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Bachelor thesis

Making Transgenic *C. elegans* with Polycistronic mCherry Vector

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Annotation

In this work, constructs pJW524, pJW526, pJW774, pJW776 were introduced to healthy adult *C. elegans* to establish stable transgenic lines by microinjection.

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České Budějovice, 10 May 2012

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Dominik Farka

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Last but not least, I would like to thank my family for their support.

Aim of the Project

In order to investigate gene function and expression in transgenic *C. elegans*, fusion of fluorescent proteins with the protein of interest is a widespread and routine approach.
However, during this approach, there is always a chance that the fused protein will be misfolded which would make it non-functional. For that reason the use of polycistronic mCherry construct was developed, bypassing the problem by translating proteins separately, though, through a single promoter. With this approach, the protein of the interest can be expressed in tissue-specific manner and the expression can be monitored by mCherry fluorescent protein.

Up until now, only few examples of the use of polycistronic mCherry constructs were reported using neuron or germline specific promoters.

In this study, I used polycistronic mCherry vector containing tissue specific promoters (*egl-17*, *grl-21*, and *wrt-2*) driving the expression of different proteins (NHR-25 and SMO-1) to establish transgenic *C. elegans* strains and monitored their expressions in live animals.

Abstract

Creation of transgenic animals has become a popular method to analyse gene function. In the nematode *Ceanorhabditis elegans* transformation is widely used and can be achieved by microinjection. For functional analyses, transgene constructs typically contain a promoter driving the expression of the protein of interest that is fused to a fluorescent protein. However, as this fusion of proteins can lead to misfolding of the protein of interest and may not reflect proper function, a modification of the expression vector has been developed; introducing a short sequence of non-coding DNA in-between the sequences of the two proteins and making the construct compatible with a polycistronic operon system.

In this study, four different polycistronic constructs were introduced into *C. elegans* by means of microinjection in order to provide new tools for the analyses of gene function. Tissue specific promoters *wrt-2* (seam cells), *grl-21* (*hyp*7), and *egl-17* (vulval precursor cells) were used to over-express either NHR-25 or SMO-1 in the corresponding tissues and the expression was visualized by independently translated mCherry red fluorescent. 10 independent transformed C. elegans strains were established and corresponding tissue-specific promoter activities were confirmed. Furthermore, in some cases, ectopic behaviour was observed e.g. ectopic mCherry expression in different tissues or specific cell differentiation defects that was most likely caused by the overexpression of NHR-25 or SMO-1. This study was the first case in our laboratory to generate transformed C. elegans utilizing the polycistronic mCherry vector system. New genetic tools were introduced in the laboratory useful for further analyses of gene function.

1. Introduction

1.1 *C. elegans*- an overview

The small, free-living soil nematode *Caenorhabditis elegans* is a commonly used model organism in genetics analysis. There are various reasons for this; amongst them is its small size, easy handling under lab-conditions, and short reproductive cycle of only 3 days at 20-25 °C. These points are important to mention as the possibility to handle large populations of animals gives strong statistic power that is needed for experimental work[1]. Addition to this, functional analyses (forward and reverse genetics) can be approached and various forms of RNA interference (RNAi) are available in the worm making RNAi screening possible[2].

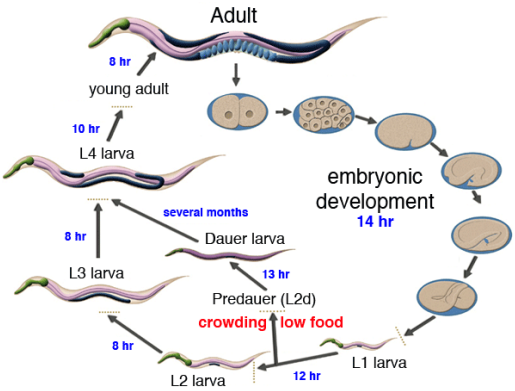

Figure 1: The life cycle of Ceanorhabditis eleagans

Found in two sexes, *C. elegans* shows hermaphrodite or male phenotype whereby the hermaphrodite produces both sperm and oocytes. The latter occurs due to normal distribution of the X chromosome (XX) whereas the male occurs due to malfunction of this chromosome (XO). This genetic error occurs once in 500 offspring. Mating between those two sexes is possible, leading to a 1:1 ratio with respect to sex of the F1 generation[1].

