IDENTIFICATION OF DRAGON, A NOVEL MEMBER OF THE SMA/MAB

TGF BETA SIGNALING PATHWAY IN *CAENORHABDITIS ELEGANS*

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By

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ABSTRACT

The *C. elegans* postembryonic lineage, the M lineage, gives rise to mesodermal cell types: body wall muscles, sex muscles and coelomocytes. Past research in the Liu lab has shown that SMA-9, the *C. elegans* homolog of *Drosophila* Schnurri, is required for establishing dorsoventral asymmetry in the M lineage, and that it does so by antagonizing the Sma/Mab TGF-β signaling pathway. To identify additional factors involved in regulating dorsoventral asymmetry, our lab has screened for mutations that suppress the M lineage defects of *sma-9* mutants. Using snip-SNP mapping, I have mapped one of these suppressor mutations, *jj4*, and identified it to be a mutation in Y71G12B.16, the hitherto uncharacterized *C. elegans* homolog of DRAGON. DRAGON belongs to the well-conserved RGM family of proteins, members of which have been shown to act as coreceptors for BMP signaling in mouse and *Xenopus*. Furthermore, I have conducted epistasis studies to place *dragon* in the context of the Sma/Mab TGF-β signaling pathway, as well as designed constructs that would help shed light on the temporal and spatial expression patterns of *dragon*. Additionally, I have designed and made fusion protein expression constructs, which can be used to generate antibodies against DRAGON.

BIOGRAPHICAL SKETCH

Debjeet Sen was born in Austin, Minnesota, and raised in Ithaca, NY and Calcutta, India. His early interest in science was fueled by his parents, and this manifested itself in his winning several scientific competitions and quizzes while in middle and high school. He then went on to complete his undergraduate Bachelor of Technology degree in Biotechnology and Biochemical Engineering from the Indian Institute of Technology (I.I.T.) at Kharagpur in India, where he worked on the optimization of biohydrogen production by *Enterobacter cloacae*. Additionally, he worked on the cloning and expression of human GATA6 at the Indian Institute of Science, Bangalore and the purification of bioactive peptides from whey wastes at the Central Food Technological Research Institute, Mysore as an Indian Academy of Sciences summer research fellow. Upon graduating from I.I.T. Kharagpur, he entered the laboratory of Dr. Kelly Liu at Cornell University as a graduate student. As a teaching assistant at Cornell University, Debjeet discovered a new-found love in teaching. His passion for teaching, together with his wish to work for the improvement of the developing world shaped the next phase of his career, as he is headed to the Maxwell School of Public Affairs at Syracuse University for his Master of Public Administration degree. Outside academics, he is interested in writing stories, dramatics, quizzing, current affairs, playing the slide guitar and drums, playing word games, English literature, skiing, cooking and traveling.

To my parents, who have always stood by me…

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I would first like to thank Dr. Kelly Liu for allowing me to work in her laboratory, and for her encouragement and motivation. I would also like to thank my committee members, Dr. Ken Kemphues and Dr. Volker Vogt for their words of advice and for being accessible at all times. I would especially like to thank Volker for being a true mentor, guide and guardian to me for nearly four years now. I have always run to him whenever things did not work properly, and never once has Volker not made me feel better after talking to him. Thank you very much for being there, always. Additionally, I am extremely grateful to my teaching advisors, Dr. Jerry Feigenson, Dr. Bik Tye, Dr. Tim Huffaker and Dr. Debra Nero for introducing me to the exciting world of teaching and for making TA-ing such an enjoyable and satisfying experience. I would also like to thank the members of the Liu lab for sharing their insights in science and for helping me out with experiments and protocols in every way possible. I am grateful to the members of the Cornell Worm Group for their ideas and advice. I also wish to thank Dr. John Lis and Dr. Mariana Wolfner for allowing me to use their respective equipments. I would like to thank my wonderful friends for their love and support, especially during trying times. Most importantly, I wish to express my gratitude to my parents for never losing faith in me and for making me into what I am today. I know often things have not turned out the way you wanted them to be, but I promise you I shall make you proud someday. Lastly, I wish to thank the Almighty for His guidance, for as long as He keeps watch, sheep may safely graze.

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CHAPTER 1

DRAGON AND TGFβ SIGNALING IN *C. ELEGANS*

Transforming Growth Factor β (TGFβ) signaling is a well-conserved signaling process in metazoans that controls a host of diverse cellular and developmental processes. In spite of the wide variety of processes mediated by TGFβ signaling, the signaling cascade is remarkably simple. The TGFβ ligand initiates signaling by recruiting type I and type II receptors into a heteromeric complex (Yamashita *et al.*, 1994). The type II receptor phosphorylates the type I receptor, which then phosphorylates and activates cytoplasmic proteins called R-Smads. R-Smads bind to Co-Smads to form a complex that translocates into the nucleus to regulate gene transcription (reviewed in Shi and Massague, 2000).

TGFβ signaling pathways in *C. elegans*

There are two canonical TGFβ signaling pathways in *C. elegans*: the Sma/Mab and dauer pathways. Mutants in the Sma/Mab pathway have reduced body sizes (Sma) and male tail abnormalities (Mab), while mutants in the dauer pathway enter the dauer stage inappropriately under favorable environmental conditions, or fail to enter the dauer stage under unfavorable conditions (reviewed in Patterson and Padgett, 2000). In addition to its role in the regulation of body size and male tail patterning, the Sma/Mab TGFβ signaling pathway has also been implicated in the development of innate immunity in *C. elegans* (Nicholas and Hodgkin, 2004). It may be noted that the dauer stage is an alternative third larval stage, which allows *C. elegans* to survive under adverse conditions (unavailability of food, overcrowding etc). Once favorable

conditions return, worms can exit the dauer stage. In addition to the two canonical TGFβ pathways in *C. elegans*, there are three other TGFβ-related genes, which have not been placed into signaling pathways: *unc-129*, *tig-2* and *tig-3* (reviewed in Savage-Dunn, 2005). *unc-129* is involved in axonal guidance along the dorsoventral axis (Colavita *et al.*, 1998), though the roles of *tig-2* and *tig-3* are yet to be elucidated.

Signaling in the Sma/Mab TGFβ pathway is initiated by the ligand DBL-1 (Morita *et al.* and Suzuki *et al.*, 1999). The type II receptor is encoded by *daf-4* and the type I receptor by *sma-6* (Krishna *et al.*, 1999). The signal is transduced by Smad proteins SMA-2, SMA-3 and SMA-4 (Savage *et al.*, 1996), which form a complex that translocates into the nucleus. Very little is known about the transcriptional cofactors that might interact with the Smad proteins in *C. elegans* to regulate gene expression, though SMA-9 (Liang *et al.*, 2003) and LIN-31 (Baird and Ellazar, 1999) have been identified as potential cofactors. In a recent study, SMA-3 and LIN-31 have been shown to physically interact with each other, though no such interaction was observed between the Smad proteins and SMA-9 (Wang *et al.*, 2005). Equally little is known about genes whose expression is regulated by the Sma/Mab TGFβ pathway. *lon-1* (Maduzia *et al.* and Morita *et al.*, 2002) and *sma-6* (Mochii *et al.*, 1999) are two genes whose expression is regulated by TGFβ signaling, the latter providing an example of a positive feedback loop.

The dauer TGFβ pathway utilizes a different set of Smads (DAF-3, DAF-8 and DAF-14) and a different type I receptor (DAF-1) and ligand (DAF-7), though it shares DAF-4 as the common type II receptor with the Sma/Mab TGFβ pathway, DAF-4 being the sole type II receptor in *C. elegans* (reviewed in Patterson and Padgett, 2000).
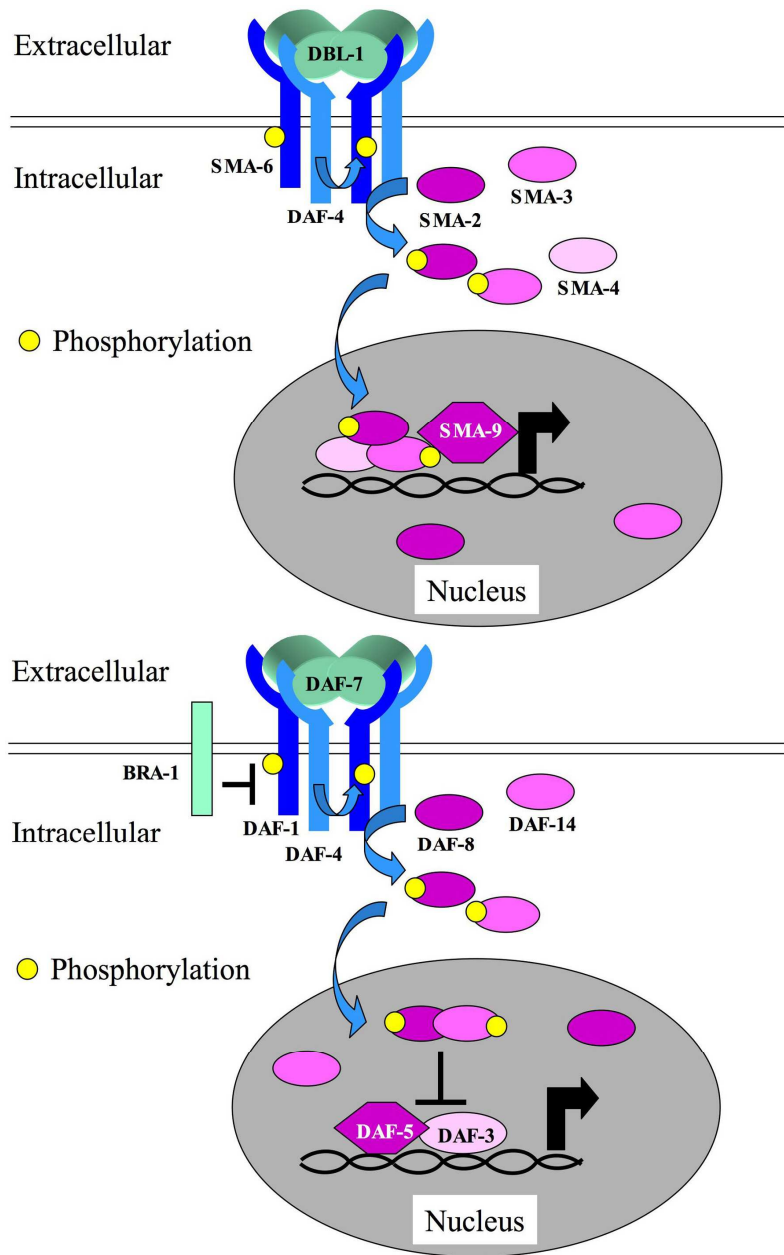
Figure 1-1: The Sma/Mab TGFβ signaling pathway (left) and the dauer TGFβ signaling pathway (right) - Savage-Dunn, 2005

Modulation of TGFβ signaling in *C. elegans*

Since TGFβ signaling is involved in a wide range of cellular processes, it must be tightly regulated. In higher organisms, TGFβ signaling is regulated by such mechanisms as ubiquitination and subsequent degradation of Smad proteins and receptors (Lin *et al.*, 2000 and Kavsak *et al.*, 2000), use of decoy receptors such as BAMBI (Onichtchouk *et al.*, 1999), use of ligand antagonists such as Noggin (Zimmerman *et al.*, 1996) and control of the recruitment of Smads to the TGFβ receptor(s) by a protein called SARA (Tsukazaki *et al.*, 1998). In *C. elegans*, none of the above mechanisms have been identified so far, though the genome sequence does reveal the presence of a SARA homolog (Patterson and Padgett, 2000). Amongst regulators that have been identified, LON-2, a GPI-anchored glypican protein, is thought to bind to the ligand DBL-1 and negatively regulate Sma/Mab TGFβ signaling by sequestering DBL-1 (Gumienny *et al.*, 2007). BRA-1, a homolog of human BMP receptor-associated molecule BRAM1, has been shown to bind to the type I receptor of the dauer TGFβ pathway and negatively regulate the dauer TGFβ signaling pathway (Morita *et al.*, 2001). There is also some evidence to suggest that BRA-2 (the other *C. elegans* homolog of BRAM1) and BIP (BRAM-interacting protein) might be involved in regulating Sma/Mab TGFβ signaling (Sugawara *et al.*, 2001): RNAi against BIP resulted in a small body phenotype (Sma), phenocopying Sma/Mab TGFβ pathway mutants. In this thesis, I shall discuss the identification of the *C. elegans* homolog of a BMP co-receptor, DRAGON, which has been shown to potentiate BMP signaling in mouse and *Xenopus* (Samad *et al.*, 2005).

