smaller than the S2 cells from previous staining. It is likely that the S2 cells became contaminated at some point and overtaken by cells of a different type, which is supported by the facts that the cells had appeared to be multiplying faster than usual and had a slightly different tinge of color. These cells may have been prokaryotic or eukaryotes with a cell wall, explaining why

they did not pick up the DAPI stain. It is not likely the cells underwent a transformation due to epigenetics, as these discrepancies were seen in the RNAi samples also.

When this experiment is tried again in the future, it would be beneficial to examine old slides of S2 cells alongside the current cell cultures for the duration that they are maintained. It would also be better to try and perform the actual experiment after maintaining the S2 cells for no more than three weeks, to reduce the risk of contamination. This experiment was performed after the cultures had been maintained for about five weeks, unnecessarily increasing the risk for contamination.

If the RNAi experiment is performed with positive results, the ultimate goal would be to examine the effects of *groucho* knockdown on *repo* expression by staining for the presence of Repo in the cells. If *repo* expression were reduced when Gro was knocked down, this would suggest that Gro interacts with Gcm in initiating *repo* expression. If *repo* expression were unaffected by Gro knockdown, this suggest that Gro and Gcm do not interact in the context of controlling *repo* expression. However, because Gcm is important to the development of other cell lineages, Gro may act as a cofactor with Gcm under different contexts, in the maintenance of a gene other than *repo*. Because Gro is a repressor, an interaction with Gcm could mean that it could be working as a repressor in one of these cell lineages, which is certainly worth being further investigated.

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