The adult hermaphrodite lays about 300 eggs during its reproductive life span. Juvenile worms hatch and develop through four larval stages referred L1-L4 and no metamorphosis occur. Every stage is preceded by moulting which makes it so crucial in *C. elegans* development. In case of food shortage the worms can change its development at L1 or early L2 towards the so called Dauer-stage in which it can survive for many months. At this stage, growth is stopped and several changes occur in the worm. The worm turns dark as granules, which are thought to be storage for energy, appear within its intestine. They have reduced metabolic rate and are more resistant to oxidative stress. Another difference lies in change in cuticle [3]. In general, this type of cuticle is thicker and more reinforced than in any other larval stages. It protects the worm from desiccation, detergent solubilisation, as well as from acidic or hypertonic conditions. The special cuticle structure called alae is formed above the seam cells on L1, dauer larva and adult worms.

Another important aspect is the worm's genetics. Consisting of 6 chromosomes, the genome of *C. elegans* is spread over 5 autosomal and one sex chromosome X. Most of the genetic information is covered only once in the genetic code and as little as 12% consists of

repeats such as the transposable element Tc1 [1]. The genome size is about 100 Mb and the whole genome was sequenced by 1998 [20]. The information is freely available at the website wormbase.org. The overall number of protein-coding genes in *C. elegans* is about 20 000 [18]. The genome also contains about 1300 functional noncoding RNAs. [19]

Yet another important part, I will mention anatomy briefly. An adult hermaphrodite *C. elegans* consists of 959 somatic nuclei whereas the adult male has 1031. Throughout the whole life cycle and all stages the worms stay transparent making developmental study at cellular level possible. Some tissues such as hypodermis are formed with synthetial cells. *C. elegans* is a typical nematode with mirror-symmetry. The cuticle-covered outer tube consisting of hypodermis, muscle and seam cell tissue as well as nerve cord contains gonad and intestine [1]. Detailed description of hypodermis, seam, and vulva cells is covered in the chapter "Tissue specific promoters".

Further, the worm is equipped with a rather simple but well functioning nervous system. This enables it to react to mechanical, thermal, chemical, and nutritional stimuli by staying in its current environment or fleeing it by undulatory movement in forward or backward direction [1].

1.2 Transcription Machinery

Within the eukaryotic cell, transcription of DNA to mRNA and translation of mRNA to protein take place. This is also true for *C. elegans*. Depending on differences in splicing of the particular gene, different products are possible. Another possibility for changes in protein structure is posttranslational modification of the mature protein.

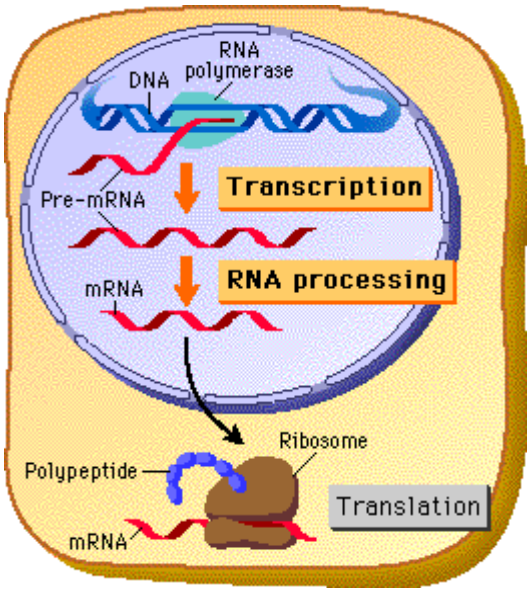

Figure 2: Transcription and translation [17]

To make transcription of a gene possible, different functional elements have to be present in the DNA sequence. A promoter region which is responsible for binding of RNA polymerase, a transcription initiation site or cap sequence at which the transcription starts and where the protective cap is localized at the mRNA, a translation initiation site or start-codon which is always ATG at which translation starts and finally exons (coding) and introns (non- coding). The end of the translated

sequence is described by a stop codon which is followed by an untranslated region which serves at mRNA level as an anchor for polyadenilation an thereby protects the mRNA in the cytosol from being digested by enzymes.