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CHAPTER 2

IDENTIFICATION OF *C. ELEGANS* DRAGON

SMA-9, the *C. elegans* homolog of *Drosophila* Schnurri, is required for establishing dorsoventral asymmetry in the *C. elegans* postembryonic mesodermal (or, M) lineage. The dorsal daughter of M (M.d) produces two coelomocytes and six body wall muscles, while the ventral daughter (M.v) produces two sex myoblasts and eight body wall muscles (Sulston and Horvitz, 1977). SMA-9 regulates this asymmetry by antagonizing the Sma/Mab TGF-β signaling pathway. *sma-9* mutant animals exhibit a loss of the two ceolomocytes and the production of two extra sex myoblasts and body wall muscles on the dorsal side (Foehr *et al*., 2006).

To identify additional factors involved in regulating this dorsoventral asymmetry, our lab screened for mutations that can suppress the M lineage defects of *sma-9* mutants, i.e. restore the two M-derived ceolomocytes absent in *sma-9* mutant animals. Five single-locus recessive suppressor mutations (*jj1*, *jj3*, *jj4*, *jj6* and *jj7*) were isolated from the suppressor screen. When these mutations were outcrossed out of the *sma-9* background, the respective single mutants were found to have small body sizes (Rachel Fairbank and Amanda Lindy, unpublished data). Three of the mutations were characterized as being mutations in core members of the Sma/Mab TGFβ signaling pathway: *jj1* was found to be a mutation in *sma-6*, *jj3* a mutation in *sma-3* (Foehr *et al.*, 2006) and *jj6* a mutation in *sma-2* (Yevgeniy Plavskin, unpublished data). Of the uncharacterized mutations, *jj4*, which suppresses the *sma-9* M-lineage mutant phenotype with a penetrance of 94% had been previously mapped to the left arm of chromosome I (Amanda Lindy and Yevgeniy Plavskin, unpublished data).
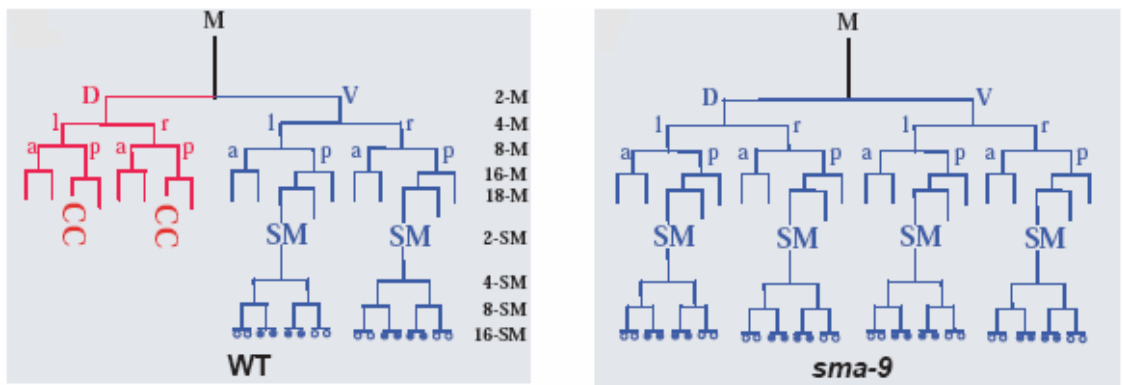
Figure 2-1: *sma-9* **mutations result in the loss of dorsal, and duplication of ventral, M lineage descendants – Foehr** *et al.*, **2006**

The absence of known members of the Sma/Mab TGFβ signaling pathway in the region to which *jj4* maps and its Sma phenotype indicated that *jj4* might correspond to a new component of TGFβ signaling. Using snip-SNP mapping, followed by RNAi, I have been able to identify *jj4* as a mutation in *dragon*, the *C. elegans* homolog of a well-conserved member of the TGFβ signaling pathway.

Materials and Methods

***C. elegans* strains**: All *C. elegans* strains were maintained using standard methods (Brenner, 1974) and kept in incubators set to 11^0C, 14^0C, 16^0C or 20^0C. All strains containing the *rrf-3* mutation were maintained at 16^0C. The strains referred to in Chapter 2 are:

LW1427: N2

LW0049: *ccIs4438 (intrinsic cc::gfp)* III*; ayIs2(egl-15::gfp)* IV*; him-5(e1467)* V; *ayIs6(hlh-8::gfp) (36x)* X

LW0085: CB4856

jj4 I*; cc::gfp*

LW0058: *unc-9(e101) sma-9(cc604) ayIs6(hlh-8::gfp) arIs39(cc::gfp)* X

LW0683: *rrf-3(pk1426)* II*; ccIs4438 (intrinsic cc::gfp)* III*; ayIs2(egl-15::gfp)* IV*; ayIs6(hlh-8::gfp)* X

LW1525 and 1526: *rrf-3(pk1426)* II*; ccIs4438 (intrinsic cc::gfp)* III*; ayIs2(egl-15::gfp)* IV; *sma-9(cc604) ayIs6(hlh-8::gfp)* X
hT2[qIs48]/tm1502

Crosses: All crosses were set up at either 16^0C or 20^0C, and involved placing 3 - 4 L4 hermaphrodites with 12 – 15 L4 larvae or young adult males. Cross progeny were collected after 4 - 5 days.

Snip-SNP mapping: SNPs or single nucleotide polymorphisms are differences between the sequences of the Bristol (N2) and Hawaiian (CB4856) strains of *C. elegans* that exist at the level of individual nucleotides. When these SNPs can be distinguished in the two strains by digestion with restriction enzymes, the SNPs are referred to as snip-SNPs. In the case of certain snip-SNPs, the restriction enzymes that would have enabled us to distinguish these SNPs were not available in the laboratory inventory. In such situations, genomic DNA was sequenced to determine the presence of the SNP. It may be noted that the present study has used both snip-SNPs that have been identified by sequencing and restriction digestion, as well as hypothetical snip-SNPs that I have tested and verified (http://genome.wustl.edu/genome/celegans/celegans_snp.cgi). In the first step of snip-SNP mapping, *jj4* hermaphrodites in the N2 background (distinguished by their small body size) were crossed with CB4856 males. The resulting F1 progeny were then allowed to self-fertilize, and F2 Sma animals collected (presumed to be homozygous for *jj4*). The latter were transferred to individual plates and allowed to self-fertilize. When the F2 animals had thrown a sufficient number of progeny, DNA was extracted from these worms. Loci containing specific SNPs were PCR-amplified from this DNA, and restriction digested (in case of snip-SNPs) or sequenced to check for the presence or absence of particular SNPs, thereby allowing one to differentiate between N2 and CB4856 DNA. The closer the SNPs lie to the mutation of interest (in this case, *jj4*), the less likely that these SNPs will recombine away from *jj4*. Thus, by scoring for

recombinants, it is possible to follow SNPs across a chromosome and map a mutation to a small region.

DNA for snip-SNP mapping was extracted from worms by first spraying the plates with sterile M9 buffer (3gm KH_2PO_4, 6gm Na_2HPO_4, 5gm NaCl and 1ml of 1M $MgSO_4$ in 1 liter water, and autoclaved) from transfer pipettes, and subsequently transferring the liquid into 2 ml of a solution of 4% sucrose with 10mM EDTA. The suspension of worms was centrifuged at 3000 rpm for 2 minutes and then washed twice with distilled water. The resulting pellet was re-suspended in 200µl of lysis buffer (50mM KCl, 10mM Tris-Cl at pH 8.3, 2.5mM $MgCl_2$, 0.45% NP40, 0.45%Tween-20, 0.01% gelatin, 60µg/ml Proteinase K) and stored at -80˚C for one hour. The suspension was then incubated at 60°C for one hour to allow the Proteinase K in the lysis buffer to lyse the worms, and then at 95°C for 15 minutes to denature the Proteinase K. The *C. elegans* genomic DNA thus obtained was used for setting up PCR reactions.

The PCR mix contained 2.5µl of the genomic DNA obtained by the above method, 2.5µl of 10X PCR buffer (500mM KCl, 100mM Tris-Cl at pH 9.0, 1% Triton X-100), 1µl of each of the two primers at concentration 25µM, 0.2µl of 10mM dNTPs, 2µl of 25mM $MgCl_2$, 0.5µl Taq polymerase and 15.3µl distilled water. The PCR cycling conditions involved an initial 2 minutes at 94˚C, followed by 46 cycles of 40 seconds at 94˚C, 50 seconds at the annealing temperature for the particular primer pair, and 1 minute at 72˚C. The reaction was completed by maintaining the PCR mix for 10 minutes at 72˚C and then holding it at 4˚C till the tubes were withdrawn from the machine. To distinguish between CB4856 and N2 DNA, the PCR products were restriction digested. Restriction digestion was carried out by adding 5µl of the PCR

mix to 0.3µl of restriction enzyme(s), 2µl 10X restriction enzyme buffer and distilled water made up to a total volume of 20µl. The restriction digests were incubated for 2 hours at temperatures that would allow their respective restriction enzymes to act most optimally (25^0C, 37^0C etc.). The restriction digests were then run on a 2% agarose gel to separate and visualize the DNA fragments.

Whenever the snip-SNP analysis involved the use of a rare enzyme that was not present in the laboratory inventory, or when the SNPs were not recognized by any restriction enzymes, the PCR fragment containing the SNP was sequenced to check for the presence of the SNP. For sequencing, 1µl of the PCR product purified by conventional gel filtration protocol was mixed with 1µl of 10µM primer and 16µl distilled water in sequencing tubes. The sequencing mixes were sent to the DNA Sequencing Facility in the Biotechnology Building of Cornell University.

Identifying a mutation in a gene by sequencing: To identify a mutation in a gene, primers were designed at ~500bp intervals across the length of the gene in both the 5' and 3' directions. For each sequencing reaction, 1µl of the gel-purified PCR product of the entire gene was mixed with 1µl of 10µM primer and 16µl of distilled water and sent to the DNA Sequencing Facility in the Biotechnology Building of Cornell University. Since each sequencing reaction provides unambiguous results for 500-700bp, one would be able to obtain a series of sequences that could be pieced together in both the 5' and 3' directions to generate the sequence of the entire gene. By comparing the pieced together sequence of the mutant gene with the sequence of the wild-type gene, one can thus identify mutations in the former.

Recovering cDNA from the ProQuest library: The Invitrogen *C. elegans* ProQuest cDNA library consists of cDNA fragments (of average length 1.4kb) inserted into vectors. I am thankful to Dave Pruyne of the Bretscher lab, who kindly provided me with the library as well as the ForwLong primer. In order to recover cDNA from the ProQuest library, a PCR was set up by mixing 0.25µl of the ProQuest library, 0.5µl ForwLong PC86 primer

(5' TATAACGCGTTTGGAATCACTACAGGGATGTTTAATACCAC 3') at concentration 1µg/ml (which anneals to the vector 5'to the cDNA insert), 1µl of 25µM reverse primer (which anneals to the 3' end of the cDNA), 1µl 10mM dNTPs, 0.5µl iProof polymerase, 10µl 5X iProof PCR buffer and 36.25µl distilled water. After incubating for 3 minutes at 98^0C, the reaction mix was cycled 10 times at 98^0C for 1 minute, 65^0C for 1 minute and 72^0C for 1 minute + 1minute/kb. This was followed by 15 cycles at 98^0C for 1 minute, 65^0C for 1 minute and 72^0C for 1 minute 10 seconds + 1 minute/kb. After allowing all product extension to finish by keeping the reaction at 72^0C for 10 minutes, the reaction was held at 4^0C till the tubes were removed from the machine. 5µl of the PCR product was then loaded on a 1% agarose gel to visualize the PCR product. cDNA could have been also recovered from the ProQuest library by using a primer annealing to the 5' end of the cDNA and the RevLong PC86 primer, which anneals to the vector downstream of the cDNA insert. This was however not done in the present study because of certain difficulties I faced in obtaining the relevant PCR product.

After recovery of cDNA from the ProQuest library, it was cloned into TOPO-Blunt II vector by using the Zero Blunt TOPO PCR Cloning kit from Invitrogen^{TM}. 2µl of the PCR product was mixed with 1µl each of salt solution (1.2M NaCl and 0.06M $MgCl_2$) and TOPO-Blunt II vector (both supplied in the Invitrogen kit), and 2µl distilled

water. This mix, known as the transformation mix was incubated at room temperature for 15 minutes. 2µl of the transformation mix was used to transform a vial of One Shot TOP10 chemically competent cells. The cells were incubated on ice for 10 minutes with the transformation mix, and then heat-shocked for 30 seconds at 42^0C. The cells were then immediately transferred to an ice bath and kept for 2 minutes. 250µl of sterile SOC media was added to the cells and the cells were then gently rocked at 37^0C for an hour. 20, 50 and 100µl of the SOC mix were plated onto individual LB plates containing 50µg/ml kanamycin and 40µl of 40mg/ml X-Gal. The plates were incubated at 37^0C overnight, and inspected for white colonies the following day. White colonies indicated that the ligation of the cDNA insert into the vector had been successful in those particular bacteria, while blue colonies indicated an unsuccessful ligation. Individual white colonies were used to inoculate 2ml of 2X YT broth containing 50µg/ml kanamycin. The tubes were shaken at 37^0C overnight, and the plasmid DNA extracted from the bacteria by a conventional Miniprep protocol.

RNAi in *C. elegans* by micro-injection: RNAi is a process of silencing a gene at the mRNA level, in which double-stranded RNA triggers the degradation of a homologous mRNA. For conducting RNAi experiments in the present study, the double-stranded RNA homologous to the target mRNA was injected into the animals by Dr. Kelly Liu using the procedure highlighted in Fire *et al.* (1998). The effects of RNAi were observed in the progeny of the injected animals.

In the first step, specific DNA sequences were PCR amplified from genomic N2 DNA using the same conditions outlined earlier in the section on snip-SNP mapping. RNA was synthesized from the PCR product by an *in vitro* transcription reaction, in which 4.5µl of PCR product was mixed with 2µl 5X T7 buffer, 3µl 25mM rNTPs and 0.5µl

T7 RNA polymerase. The transcription reaction was allowed to go on for 3 hours at 37^0C. 0.5µl of the transcribed RNA was kept aside, while the rest was purified using the standard phenol-chloroform extraction protocol that is used to purify PCR products. The final RNA pellet was resuspended in 10µl DEPC water. The purified RNA was run alongside the unpurified RNA on a 2% agarose gel to verify that little or no RNA had been lost during the purification process. Since RNA is extremely susceptible to degradation, care was taken to ensure that the experimental work-space was free of RNases. Sterility was maintained at every juncture of the experiment. The dsRNA thus generated was used for micro-injection. If the yield of dsRNA was extremely high, it was diluted 1:2 before being injected.

RNAi in *C. elegans* by feeding: Specific genes in *C. elegans* can be silenced following the ingestion of dsRNA-expressing bacteria (Timmons and Fire, 1998). A genomic fragment of *dragon* was PCR amplified and cloned into the L4440 feeding vector. L4440 is a modified Bluescript vector with a T7 promoter on each side of the multiple cloning site driving transcription of each DNA strand. The primers used for the PCR amplification of *dragon* were DS-151 and DS-152, and after the PCR product was purified by standard phenol-chloroform extraction protocol, the pellet obtained from the latter was dissolved in 8µl distilled water. 2µl of the PCR product was digested at 37^0C with 0.3µl each of HinDIII and SpeI in 2µl NEB #2 buffer and 15.4µl water. 2µl of the L4440 vector was digested similarly with HinDIII, PstI and SpeI. The restriction digests were loaded onto a 1% agarose gel and the relevant bands cut out of the gel and gel purified. The pellet of the digested PCR product was dissolved in 5µl water, while that of the digested vector was dissolved in 7µl water. A ligation reaction was set up at 16^0C overnight by mixing 5µl of the digested PCR product with 1µl of the digested vector, 1µl 10X ligation buffer, 1µl T4 ligase and 2µl water. DH5α cells

were transformed with the ligation mix by a standard heat shock protocol and plated onto LB-Ampicillin plates. Single colonies from the latter were used to inoculate 2ml 2X YT, from which plasmid DNA was extracted by a conventional Miniprep protocol. The feeding vector containing the *dragon* insert was denoted pDS11. The plasmid DNA was checked by restriction digestion with HinDIII and SpeI, as well as by sequencing. HT115(DE3) competent cells were then transformed with pDS11. HT115(DE3) is an RNase III-deficient *Escherichia coli* strain with IPTG-inducible T7 polymerase activity (Timmons *et al.*, 2001). As controls, competent cells were also transformed with the blank L4440 vector, a construct containing a fragment of *sma-2* cloned into L4440 (III-4N03) and another containing a fragment of *sma-3* cloned into L4440 (III-3J09). The transformed bacteria were plated onto LB-Ampicillin-Tetracyclin plates and allowed to grow overnight at 37^0C. Single cells were used to inoculate LB with 75μg/ml Ampicillin and 12.5μg/ml Tetracyclin. 200μl of this bacterial inoculum was used to seed each IPTG-containing NGM plate. The plates were allowed to dry overnight and stored at 4^0C.

RNAi by feeding can be done with either L1 or L4 animals. In the former case, the phenotypes of the adult animals are observed to determine the effect of the RNAi, while in the latter, the effects of the RNAi are observed in the next generation. To obtain a synchronized population of L1 animals, gravid adults were collected in M9 buffer. To this was added 1ml of Clorox and 1ml of 10M sodium hydroxide, with the final volume made up to 10ml with water. The tubes were gently shaken and allowed to stand for 2 minutes. The tubes were then centrifuged at 3000 rpm for 2 minutes and the supernatant drained off. The pellet was washed twice in water/ M9 buffer and finally resuspended in 1ml M9 buffer. The sodium hypochlorite in the Clorox lyses the worms, and since the eggs are resistant to hypochlorite treatment, one can collect the

eggs in the M9 buffer at the end. The eggs were allowed to hatch by gently rocking the tubes overnight at 16^0C. Since the M9 buffer contains no nutrients, the hatched larvae are arrested at the L1 stage. 50 - 100 L1 larvae were placed on each IPTG-NGM plate and the plates were kept at 16^0C.

For carrying out RNAi by feeding L4 larvae, 4 – 6 L4 larvae were placed on each IPTG-NGM plate and allowed to lay eggs. When the worms had laid 50 - 100 eggs in total, they were transferred to another IPTG-NGM plate. The worms were allowed to lay eggs over a total of four IPTG-NGM plates. The plates were kept at 16^0C and the progeny observed for effects of the RNAi.

Results and Discussion

By carrying out snip-SNP mapping on a sample size of 629 worms, I mapped *jj4* to an interval between -14.6 and -13.15 map units on chromosome I, a region spanning 231 kb of DNA. Table 2-1 lists the primers that I had used for the purpose of mapping, together with the map positions of the SNPs that they amplified on chromosome I.

To confirm this map location, I took two recombinant *jj4* strains that I had generated during the course of my mapping experiments: #19, which had CB4856 DNA till -14.5828 map units on chromosome I (and N2 DNA all the way to the right end of the chromosome); and #27, which had CB4856 DNA till -13.1227 map units (and N2 DNA all the way to the left end of the chromosome).

Table 2-1: List of primers used for mapping *jj4* with the map positions of the SNPs that they amplify on chromosome I

PRIMER PAIR	LOCUS ON I	PRIMER PAIR	LOCUS ON I
DS-9/10	-18.4361	DS-11/12	-8.11021
DS-13/14	-1.646	DS-15/16	-4.483
DS-17/18	-5.209	DS-19/20	-19.4382
DS-21/22	-16.7152	DS-23/24	-11.9964
DS-25/26	-15.3783	DS-27/28	-14.7492
DS-29/30	-15.3783	DS-31/32	-11.9964
DS-33/34	-13.1227	DS-35/36	-11.9964
DS-37/38	-10.5945	DS-39/40	-4.47575
DS-41/42	-3.90174	DS-43/44	-14.5828
DS-45/46	-12.3596	DS-47/48	-13.8168
DS-49/50	-13.1227	DS-51/52	-14.7482
DS-53/54	-14.669	DS-55/56	-14.6433
DS-57/58	-13.8168	DS-59/60	-13.1227
DS-61/62	-13.8168	DS-63/64	-13.8168
DS-65/66	-13.8168	DS-67/68	-15.3783
DS-117/118	-14.385	DS-119/120	-13.405
DS-121/122	-13.319	DS-123/124	-13.492

Using the series of crosses outlined in Figure 2-2, I introduced the recombinant chromosome I into a *sma-9 unc-9* background and saw that it was able to suppress the *sma-9* mutant phenotype, restoring the two M-derived ceolomocytes absent in *sma-9* mutant animals. Because the only N2 DNA on chromosome I that is common to both #19 and #27 lies between -14.5828 and -13.1227, these results suggest that the *jj4* mutation must be located in this interval.

The region that I had mapped *jj4* to contains 26 predicted genes. RNAi by micro-injection in a *unc-9 sma-9* mutant background was carried out by Dr. Kelly Liu against 23 genes in this region, in order to test whether RNAi of one of these genes would phenocopy the suppression of the *sma-9* mutant phenotype by *jj4*. The 3 genes at the periphery of this region were not included in the RNAi analysis since the results of the snip-SNP mapping suggested that *jj4* was located in the center of the region. The list of these predicted genes, along with the primers that were used to amplify the genomic DNA for synthesizing gene-specific dsRNAs is given in Table 2-2.

We found that RNAi against Y71G12B.16 resulted in a suppression of the *sma-9* mutant phenotype and was able to restore the 2 M-derived coelomocytes absent in *sma-9* mutants with a penetrance of nearly 60%. Y71G12B.16 codes for the *C. elegans* homolog of DRAGON, a protein that has been found to act as a co-receptor for BMP signaling in mouse and *Xenopus* (Samad *et al.*, 2005).

Figure 2-2: Cross scheme for introducing a recombinant chromosome I into a *sma-9 unc-9* background

#19 *jj4*[*] CB   *jj4*   N2
            ▬▬▬▬▬▬▬▬▬▬────────────────────

                    -14.5828

#27 *jj4*[*] N2   *jj4*   CB
            ────────────────────▬▬▬▬▬▬▬▬▬▬▬

                    -13.1227

hT2 : gfp ♀   X   *him-5* ♂
tm1502   *him-5*
            │
            ▼

        ht2 : gfp ♂   X   *jj4*[*] ♀
            +   *jj4*[*]
                        │
                        ▼

            ht2 : gfp ♂   X   *sma-9 unc-9* ♀
                jj4[*]   *sma-9 unc-9*
                            │
                            ▼
                    (See next page)

                    22

Figure 2-2 (Continued)

$$\frac{jj4^{*} \qquad \underline{sma\text{-}9\ unc\text{-}9}}{+ \qquad\qquad +} \;\female\!\!\male$$

$$\downarrow$$

$$\frac{jj4^{*} \qquad \underline{sma\text{-}9\ unc\text{-}9}}{+ \qquad\qquad sma\text{-}9\ unc\text{-}9} \;\female\!\!\male$$

$$\downarrow$$

$$\frac{jj4^{*} \qquad \underline{sma\text{-}9\ unc\text{-}9}}{jj4^{*} \qquad\qquad sma\text{-}9\ unc\text{-}9} \;\female\!\!\male$$
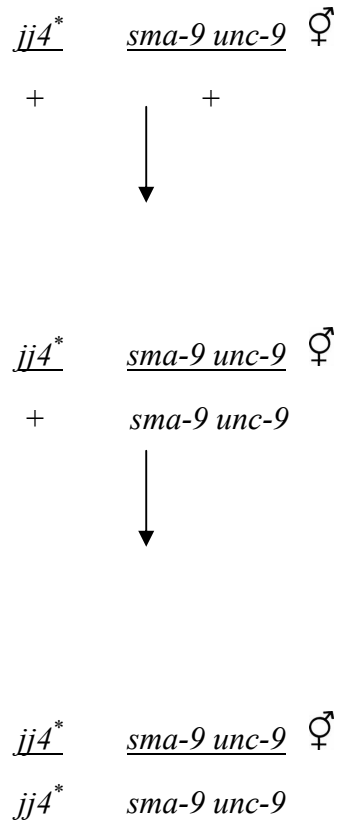
Table 2-2: List of primers used to amplify genomic DNA for synthesizing gene-specific dsRNAs for RNAi (gene names on the left hand side of each cell)