Promoters and enhancers of transcription, are usually localized upstream of the site where transcription is initialized but can be as well at its end or even within the sequence. Most of these promoters contain the sequence TATA which is known as the TATA-box.
During transcription, the DNA inside of the nucleus is firstly transcribed to nuclear RNA cutting the promoter sequence off, leaving the cap, exons, introns, and transcription initiation and initiation sites in the sequence, as well as the polyadenylation site. In the next step of transcription, introns are spliced out as and mRNA is formed. This is then released from the nucleus to the cytosol. With the help of ribosomes, a cluster of rRNA and protein, the mature mRNA is translated into protein and can be further modified to change its folding and thereby its function [5].

1.3 Operon/Polycistronic System

In multicellular organisms and many other eukaryotes, usually one promoter starts the transcription of one gene, and thus one protein. In bacteria and some protozoa such as *Trypanosoma brucei*, a different mechanism called an operon system is commonly used. In an operon system a number of genes, which are often related, are transcribed at once due to a single promoter. This produces a long piece of polycistronic mRNA which is further processed by means of translational reinitiation whereby the appropriate single messages are made. The rise of this kind of promoter is tried to be explained by the Selfish Operon Model which states an advantage of cooperating clusters to single genes during horizontal gene transfer.

Though the presence of clustering of genes in eukarya was proved to be much more common than firstly expected, widespread polycistronic transcription is limited to a few phyla only; trypanosomes and nematodes. In *C. elegans*, the polycistronic mRNA is processed to monocistronic units by both, cis- and trans-splicing making use of so called short splice-leader (SL) sequences 1 and 2. SL2 is typically used for downstream genes in operons and about 15% of all mRNAs are found in operons. Global microarray analysis of *C. elegans* mRNA by Blumenthal et al. showed a significant enrichment in SL2 in about 1200 genes of which more than 90% were falling into 790 operons. These operons contain in average 2.6 genes, 8 at most [6].

Recently an expression vector carrying polycistronic fluorescent (mCherry) cassette optimized for expression in *C. elegans* was established [21]. As conventional fluorescent fusion construct may disrupt proper function of the protein of interest, polycistronic construct may be advantageous. Thus far, this system has been used to monitor gene expression in neurons and germline [21, 22].

1.4 Tissue Specific Drivers

Gene regulation in various tissues in *C. elegans* is inevitable for proper development. To monitor tissue specific function of a protein, tissue specific promoter could be utilized. We choose widely used promoters; *egl-17* (vulva cells), *grl-21* (hyp7) and *wrt-2* (seam cells).
EGL-17 is a fibroblast growth factor-like protein and its promoter is reported to be initially active in the vulva precursor cells namely P5.p, P6.p, and P7.p but the expression fades away during the next stages first in P5.p and P7.p. During L4 and adult stage, expression is restricted mainly in VulC and VulD though weak expressions in other tissues are reported [7].

Hedgehog like protein, *GRL-21* is expressed in arcade cells and in the hypodermis (hyp7). For that reason *grl-21* promoter is used as a tissue specific promoter for hypodermal cells. It is expressed during larval stages as well as in adult stage [8].

wrt-2 encodes another hedgehog-like protein and expressed in the seam cells and in the hypodermal syncytia (hyp7). It is involved in intercellular signalling. As the expression is much higher in the seam cells, the promoter is used for tissue specific expression for the seam cells [9].

1.5 Transgenesis of the Worm

In *C. elegans*, two different ways of transformation were developed, two of them being microinjection and particle bombardment. Both methods take use of introducing exogenous DNA into adult worm's gonads to produce genetically modified individuals among their progeny.