LOCUS	PRIMER PAIR	LOCUS	PRIMER PAIR
Y91H12A.1	DS-99/100	Y71G12B.21	DS-89/90
Y73E7A.3	DS-95/96	Y71G12B.33	DS-91/92
Y73E7A.4	DS-69/70	Y71G12B.22	DS-93/94
Y73E7A.5	DS-71/72	Y71G12B.17	DS-97/98
Y73E7A.2	DS-73/74	Y71G12B.23	DS-101/102
Y73E7A.6	DS-75/76	Y71G12B.16	DS-103/104
Y73E7A.9	DS-77/78	Y71G12B.31	DS-105/106
Y73E7A.7	DS-79/80	Y71G12B.15	DS-107/108
Y73E7A.1	DS-81/82	Y71G12B.14	DS-109/110
Y73E7A.8	DS-83/84	Y71G12B.13	DS-111/112
Y71G12B.20	DS-85/86	Y71G12B.12	DS-113/114
Y71G12B.18	DS-87/88	Y71G12B.21	DS-115/116

To verify the results of the RNAi by micro-injection, I also carried out RNAi by feeding. L1 and L4 larvae of an *rrf-3(pk1426)* II*; ccIs4438 (intrinsic cc::gfp)* III*; ayIs2(egl-15::gfp)* IV*; ayIs6(hlh-8::gfp)* X strain and an *rrf-3(pk1426)* II*; ccIs4438 (intrinsic cc::gfp)* III*; ayIs2(egl-15::gfp)* IV*; sma-9(cc604) ayIs6(hlh-8::gfp)* X strain were fed with bacteria containing RNAi constructs for L4440, *sma-2*, *sma-3* or *dragon*. L4440 was the negative control and *sma-2* and *sma-3* were the positive controls. It may be noted that *rrf-3* mutants are hypersensitive to RNAi treatment (Simmer *et al.*, 2002). 3.3% of the progeny thrown by *rrf-3(pk1426)* II*; ccIs4438 (intrinsic cc::gfp)* III*; ayIs2(egl-15::gfp)* IV*; ayIs6(hlh-8::gfp)* X L4 larvae fed on L4440 were found to have a small body size(N=453), as did 26.4% of the progeny of L4 larvae fed on *sma-2* RNAi bacteria (N=344), 37.8% of the progeny of L4 larvae fed on *sma-3* RNAi bacteria (N=354) and 21.7% of the progeny of L4 larvae fed on *dragon* RNAi bacteria (N=377). Since *sma-2*, *sma-3* and *jj4* mutants are small in size, the results of the feeding experiment provides further evidence that *jj4* is indeed a mutation in *dragon*. None of the progeny thrown by *rrf-3(pk1426)* II*; ccIs4438 (intrinsic cc::gfp)* III*; ayIs2(egl-15::gfp)* IV*; sma-9(cc604) ayIs6(hlh-8::gfp)* X L4 larvae fed on L4440 (N=439) were found to have 6 coelomocytes. However, 1% of the progeny of L4 larvae fed on *sma-2* RNAi bacteria (N=386), 1.5% of the progeny of L4 larvae fed on *sma-3* bacteria (N=264) and 52.3% of the progeny of L4 larvae fed on *dragon* RNAi bacteria (N=375) were found to have 6 coelomocytes. The low penetrance of suppression of the *sma-9* mutation by *sma-2* and *sma-3* RNAi could be due to the fact that RNAi was not efficient. That RNAi against *dragon* is able to suppress the *sma-9* mutation is encouraging since it provides us with additional proof that *jj4* is a *dragon* mutant. These results also suggest that *jj4* might be a loss-of-function mutant of *dragon* since RNAi results in the degradation of the mRNA message and RNAi of *dragon* is able to phenocopy the *jj4* mutant phenotype.

6% of the *rrf-3(pk1426)* II*; ccIs4438 (intrinsic cc::gfp)* III*; ayIs2(egl-15::gfp)* IV; *ayIs6(hlh-8::gfp)* X adults that had been fed on L4440 (N=66) since the L1 stage were found to be small, as were 18.7% of the adults fed on *sma-2* RNAi bacteria (N=166), 35.3% of the adults fed on *sma-3* RNAi bacteria (N=116) and 25% of the adults fed on *dragon* RNAi bacteria (N=132). As noted before, *jj4*, sma-*2* and *sma-3* mutants are small in size, and the results of the RNAi experiment once again suggested that *jj4* is a *dragon* mutant. Of the *rrf-3(pk1426)* II*; ccIs4438 (intrinsic cc::gfp)* III*; ayIs2(egl-15::gfp)* IV; *sma-9(cc604) ayIs6(hlh-8::gfp)* X adults that had been fed on L4440 and *sma-3* RNAi bacteria since the L1 stage, none had 6 coelomocytes (N=66 and 177, respectively), while 1.8% of the adults that had been fed *sma-2* RNAi bacteria (N=164) and 39.3% of the adults that had been fed *dragon* RNAi bacteria (N=196) were found to have 6 coelomocytes. These results lend further credence to the fact that *jj4* is indeed a mutation in *dragon*.

In order to verify the gene annotation of Y71G12B.16 on WormBase (www.wormbase.org), I recovered cDNAs corresponding to the gene from the ProQuest *C. elegans* cDNA library by using the ForwLong and DS-147 primers. DS-147 anneals to the 3' end of Y71G12B.16 (*dragon*). I then sequenced the cDNAs after cloning them into TOPO-Blunt II vectors. Surprisingly, the start site was found to be 1363bp upstream of that given in WormBase, and there was one additional intron, one additional exon and a 60bp 5' UTR upstream of the newly annotated start site. The first exon was found to be 49bp long, while the first intron immediately following it was 1069bp in length. The start site as originally annotated on WormBase was located in the second exon, 185bp after the 3' end of the first intron.

After proper annotation, *C. elegans* DRAGON was found to be a well-conserved protein 408 amino acids long: it shares approximately 80% amino acid identity with its zebrafish, human and mouse homologs. Surprisingly, there is no corresponding homolog in *Drosophila.* DRAGON has a predicted N-terminal signal peptide sequence, a C-terminal hydrophobic tail and a putative GPI-anchor site. I first sought to establish the presence of a GPI-anchor site in *C. elegans* DRAGON by using a GPI-anchor prediction program available online at

http://mendel.imp.ac.at/gpi/gpi_server.html (Eisenhaber *et al.*, 1999). The software failed to predict the definite presence of a GPI-anchor site in the *C. elegans* DRAGON sequence and indicated that the most likely site of GPI-anchorage in *C. elegans* DRAGON would be a Ser residue at position 387. However, when I entered the mouse DRAGON sequence, the program also failed to definitively predict the presence of a GPI-anchor. Since it has been previously shown that DRAGON homologs contain a GPI-anchor site (Monnier *et al.*, 2002; Samad *et al.*, 2005), I can likely conclude that *C. elegans* DRAGON also contains a GPI-anchor site, notwithstanding the negative prediction returned by the program.

DRAGON belongs to the repulsive guidance molecule (RGM) family of proteins, which includes such members as hemojuvelin (Babitt *et al.*, 2006) and RGMa (Babitt *et al.*, 2005) in higher organisms. Hemojuvelin is involved in iron metabolism (Babitt *et al.*, 2006), while RGMa has been implicated in neuronal differentiation (Matsunaga *et al.*, 2006). DRAGON is the only member of the family present in *C. elegans*.

Finally, I sequenced the Y71G12B.16 (*C. elegans dragon*) locus in *jj4* mutant animals in order to identify the molecular lesion. The list of primers used for sequencing is given in Table 2-3. All distances are from the start site annotated on WormBase.
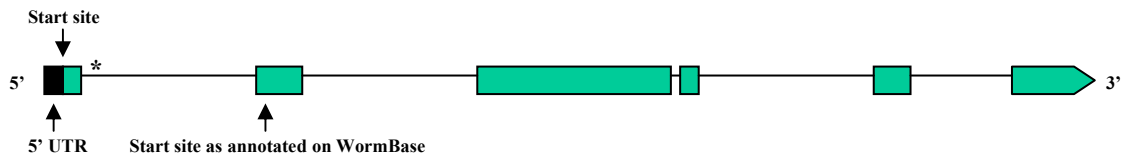
Figure 2-3: Gene structure of *C. elegans dragon* showing exons and introns. The green boxes represent exons, while the black lines represent introns. The 5' UTR is represented by the black box. The location of the molecular lesion in *jj4* is denoted by the asterisk (*).
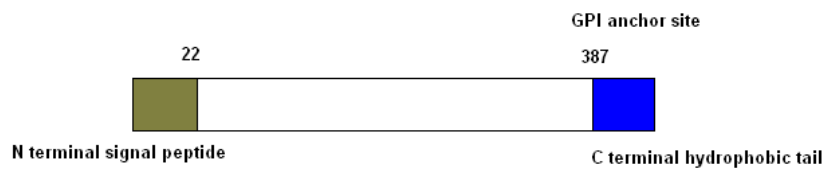
Figure 2-4: Schematic of *C. elegans* DRAGON

Table 2-3: List of primers used to sequence the Y71G12B.16 locus (primer positions on the left hand side of each cell are relative to the start site annotated on WormBase)

5' PRIMER	POSITION	3' PRIMER	POSITION
DS-125	-195bp	DS-133	~3.4kb
DS-126	217bp	DS-134	3127bp
DS-127	774bp	DS-135	2648bp
DS-128	1232bp	DS-136	2228bp
DS-129	1728bp	DS-137	1786bp
DS-130	2208bp	DS-138	1347bp
DS-131	2688bp	DS-139	794bp
DS-132	3095bp	DS-140	319bp
DS-153	~ -1kb	DS-158	~ -900bp
DS-154	~ -500bp	DS-159	~ -500bp
DS-157	~ -1.5kb	DS-161	~ -120bp
DS-160	~ -500bp		
DS-162	~ -190bp		

From the results of sequencing, *jj4* is found to have a mutation in which the G at the start of the first intron is mutated to an A. This will likely prevent splicing of the first intron, and ultimately result in a truncated protein containing only the first exon. Thus, *jj4* is probably a null mutant of *C. elegans dragon*.

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CHAPTER 3

EXPRESSION OF *DRAGON* cDNA TO GENERATE ANTIBODIES AGAINST

DRAGON

Generating antibodies against a protein is of paramount importance for localization and biochemical assays. GFP-tagging a protein to study its expression pattern, while convenient, has its disadvantages, the most important of which is the potential interference of the GFP tag with its fusion partner, thereby leading to its misfolding and possible mislocalization. To verify the expression patterns of a DRAGON::GFP fusion protein, immunostaining using antibodies against DRAGON is thus necessary. Additionally, the antibody can be used for biochemical assays such as Western blot and immunoprecipitation.

Materials and Methods

Expression of GST::DRAGON and optimizing its solubility: Two GST::DRAGON fusion constructs were generated by cloning a 1086 bp and a 688 bp fragment of DRAGON cDNA into the GST-expression vector PGEX-6P-1. Both of these constructs encompassed regions toward the N-terminus of the protein. The 1068bp sequence was immediately 3' to the N-terminal signal peptide sequence, while the 688bp fragment was generated by restriction digestion of the 1068bp sequence. The 1068 bp sequence was PCR amplified from pDS9.5- a plasmid containing full-length *dragon* cDNA, by using primers DS-172 and DS-168. After checking 5μl of the PCR product on a 2% agarose gel, 3μl of the PCR product and 1μl of the PGEX-6P-1 vector were restriction digested at 37^0Cwith 0.3μl each of EcoRI and SalI in 2μl of

NEB EcoRI buffer, with the final volume made up to 20μl with distilled water. Simultaneously, 3μl of the PCR product was restriction digested at 37^0C with 0.3μl each of EcoRI and HaeIII in NEB #2 buffer, with the final volume made up to 20μl with distilled water. Additionally, 1μl of the PGEX-6P-1 vector was first digested with 0.3μl of EcoRI in 2μl of NEB EcoRI buffer and 16.7μl distilled water, and then digested at 25^0C with 0.3μl SmaI in 5μl NEB #4 buffer and 24.7μl distilled water. The restriction digests were loaded on a 1% agarose gel and the relevant bands cut out and gel-purified. The resulting DNA pellets were each dissolved in 8μl of distilled water. One ligation reaction was set up at 16^0C overnight by mixing 1μl of the resuspended DNA pellet of the EcoRI/SalI-digested PGEX-6P-1, 4μl of the resuspended DNA pellet of the similarly digested PCR product of DS-172 and DS-168., 1μl 10X ligation buffer, 1μl T4 ligase and 3μl distilled water. The other ligation reaction involved mixing 1μl of the resuspended DNA pellet of the EcoRI/SmaI-digested PGEX-6P-1, 4μl of the resuspended DNA pellet of the EcoRI/HaeIII-digested PCR product of DS-172 and DS-168, 1μl 10X ligation buffer, 1μl T4 ligase and 3μl distilled water. The former ligation reaction was used to generate pDS16, the plasmid containing GST fused to a 1086 bp fragment of *dragon* cDNA, while the latter reaction generated pDS17, the plasmid containing GST fused to a 688 bp fragment of *dragon* cDNA.

The ligation mix was used to transform *Escherichia coli* DH5α competent cells, which were then plated on LB-Ampicillin plates and incubated overnight at 37^0C. Individual colonies from the plates were used to inoculate 2ml of 2X YT containing 75 μg/ml ampicillin, and the tubes shaken overnight at 37^0C. Plasmid DNA was extracted from these bacterial cultures by standard Miniprep protocol, and the resulting DNA pellets each dissolved in 15μl distilled water. After checking 1μl of the plasmid DNA by restriction digestion with 0.3μl of each of SalI and EcoRI in 2μl NEB EcoRI buffer

and 16.4μl water, and loading the digests on a 1% agarose gel, two of the samples, pDS16.2 and pDS17.5 were selected for expression studies. The two samples were also sequenced to ensure that the cDNA inserts were in frame with the rest of the vector.

0.5μl of each prep of pDS16.2 and pDS17.5 was used to transform *Escherichia coli* BL21[*] competent cells. The transformed cells were plated onto LB-Ampicillin plates and incubated overnight at 37^0C. Single colonies were each used to inoculate 2ml LB-Ampicillin and the tubes were shaken overnight at 37^0C. 100μl overnight culture was used to inoculate 4ml LB-Ampicillin, which was shaken for 3 hours at 37^0C. The optical density at 600nm (A_{600}) was noted and the samples then induced with 1mM IPTG (to induce expression of the GST-fused protein), and shaken at 37^0C for 3 more hours. Prior to induction, the A_{600} should preferably be in the range 0.4 - 0.8.

To verify that the samples had been induced properly, 1ml of each induced sample was spun down at 13000 rpm for 2 minutes. The pellets were resuspended in 100μl of ice-cold 1X PBS. 100μl of 2X SDS buffer was added and the samples boiled for 10 minutes. The samples were then spun down for 10 minutes at 13000 rpm, and 20μl of each sample loaded onto 10% SDS-PAGE. The latter comprised a 10% resolving gel and a stacking gel. The former was created by mixing 3.75ml 40% acrylamide liquigel, 5ml 1.5M Tris (pH 8.8), 75μl 20% SDS, 4.15ml water, 25μl TEMED and 75μl APS, and then pouring the mixture into gel plates to allow it to solidify. The stacking gel that was poured on top of the resolving gel was created by mixing 0.62ml 40% acrylamide liquigel, 0.615ml 0.5M Tris (pH 6.8), 25μl 20% SDS, 4.025ml water, 5μl TEMED and 50μl APS. The gel was stained with Coomassie in acetic acid and methanol for 30 minutes and destained in water till the protein bands were visible.

Upon verifying that the samples had been induced, I checked if the GST::DRAGON fusion proteins were being expressed in the soluble or insoluble fraction. Each colony of transformed BL21[*] bacteria was used to inoculate 5ml LB-Ampicillin, and the tubes were shaken overnight at 37^0C. 1ml of the overnight inoculum was used to inoculate 50ml LB-Ampicillin and the A_{600} noted after 3 hours. The cells were then induced with 1mM IPTG and allowed to grow for a further 3 hours. 1ml of each culture was kept aside as a pre-treatment control, while the remaining cells were spun down for 10 minutes at 10000 rpm. The resulting pellets were washed twice with ice-cold 1X PBS and resuspended in 1.5 - 5ml ice-cold 1X ice-cold PBS (depending on the size of the pellet). Sonication was carried out on the samples (6 - 8 bursts of 20 seconds each) and the sonicates then spun down for 10 minutes at 10000 rpm. The supernatant was removed and the pellets resuspended in 1 – 1.5ml ice-cold 1X PBS. 15μl of each of the supernatant and pellet were mixed with 15μl 2X SDS buffer and boiled for 10 minutes. The samples were spun down at 13000 rpm for 5 minutes and loaded onto 10% SDS-PAGE.

In order to increase the solubility of the expressed fusion proteins and allow them to be expressed in the soluble fraction, 10 mg/ml lysozyme was added after resuspending the pellet prior to the sonication step. The volume of lysozyme was equal to 1/10 the volume of ice-cold 1X PBS added to resuspend the pellet. Additionally, Triton X-100 was added to the sonicate after the sonication step to a final concentration of 1%. In order to better solublize the expressed protein, the fusion proteins were induced for 1.5 hours instead of 3 hours, or induced at lower temperatures (30^0C for 6 hours and 18^0C for 12 hours), or grown for only 1 hour 40 minutes prior to induction instead of 3 hours.

Extracting GST::DRAGON from inclusion bodies: Often, fusion proteins are expressed in the insoluble fraction in the form of inclusion bodies, which can be solublized by treating them with urea. After sonication and separating the supernatant from the pellet, and resuspending the latter in ice-cold 1X PBS, four 100μl aliquots were taken in separate tubes. The tubes were spun down for 10 minutes at 13000 rpm and the supernatant discarded. The pellets were resuspended in 100μl of 0.1M Tris-Cl (pH 8.5) containing varying concentrations of urea (1M, 2M, 5M and 8M). The tubes were spun at 13000 rpm for 10 minutes and the supernatant set aside. The pellets were resuspended in 50μl ice-cold 1X PBS, and both the pellets and supernatants were loaded onto 10% SDS-PAGE.

Western Blot analysis of GST::DRAGON with antibodies against GST: In order to verify the identity of the overexpressed protein as GST::DRAGON, Western Blot analysis was carried out using an antibody against the GST tag. In the first step, GST::DRAGON was run on 10% SDS-PAGE using the protocol described earlier. The gel was not stained and destained to visualize the bands. A piece of nitrocellulose membrane the same size as the gel was cut and soaked in methanol for 5 seconds, and then in water for 5 minutes. The membrane was kept in cold transfer buffer (12gm Trizma base, 57.6gm glycine, 700ml methanol and 20ml 20% SDS made up to 4 liters with water). Two pieces of Whatman paper of the same size as the gel and membrane were also cut out and soaked in transfer buffer. Finally, two pieces of scotchbrite pad were washed with water and soaked in transfer buffer. A sandwich was made under the transfer buffer, where the following were assembled in the transfer cassette in the given order (from the black to white side of the cassette): scotchbrite pad, Whatman paper, gel, nitrocellulose membrane, Whatman paper, scotchbrite pad. Care was taken

to ensure there were no bubbles in the cassette, which was then locked and loaded onto the gel transfer box. An ice tray was kept in place on one side of the cassette, and transfer buffer was poured into the gel box. The transfer was allowed to take place at 70V for one hour.

After an hour, the cassette was removed from the apparatus and the transfer sandwich carefully disassembled. The nitrocellulose membrane was incubated in blocking solution (10% milk in 1X PBS with 0.1% Tween-20) at room temperature for one hour, and then washed thrice in 1X PBS with 0.1% Tween-20 (1X PBST) for 15 minutes each. After this step, the membrane was incubated overnight at 4^0C (or, for an hour at room temperature) with goat anti-GST antibody (final concentration 1:2000) in 10ml of a solution containing 5% milk and 0.1% Tween-20 in 1X PBS. The membrane was then washed thrice for 15 minutes each with 1X PBST, and incubated overnight at 4^0C (or, for an hour at room temperature) with HRP donkey anti-goat antibody (final concentration 1:10000) in 10ml of a solution containing 5% milk and 0.1% Tween-20 in 1X PBS. The membrane was washed thrice for 15 minutes each with 1X PBST and drained of excess liquid by touching it to a scrap piece of Whatman paper. The membrane was then placed on an acetate sheet and 250µl of each ECL solution added to it. The membrane was covered with another acetate sheet and placed in a cassette. An X-ray film was placed on the acetate sheet and exposed for varying amounts of time. The film was developed to visualize the bands.

Obtaining DRAGON antibodies by direct injection of SDS-PAGE gel pieces: Should the GST::DRAGON fusion protein be solublized only at high concentrations of urea (for example: 5M, 8M etc), it would not be possible to affinity purify the fusion protein using glutathione since high concentrations of urea would interfere with

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the binding of the fusion protein to the glutathione beads. In such a case, it would be expedient to produce antibodies against DRAGON by excising the corresponding band(s) out of an SDS-PAGE gel and sending the excised band(s) to Cocalico Biologicals Inc. of Reamstown PA, the company from which the Liu lab usually obtains its antibodies. To generate antibodies in rats, 175μg of protein antigen is required for each animal. Since it is usually customary to send double the amount of protein antigen in order to be on the safe side, 350μg of protein antigen per animal would be a good estimate.

The protein samples were first loaded onto a 10% SDS-PAGE placed in a large gel electrophoresis unit from Bio-Rad. The composition of the stacking and resolving gels were the same as before. The protein samples were allowed to run through the stacking gel at 11mA for 1 hour, and through the resolving gel at 20mA for 8 hours. The gel was stained with Coomassie in water for 10 minutes and destained in water for 20 minutes. The bands of interest were cut out and put into tubes. The tubes were dipped into liquid nitrogen and stored at -80^0C. They were shipped to Cocalico Biologicals Inc. over dry ice.

Results and Discussion

pDS16.2 and pDS17.5 were both expressed robustly at their expected molecular weights: 68 kD and 53 kD, respectively, albeit in the insoluble fraction. Reducing the induction temperature to 18^0C or 30^0C, or reducing the induction time to 1.5 hours did not result in the protein being expressed in the soluble fraction. Reducing the growth period prior to induction to 1 hour 40 minutes, or treating with Triton X-100 and

lysozyme did not help either. The fusion proteins were extracted into the soluble fraction only when the insoluble fraction was washed with 8M urea, a concentration that would interfere with the affinity purification of the fusion proteins. As a consequence, I decided to cut the relevant band out of a polyacrylamide gel and directly send it to Cocalico Biologicals Inc.

In order to verify the identity of the fusion protein, I carried out a Western Blot using an antibody against the GST tag of the GST::DRAGON fusion protein expressed by pDS17.5. 0.5, 1 and 2μl of the supernatant obtained after washing with 8M urea were analyzed in the Western blot. 7μl each of uninduced and induced PGEX-6P-1 vector, and 10μl of uninduced pDS17.5 were used as controls. The X-ray film was exposed for 15 minutes.