The most widely used method for transformation is microinjection. DNA fragments or plasmids are injected into adult gonads of hermaphrodites. The injected DNA forms extra chromosomal arrays of 50-300 copies. The way of inheriting this genetic information follows non-Mendelian fashion and is of varying efficiency. As gradual loss of DNA over generations is nothing uncommon and the mosaic expression is sometimes advantageous but sometimes rather complicates the gene analyses. Integration of the arrays into the genomic DNA offers a

great tool for facilitating work with transgenic stable lines. For that purpose, mutagenic chemicals, irradiation by UV-, X- or γ-rays are used to cause chromosomal breaks and thereby trigger incorporation of the injected genes.

The second and lately more popular method is the particle bombardment. In this technique, gold microparticles are used to coat the DNA and shoot into the worms by means of a "gene gun". There are a number of advantages over microinjection such as to obtain stable integrated DNA in the animals rather than unstable mosaic. It can be integrated as very low copy number and it is favored when high copy number of the array causes a problem in the worm. It also allows us to carry out knock-in and knock-out methodologies by its easy scalability [10].

1.6 NHR-25

Nuclear receptors (NRs) are an important family of transcription factors. They regulate gene transcription and they are often found to be connected to diseases in humans including diabetes and obesity.

Nuclear receptors have the DNA binding capacity and work as simple switches in gene regulation. They always consist of a DNA-binding domain (DBD) which targets a certain portion of DNA known as hormone response elements (HRE). Two highly conserved zinc fingers in DBD ensure the binding between DBD and HRE. On the C-terminal half a ligand binding domain (LBD) is situated which ensures the ligand recognition and high specificity in function [11].

NHR-25 belongs to NR5A(Ftz-F1/SF-1) family and serves in many tissues for various purposes. It functions in embryogenesis, molting, vulva and gonadal development, and it is known to play a role also in cell migration, locomotion and lipid metabolism. It interacts with various pathways [12, 13 23, 24].

Its molting role is thought to be evolutionary conserved between nematodes and arthropods where NHR-25's ortholog Ftz-F1 is present [12]. However, the molecular mechanisms of Ftz-F1 in other organisms may vary. In mammals, SF-1 is responsible for sexual differentiation, steroid hormone synthesis in primary steroidogenic tissues, and cholesterol homeostasis. In mice deficiencies lead to lethality soon after birth [13]. In *Drosophila*, there are two isoforms encoded and play important roles for segmentation during embryo stage and molting, metamorphosis, and cuticle synthesis during postembryonic stage [13].

1.7 SMO-1

It is now almost 15 years after the discovery of the four classes of SUMO (small ubiquitin-like modifier) proteins and their roles as crucial players in regulation of protein function becomes more and more obvious. An acronym for small ubiquitin-like protein modifier, SUMO was soon accepted as an official term not only as it phonetically suggests to be related to the ubiquitin family but also as it is pronounced the same way as the Japanese-style wrestling. Sumoylation, the process of adding itself to other proteins and thereby changing their properties, involves three different enzyme classes (E1, E2, E3). During the modifications, various kinds of enzymes are available for the each step. The first step involves an ATP dependent activator enzyme (E1), the second step needs an enzyme for conjugation (E2), and the last step involves a ligase (E3) that helps its addition to target protein [14].

While showing similarities in their size and in the kind of enzymes in their reaction pathway, ubiquitination and sumoylation differ greatly in other aspects. First, ubiquitination serves as a marker for proteins that are to be degraded whereas sumoylation rather affects their function or substrate activity. Second, the number of possible enzymes that can be involved in the pathway ubiquitination grows from the first to the last drastically, whereas the number of different enzymes available for the three steps of sumoylation does not, e.g. there is only one single E2. On top of that, ubiquitin enzymes are located all over the cell, whereas the enzymes responsible for modifications by sumoylation are limited to specific subcellular compartments giving a hint towards the specificity of sumoylation [14].

Although SUMO is such a small protein, defects in SUMO function are known to lead to severe problems such as cleft lip and palate (defects in sumo-1) or type-1 diabetes in the case of mutation in SUMO. However, this is not all as deficiencies or defects in one of the three enzymes involved can cause severe problems. Ubc9, the SUMO E2 enzyme plays an important role in embryonic development and deficiencies in it were shown to cause severe disruptions in cellular organisation. In *C. elegans* defect in UBC-9 causes embryonic lethality [14].