From the Western Blot, one can see that the PGEX-6P-1 was induced robustly, though there appeared to be some nominal expression of GST in the uninduced vector as well. The latter can be explained by spillover from the adjacent lane. The uninduced pDS17.5 vector did not give a signal. The signal from 0.5μl of the 8M urea wash was too faint to be detected, while 1μl and 2μl of the 8M urea wash gave distinct bands. Surprisingly, the signal from probing 2μl of the wash was fainter than the signal from 1μl of the wash. The expected molecular weight of the expressed GST should have been 26 kD and that of the expressed GST::DRAGON fusion protein 53 kD. The molecular weights of the bands corresponding to GST and GST::DRAGON observed in the Western Blot tallied closely with the expected values.
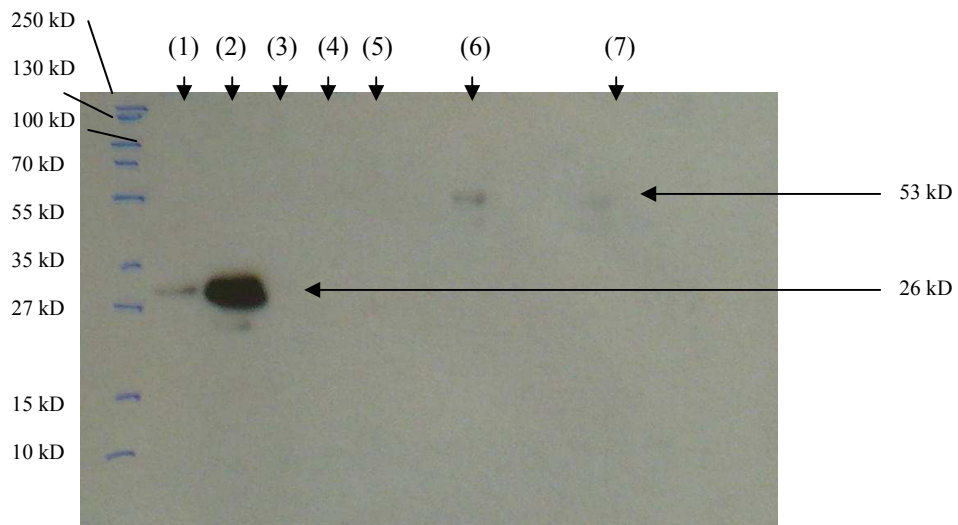
Figure 3-1: Western Blot of expressed fusion protein using antibody against GST. The lanes from left to right: (1) MW marker (2) Uninduced PGEX-6P-1, (3) Induced PGEX-6P-1, (4) Uninduced pDS17.5, (5) 0.5µl Induced pDS17.5, (6) 1µl Induced pDS17.5, (7) 2µl Induced pDS17.5. The molecular weights of the marker bands are shown in the figure.
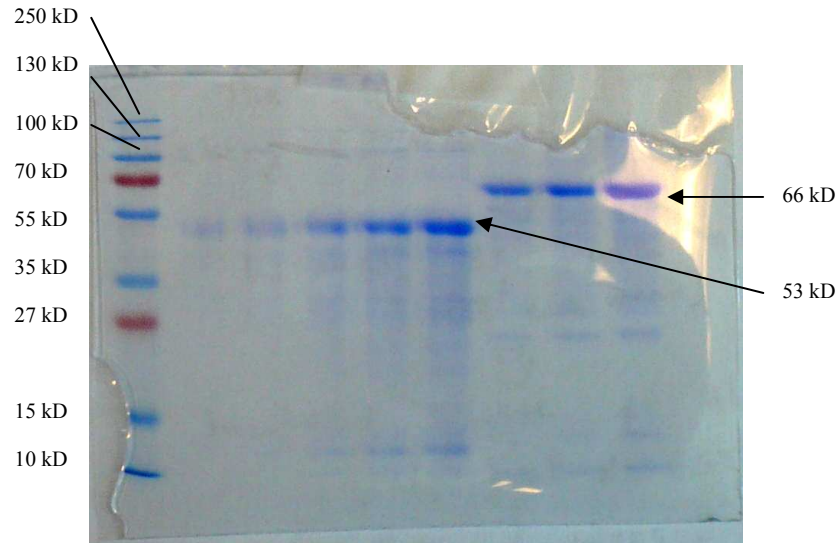
Figure 3-2: Quantifying the amount of GST::DRAGON expressed by pDS17.5. The lanes from left to right: (1) MW marker, (2) 1μl of a 1:10 dilution of a 8M urea wash of GST::DRAGON, (3) 2μl of a 1:10 dilution of a 8M urea wash of GST::DRAGON, (4) 5μl of a 1:10 dilution of a 8M urea wash of GST::DRAGON, (5) 1μl of a 8M urea wash of GST::DRAGON, (6) 2μl of a 8M urea wash of GST::DRAGON, (7) 5μl of 0.5 mg/ml BSA, (8) 5μl of 1 mg/ml BSA, (9) 5μl of 2 mg/ml BSA. The molecular weights of the marker bands, expressed fusion protein and BSA are shown in the figure.

To generate anti-DRAGON antibodies in a rat, the company needed 175μg protein antigen per animal. Since we had decided to use two animals, and since our lab usually sends double the amount of protein antigen required by the company, I ran a total of 700μg of the fusion protein expressed by pDS17.5 on a polyacrylamide gel and cut out the bands corresponding to the fusion protein of interest. The fusion protein expressed by pDS16.2 was not used to generate antibodies since its expression yield was considerably lower than that of pDS17.5. I quantified the amount of GST::DRAGON by running 1μl, 2μl and 5μl of a 1:10 dilution, and 1μl and 2μl of the supernatant obtained after extracting with 8M urea, along with 5μl each of 0.5 mg/ml, 1 mg/ml and 2 mg/ml of BSA.

By comparing the intensities of the bands, I was able to determine that the concentration of GST::DRAGON in the supernatant obtained after washing with 8M urea was 2.5 mg/ml. For 700μg of GST::DRAGON, I thus needed to load 280μl of the supernatant obtained after washing with 8M urea. To be on the safe side, I loaded 320μl. Once the antibody is available from Cocalico Biologicals Inc., in the first round of purification, the antibody could be purified against the bacterially expressed fusion protein bound to nitrocellulose (Smith and Fisher, 1984). In the second round of purification, the antibody can be further purified by pre-adsorption with extracts from *jj4* larvae (Maloof and Kenyon, 1998). The purified antibody can then be used for biochemical analysis, as well as to determine the cellular localization of DRAGON.

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CHAPTER 4

GENERATION OF STRAINS FOR EPISTATIS ANALYSIS OF *DRAGON*

dbl-1 codes for the ligand of the Sma/Mab TGFβ signaling pathway, while LON-2 binds to DBL-1 and negatively regulates the Sma/Mab TGFβ signaling pathway by attenuating the amount of DBL-1 (Gumienny *et al.*, 2007; Suzuki *et al.*, 1999). *sma-6* codes for the type I receptor that receives the signal from the ligand. Both *dbl-1(wk70)* and *sma-6(jj1)* animals are much shorter than wild-type N2 worms, while *lon-2(e678)* animals are longer. Maduzia *et al.* and Morita *et al.* (2002) identified *lon-1*as a target of the Sma/Mab TGFβ signaling pathway. *lon-1(e185)* animals are longer than wild-type N2 worms. Maduzia *et al.* also showed that *lon-1* mRNA levels were up-regulated in *sma-6(0)* animals, thus providing evidence that *lon-1* is negatively regulated by the Sma/Mab TGFβ pathway.

The *jj4* mutant phenotype is characterized by a small body size, similar to that observed in other Sma/Mab TGFβ pathway mutants such as *dbl-1(wk70)* and *sma-6(jj1)*. In this chapter, I will discuss the generation of double mutants of *jj4* with several other mutants in the Sma/Mab TGFβ pathway (*dbl-1(wk70)*, *ctIs40(dbl-1++)*, *lon-1(e185)*, *lon-2(e678)* and *sma-6(jj1)*) to place *jj4* in the context of the pathway.

Mutations in the Sma/Mab TGFβ pathway also result in a male tail patterning defect, in which rays 4 and 5, 6 and 7, and 8 and 9 fuse together (Suzuki *et al.*, 1999; Krishna *et al.*, 1999; Savage-Dunn *et al.*, 2000). It would be interesting to study the male tail in *jj4* mutants to see if its patterning bears any similarity to that of other mutants in the pathway. Additionally, while conducting epistasis analysis between *jj4* and other members of the TGFβ signaling pathway, one needs to measure the body sizes of the

various mutant strains. Previous work in the lab has indicated that the presence of a GFP transgene in a strain could affect the body size of that strain. Thus, the GFP needs to be removed. The following section will also discuss strategies to outcross GFP from strains, as well as generate a *jj4* male strain.

Materials and Methods

C. elegans strains: All *C. elegans* strains were maintained using standard methods (Brenner, 1974) and kept in incubators set to 11^0C, 14^0C, 16^0C or 20^0C. The strains referred to in Chapter 4 are as follows:

LW1427: N2

LW0049: *ccIs4438 (intrinsic cc::gfp)* III*; ayIs2(egl-15::gfp)* IV*; him-5(e1467)* V; *ayIs6(hlh-8::gfp) (36x)* X

LW0058: *unc-9(e101) sma-9(cc604) ayIs6(hlh-8::gfp) arIs39(cc::gfp)* X

LW0614: *cup-5(ar465) sma-3(jj3)* III*; cc::gfp*

LW1085: *ccIs4438 (intrinsic cc::gfp)* III*; ayIs2(egl-15::gfp)* IV; *dbl-1(wk70)* V*; ayIs6(hlh-8::gfp)* X

LW1288: *sma-6(jj1)* II*; cc::gfp*

BW1940 *ctIs40(dbl-1++) [ZC421(+); sur-5::gfp]* X

jj4 I*; cc::gfp*

hT2[qIs48]/tm1502

lon-1(e185) III

lon-2(e678) X

Crosses: All crosses were set up at either 16^0C or 20^0C, and involved placing 3 - 4 L4 hermaphrodites with 12 – 15 L4 larvae or young adult males. Cross progeny were collected after 4 - 5 days.

Generating the *jj4* male strain, and *jj1*, *jj3* and *jj4* without GFP: The cross schemes for generating a *jj4* male strain and a *jj4* strain without GFP are shown in Figure 4-1. It may be noted the presence of the *him-5* mutation results in an increased frequency of X chromosome non-disjunction, and therefore an increased frequency of males.

Generating the *lon-1(e185) jj4* double mutant: Double mutants of *jj4* with *lon-1* were generated in order to place *jj4* in the context of the Sma/Mab TGFβ signaling pathway. *lon-1* animals have body sizes longer than wild-type N2 worms. If *jj4* were acting downstream of *lon-1*, the corresponding double mutant would have a small body phenotype. Should *jj4* be acting upstream of *lon-1*, the corresponding double mutant would have a long body size phenotype. Conversely, if *jj4* were acting in parallel with *lon-1*, the corresponding double mutant would have an intermediate body size phenotype. The cross scheme for generating a double mutant between *jj4 and lon-1(e185)* is illustrated in Figure 4-2.

$$\underline{him\text{-}5} \ \male \quad X \quad \underline{jj4} \quad \underline{cc:gfp} \ \female$$
$$him\text{-}5 \qquad\qquad\quad jj4 \quad cc:gfp$$

$$\underline{him\text{-}5} \qquad \underline{jj4} \quad \underline{cc:gfp} \ \male\!\!\!\female$$
$$+ \qquad\quad + \qquad +$$

$$\underline{him\text{-}5} \qquad \underline{jj4} \ \underline{+} \ \male\!\!\!\female \qquad\qquad \underline{+} \qquad \underline{jj4} \ \underline{+} \ \male\!\!\!\female$$
$$him\text{-}5 \qquad\quad jj4 \ + \qquad\qquad\qquad + \qquad\quad jj4 \ +$$

A similar cross scheme was used to generate *jj1* and *jj3* worms without GFP.

Figure 4-1: Cross scheme for generating the *jj4* male strain, and *jj1, jj3 and jj4* strains without GFP

lon-1 ♀   X   him-5 ♂

lon-1   him-5

↓

lon-1 ♂   X   jj4 ♀

  +   jj4

↓

Pick 25 worms and allow them to self-fertilize in individual plates. Of these 25 plates, pick 2 plates which give all long (i.e. homozygous *lon-1*) progeny. Allow one worm from each of the 2 plates to self-fertilize.

lon-1   jj4 ☿   OR   +   jj4 ☿

  +   +   +   +

↓

lon-1   +

lon-1   +

Pick a total of 24 worms from the progeny of the above 2 worms. Allow the 24 worms to self-fertilize, and then sequence for *jj4*. Select worms that are homozygous for *jj4*.

lon-1   jj4
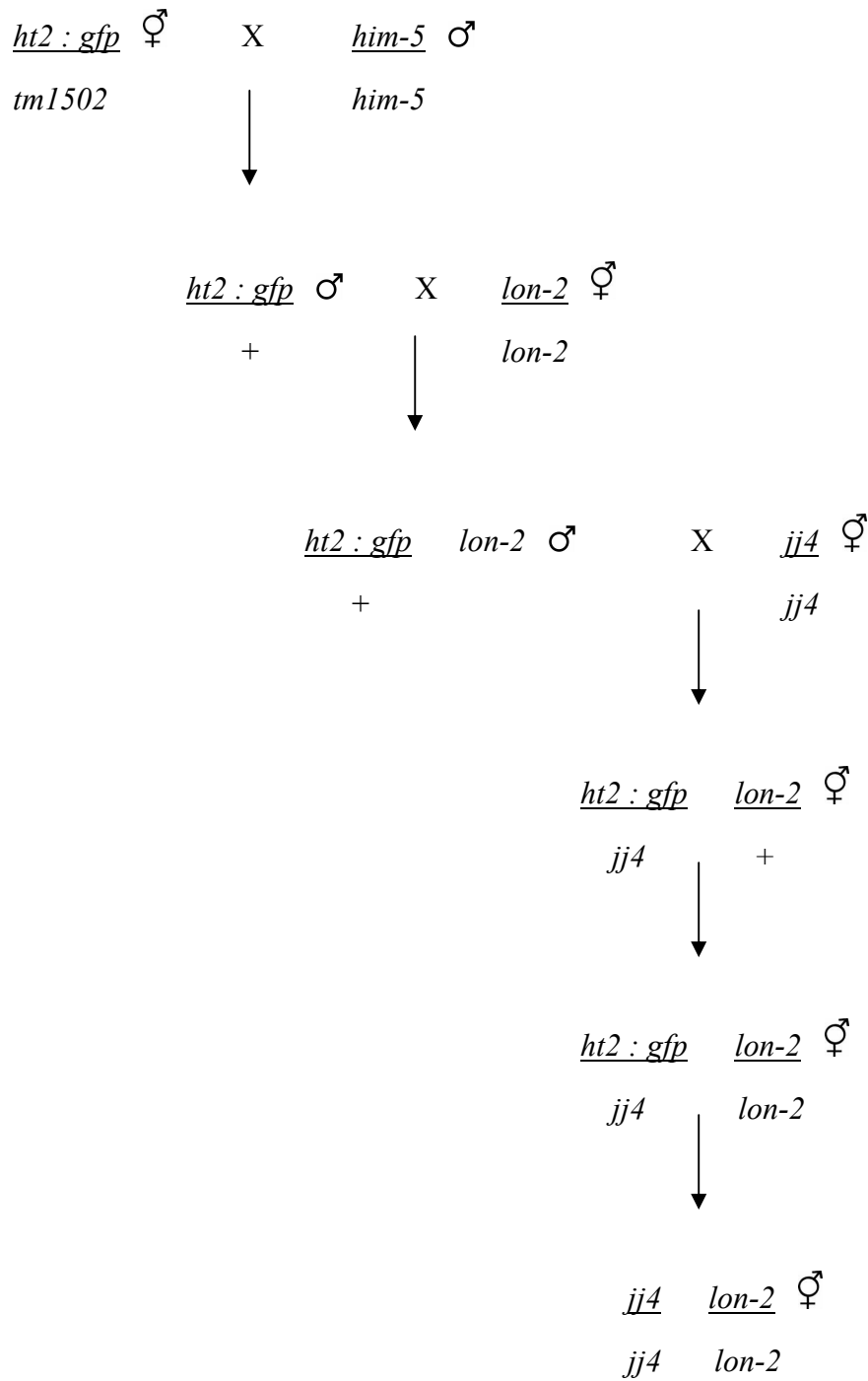
lon-1   +

lon-1   jj4

lon-1   jj4

Figure 4-2: Cross scheme for generating the *lon-1(e185) jj4* double mutant
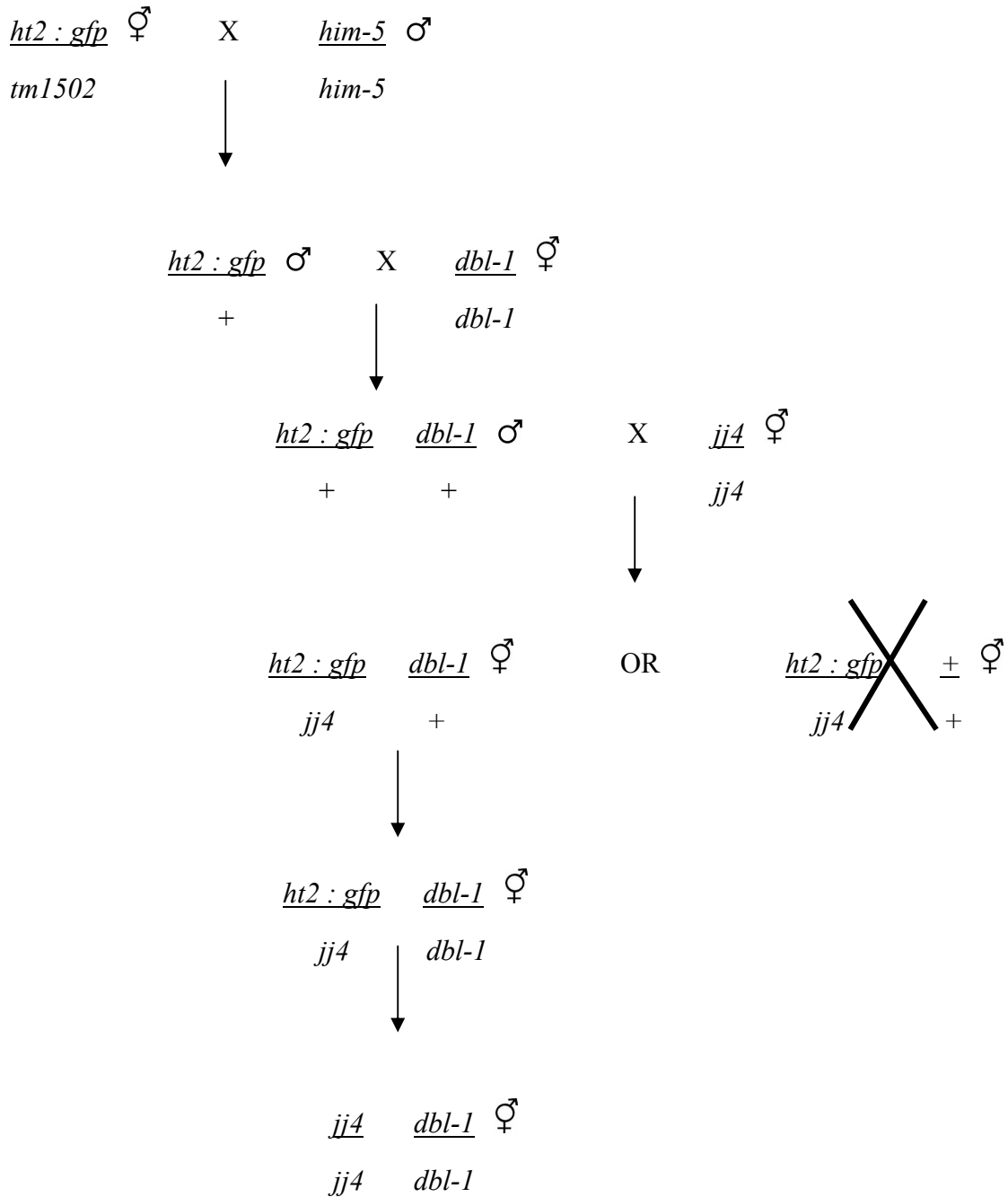
Generating the *lon-2(e678) jj4* and *ctIs40(dbl-1++) jj4* double mutants: *ctIs40(dbl-1++)* animals, which overexpress DBL-1 contain an integrated array of multiple copies of *dbl-1*. Both *ctIs40* and *lon-2(e678)* animals have extended body lengths. If *jj4* were acting downstream of *lon-2* or *dbl-1*, the corresponding double mutant would have a small body phenotype. Should *jj4* be acting upstream of *lon-2* or *dbl-1*, the corresponding double mutant would have a long body size phenotype. Conversely, if *jj4* were acting in parallel with *lon-2* or *dbl-1*, the corresponding double mutant would have an intermediate body size phenotype. The cross scheme for generating double mutants between *jj4 and lon-2(e678),* and *jj4* and *ctIs40(dbl-1++)* is illustrated in Figure 4-3.

Generating the *dbl-1(wk70) jj4* and *sma-6(jj1) jj4* double mutants: The cross scheme for generating double mutants between *jj4 and dbl-1(wk70),* and *jj4* and *sma-6(jj1)* is illustrated in Figure 4-4.

ht2 : gfp ♀   X   him-5 ♂

tm1502   him-5

        ↓

    ht2 : gfp ♂   X   lon-2 ♀

     +   lon-2

        ↓

     ht2 : gfp   lon-2 ♂   X   jj4 ♀

       +   jj4

           ↓

        ht2 : gfp   lon-2 ♀

         jj4   +

          ↓

        ht2 : gfp   lon-2 ♀

         jj4   lon-2

          ↓

        jj4   lon-2 ♀

        jj4   lon-2

A similar cross scheme was used to generate
ctIs40(*dbl-1++*) *jj4* double mutants

Figure 4-3: Cross scheme for generating the *lon-2(e678) jj4 and ctIs40(dbl-1++)* *jj4* double mutants

ht2 : gfp ♀   X   him-5 ♂

tm1502   him-5

ht2 : gfp ♂   X   dbl-1 ♀

+   dbl-1

ht2 : gfp   dbl-1 ♂   X   jj4 ♀

+   +   jj4

ht2 : gfp   dbl-1 ♀   OR   ht2 : gfp   ± ♀

jj4   +   jj4   +

ht2 : gfp   dbl-1 ♀

jj4   dbl-1

jj4   dbl-1 ♀

jj4   dbl-1

A similar cross scheme was used to
generate *sma-6* (*jj1*) *jj4* double mutants

Figure 4-4: Cross scheme for generating the *dbl-1(wk70) jj4 and sma-6(jj1) jj4* double mutants

Results and Discussion

Using the cross schemes outlined in methods, I generated *jj1*, *jj3* and *jj4* strains without GFP. There were three isolates for each of *jj1* and *jj3* strains without GFP: 1A1, 1D1 and 1D3, and 2A3, 2D3 and 2C3 respectively. The single isolate of *jj4* without GFP was 2B, while there were nine isolates of the *jj4* male strain: 2B2, 2B1, 1A1, 1B1, 1A5, 2A2, 2A1, 1A4 and 1A3. In preliminary experiments, I assessed the length of the double mutant worms by eye. The three isolates of the *lon-1(e185) jj4* double mutant, B4, B5 and B18 (without GFP) seemed to resemble the long *lon-1(e185)* single mutant, suggesting that *lon-1* might be acting downstream of *jj4*. The *ctIs40(dbl-1++) jj4* and *lon-2 jj4* double mutants seemed to be intermediate in size between the *lon-2(e678)* and *ctIs40(dbl-1++)* single mutants, and *jj4*. This suggests that *jj4* might act parallel to the Sma/Mab TGFβ signaling pathway at the level of *lon-2* and *dbl-1*. The two isolates of the *lon-2(e678) jj4* double mutant did not carry any GFP and were designated as AI and BIII. The two isolates of the ctIs40(*dbl-1++) jj4* double mutant, #2 and #4 carried coelomocyte GFP as well as sur-5::GFP integrated into the transgene array carrying multiple copies of *dbl-1*. The *sma-6(jj1) jj4* and *dbl-1(wk70) jj4* double mutants resembled the single mutants, suggesting that *jj4* might be acting within the Sma/Mab TGFβ pathway at the level of the receptor SMA-6 and ligand DBL-1. These preliminary results need to be verified by repeating the crosses and obtaining exact measurements of the single mutants and double mutants. The body sizes of the single mutants and double mutants are summarized in Table 4-1. The three isolates of the *dbl-1(wk70) jj4* double mutant without GFP were 1A, 1B and 2A, while the single isolate with ceolomocyte GFP was 2B. There were three isolates apiece of the *sma-6(jj1) jj4* double mutant with and without ceolomocyte GFP: 1C, 2B and 2C, and 1A, 1B and 2A respectively.

Table 4-1: Summary of *C. elegans* strain sizes used in epistasis analysis, as estimated by visual observation

STRAIN	ESTIMATED SIZE
ctIs40(dbl-1++)	LONG
lon-1(e185)	LONG
lon-2(e678)	LONG
dbl-1(wk70)	SMALL
sma-6(jj1)	SMALL
jj4 ctIs40(dbl-1++)	INTERMEDIATE
jj4 lon-2(e678)	INTERMEDIATE
jj4 dbl-1(wk70)	SMALL
jj4 sma-6(jj1)	SMALL
jj4 lon-1(e185)	LONG

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CHAPTER 5

GENERATION OF PLASMID CONSTRUCTS FOR TRANSGENE RESCUE
EXPERIMENTS

The RNAi and sequencing experiments described in Chapter 2 identified *jj4* as a
mutation in Y71G12B.16- the *C. elegans* homolog of DRAGON, a potential co-
receptor of the Sma/Mab TGFβ signaling pathway that has been shown to potentiate
BMP signaling in mouse and *Xenopus* (Samad *et al.*, 2005). In order to verify that *jj4*
is indeed a mutation in *dragon*, a construct containing full-length *dragon* driven by its
own promoter must be introduced into mutant animals in order to test for rescue of the
mutant phenotype. Additionally, to gain further insights into the function of *dragon*,
its temporal and spatial expression patterns must be determined. The former can be
determined by expressing *dragon* cDNA under the control of a heat-shock promoter at
different times in the life cycle and determining when it must be expressed for its
proper function. The latter can be determined by a transcriptional fusion construct,
which will shed light on the subset of cells and tissues in which DRAGON is
expressed. One can also determine if *C. elegans* DRAGON needs to be expressed in
the M lineage for its proper function by forcing its expression under the control of a
M-lineage specific promoter. In the following section, I shall discuss the schemes for
generating the constructs that can be used for carrying out the above experiments.

Materials and Methods

Generation of the transcriptional fusion construct: The transcriptional fusion
construct was generated by introducing a 4kb fragment of the *dragon* promoter along

with its first exon and intron upstream of GFP containing a nuclear localization signal (NLS). lacZ and an unc-54 3'UTR were located downstream of the GFP. The lacZ moiety, on account of its large size would ensure that the expressed GFP would remain within the nucleus. The first exon and intron were incorporated in the construct since regulatory sequences are often present in the first exon and intron of *C. elegans*. ATG was removed from the first exon to prevent its translation.

The 4kb promoter fragment was PCR amplified from N2 DNA by using primers DS-163 and DS-164. 10µl of 5X iProof High GC Buffer was mixed with 0.5µl of 25µM of each primer, 1µl 10mM dNTPs, 0.2µl N2 DNA, 0.5µl iProof polymerase and 37.3µl water. The PCR mix was initially incubated for 30 seconds at 98^0C, and then taken through 30 cycles of 10 seconds at 98^0C, 30 seconds at 59^0C and 2 minutes at 72^0C. The PCR was then taken through 10 minutes at 72^0C and held at 4^0C. The PCR product was purified by a standard phenol-chloroform extraction protocol and the final pellet dissolved in 10µl water. 4µl of the latter was digested at 37^0C with 0.3µl of each of PstI and SalI, 2µl NEB #3 buffer and water made up to a final volume of 20µl. Concomitantly, 1µl of pPD96.04 (a gift from Dr. Andrew Fire) diluted 1:10 was also digested in a similar fashion. The digests were run on a 1% agarose gel and the relevant bands cut out and gel purified. The digested PCR product extracted from gel purification was dissolved in 5µl water, while the digested plasmid was dissolved in 6µl water. A ligation reaction was set up overnight at 12^0C with 1µl of the digested plasmid, 5µl of the digested PCR product, 1µl T4 ligase, 1µl 10X ligation buffer and 2µl water. The ligation mix was used to transform DH5α cells, which were then spread over LB-Ampicillin plates. Single colonies were used to inoculate 2X YT with 75 µg/ml Ampicillin, and the plasmid DNA was extracted by standard Miniprep procedure. The plasmid DNA was checked both by restriction digestion as well as by

sequencing. The plasmid containing 4kb of the promoter upstream of GFP, NLS, LacZ and unc-54 3'UTR was denoted as pDS14.

DS-180 and DS-166 were used to PCR amplify the first exon (minus ATG) and intron from N2 DNA using a protocol similar to that used for amplifying the promoter fragment. However, instead of allowing the primers to anneal at 59^0C, annealing was done at 61^0C and the 72^0C extension was carried out for 1 minute 10 seconds only. Since the yield of the PCR product was extremely low, the PCR product was TOPO-cloned into One Shot TOP10 competent cells using a protocol described in a previous section. The TOPO plasmid containing the PCR product insert was initially restriction digested with 0.3µl SalI, 2µl NEB #3 buffer and 16.7µl water, and then with 0.5µl SmaI, 5µl NEB #4 and 24.5µl water. pDS14 was also similarly digested. The restriction digests were loaded onto a 1% agarose gel, and the relevant bands cut out. The DNA was extracted from these bands and ligated as described previously. The ligation mix was used to transform DH5α competent cells and plasmid DNA was extracted by a standard Miniprep procedure. The plasmid DNA was restriction digested as well as sequenced to verify its identity. The transcriptional fusion construct containing 4kb of the promoter fragment, the first exon (minus ATG), the first intron, GFP, NLS, LacZ and unc-54 3'UTR was denoted as pDS15.

Generation of the hlh-8p::*dragon* cDNA::unc-54 3'UTR construct: The construct containing *dragon* cDNA driven by the hlh-8 promoter was generated by inserting full-length *dragon* cDNA downstream of the hlh-8 promoter in the vector pJKL502 (obtained from Dr. Kelly Liu). Full length cDNA was amplified from pDS9 by primers DS-169 and DS-171 by making a PCR mix containing 10µl 5X iProof Hi Fi Buffer, 0.5µl of 25µM of each primer, 1µl 10mM dNTPs, 1µl pDS9 diluted 1:10,

0.25μl iProof polymerase and 36.25μl water. The iProof PCR protocol discussed previously was used for the PCR, with the annealing temperature maintained at 61^0C and extension allowed for 40 seconds in each cycle. The PCR product was purified and the final pellet was dissolved in 10μl water. 3μl of the latter was digested with 0.3μl each of XbaI and EcoRI in NEB #2 buffer. 1μl pJKL502 was digested similarly and both restriction digests loaded onto a 1% agarose gel. The relevant bands were cut out of the gel and the DNA gel purified from them. The digested plasmid was resuspended in 4μl water, while the digested PCR product was resuspended in 8μl water. A ligation reaction was set up as described previously with 1μl of the digested pJKL502 and 4μl digested PCR product. The ligation mix was used to transform DH5α cells, which were then plated. The plasmid DNA extracted by a conventional Miniprep protocol was checked by restriction digestion and sequencing. The hlh-8p::*dragon* cDNA::unc-54 3'UTR construct was named pDS20.

Generation of hsp-16p::*dragon* cDNA::unc-54 3'UTR construct: The hsp-16 promoter is contained in a plasmid lig779 (obtained from Dr. Andrew Fire). 1μl each of lig779 and pDS20 were digested with 0.3μl each of HinDIII and NheI in 2μl NEB #2 buffer and water. The digests were loaded onto a 1% agarose gel, and the relevant bands were cut out and the DNA was gel purified out of the bands. 5μl of the digested lig779 and 1μl of the digested pDS20 were ligated as described previously. The ligation mix was then used to transform DH5α competent cells, which were then plated. The plasmid DNA extracted by the Miniprep protocol was checked by restriction digestion and sequencing. The plasmid containing hsp-16p::*dragon* cDNA::unc-54 3'UTR was denoted as pDS21.

Generation of the full-length rescue construct: The full-length rescue construct contains the entire ORF of *dragon*, 4kb of the upstream promoter sequence and 1kb of a downstream sequence (hypothesized to contain the 3'UTR). This DNA sequence was PCR-amplified out of N2 DNA. The PCR mix contained 10μl of 5X iProof Hi Fi Buffer, 1μl 10mM dNTPs, 0.5μl of each of DS-163 and DS-142 (at 25μM each), 0.5μl N2 DNA, 0.5μl iProof polymerase and 36μl water. The reaction was initially incubated at 98^0C for 30 seconds. It was then taken through 10 cycles of 10 seconds at 98^0C, 30 seconds at 60^0C and 5 minutes at 72^0C. Following this, the reaction was taken through 20 cycles of 10 seconds at 98^0C, 30 seconds of 60^0C and 5 minutes + 20 seconds over the previous cycle at 72^0C. The reaction was finished by keeping it at 72^0C for 10 minutes and holding it at 4^0C. The PCR product was checked by running it on a 1% agarose gel. It was then TOPO-cloned into One Shot TOP10 chemically competent cells. Plasmid DNA was extracted as described in a previous section and checked by sequencing and restriction digestion. However, upon sequencing, it was discovered that there was extra T nucleotide at the 3' end of the ORF. This plasmid was denoted as pDS27. To eliminate this problem, a 1201bp portion of the 3' end of the ORF was excised by restriction digestion and replaced with an identical sequence from pDS22, which did not contain the aberrant nucleotide. pDS22, it may be noted is a plasmid that contains the *dragon* ORF and 1kb of sequence downstream of it, in a pJKL502 backbone. 1μl of pDS27 was first digested for 2 hours at 37^0C with 0.3μl HpaI in 2μl NEB #4 buffer and 16.7μl water, and then with 0.5μl MluI in 5μl NEB #3 buffer and 24.5μl water. 1μl of pDS22 was also digested thus. The restriction digests were loaded onto a 1% agarose gel, and the relevant bands were out. The DNA was extracted from the bands by conventional gel purification, and the final DNA pellet was dissolved in 20μl in case of the digested pDS27 and 5μl in case of the digested pDS22. A ligation reaction was set up as described previously with 1μl of pDS27 and

5μl of pDS22. The ligation mix was used to transform DH5α competent cells, and the final plasmid that was obtained was sequenced to verify that there were no mutations present.

Results and Discussion

The constructs described above were all restriction digested and sequenced to verify their identity. These constructs will provide valuable information about the temporal and spatial expression patterns of *C. elegans dragon*. They shall be co-injected into gravid *C. elegans* adults with a plasmid pRF4 containing a dominant *rol-6(su1006)* marker (after Mello *et al.*, 1991). Transformants can be then identified by looking for the roller phenotype in the progeny, induced by the presence of the *rol-6* marker.

With the transcriptional fusion construct, one can observe expression of GFP in the nuclei of various cell types, where its expression is being driven by the *dragon* promoter. Additionally, one can also observe GFP expression in different larval stages as well as in adult worms to check for any change in the pattern of expression over time.

Foehr and colleagues (2006) were able to show that the antagonism between *sma-9* and the Sma/Mab TGFβ signaling pathway takes place in the M-lineage. Since *dragon* is thought to act as a co-receptor in the Sma/Mab TGFβ signaling pathway, it would be interesting to see if it too can suppress *sma-9* when expressed specifically in the M-lineage. This experiment can be carried out by injecting the hlh-8p::*dragon* cDNA::unc-54 3'UTR construct in *sma-9 jj4* double mutants.

To determine what stage in the life cycle of *C. elegans* must *dragon* be expressed for its proper function, one can inject the hsp-16p::*dragon* cDNA::unc-54 3'UTR construct into *jj4* mutants. The hsp-16 promoter can be induced by subjecting worms to heat shock treatment at 37^0C for one hour. By inducing the hsp-16 promoter at different stages in the life cycle of *C. elegans* and observing for rescue of the *jj4* mutant phenotype (i.e. small body size), one can determine the time period in which *dragon* needs to be expressed for its function of regulating body size. Conversely, the construct can also be injected into *sma-9 jj4* double mutants, and after inducing the expression of *dragon*, one can look for reversal of the suppression of *sma-9* by *jj4*. Thus, one can determine the stage at which *dragon* needs to be expressed in order to be able to suppress the *sma-9* mutant phenotype.

Lastly, the full-length rescue construct can be used to verify the identity of *jj4* as a mutation in *dragon*. Should the full-length rescue construct be able to rescue the small body size phenotype of *jj4* mutants, this piece of data, together with the results of the RNAi and sequencing experiments (discussed in the second chapter) shall definitively confirm *jj4* to be a mutation in *dragon*.

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