So, how does sumoylation affect the function of substrate protein? The answer is, we do not exactly know but it is thought that it is all about changing the proteins interaction with its reaction partners. This, for example, can mean occupation of a reaction site by the SUMO protein which would usually serve as a region of interaction with target proteins partner [14].

2. Material and Methods

2.1 *C. elegans* Strains

For all experiments, N2 (wild type) *C. elegans* worms were used. New transgenic strains were generated in this study (see detail in Results). HL107, HL108, HL109, HL110 carrying plasmids pJW774 [P_*egl-17*::Myc::*NHR-25(3KR)*_polycistronic mCherry] + pRF4 [*rol-6 (su1006)*], HL111, HL112 carrying plasmids pJW526[P_*grl-21*::*Myc::NHR-25*_ polycistronic mCherry] + pRF4[*rol-6 (su1006)*], HL113, HL114 carrying plasmids pJW524 [P_*wrt-2*::Myc::*NHR-25*_polycistronic mCherry] + pRF4[*rol-6 (su1006)*], HL115 and HL116 carrying plasmids pJW776[P_*wrt-2*::*Myc::SMO-1*_ polycistronic mCherry] + pRF4[*rol-6 (su1006)*].

2.2 Maintenance of *C. elegans* Stock

All worm stocks were maintained at 20 °C on rich nematode growth medium (RNGM) plates and fed with OP50, E. coli strain [15]. To keep worms healthy, well-fed worms were prepared for injection; 8 healthy adult hermaphrodites were transferred every 4th day to a new plate as mentioned above. This is different to what is stated to literature [16] but was found to be ideal for my purpose. For transferring a platinum worm picker was used (Pt wire mounted on a glass Pasteur pipette).

In case that a strain was not expected to be used for experiment within weeks, e.g. after finishing phenotypic analysis, a 15 °C stock and a frozen stock was prepared. The former is easily achieved by transferring the plates to a 15 °C incubator.

For the latter, however, some more elaborate rules had to be followed. First of all, only well starved worms of L1 and L2 stage are suitable for freezing. For this purpose worms were transferred to 3-4 new RNGM plates per strain and set aside until they were just starving (few eggs on the plate and many L1/L2 worms). It is of highest interest to have contamination-free plates.

A plate fulfilling these criteria was harvested using 1600 µL of S basal solution into a microtube and centrifuged at 1500 rpm for 1 min. Supernatant was removed until only 600 µL were left and an equal amount of freezing solution added. Upon brief mixing the liquid was equally distributed to 4 cryotubes, each marked with strain name, genotype, date and initials and put to freeze in an isopropanol filled container at -80 °C. The isopropanol reduces the

shock for the worms by gradually cooling by 1 °C per minute. After one day, the tubes were removed and from the container and put into a regular box and placed again to -80 °C.

Recovery of stock is possible even after long period of time. In case of 15 °C stock they have to be recovered ideally within 3 months though even longer periods of storage are possible but the more time passes the fewer worms will survive and the higher the chances of them loosing plasmids of injected DNA. In case of -80 °C the storage time can be prolonged nearly infinitely. In the case of 15 °C stock, simple harvesting with S basal is sufficient for recovery. In the case of frozen stock, one has to remove an adequate part of freshly thawed solution from the vial previously stored at -80 °C and put it on a plate with an OP50-lawn and let to recover.

For microinjections, it is necessary to have a synchronized population of worms ready. On that purpose a plate of worms with as many eggs as possible was harvested as described above, spun down for 1 min at 1500 rpm and the supernatant was removed. After this, 500 µL of bleaching solution was added. Upon constant checking, the solution was left to react until the mother started to brake apart. This served as the signal to vortex and spin down the solution. The supernatant was removed and the solution was washed twice with S basal with intermediate spinning steps. Now, only eggs survived and these were put on a plate of RNGM with bacterial lawn to be ready 3 days later for microinjection.

2.3 Microinjection

For microinjection, the plasmids carrying various constructs were transformed into competent bacterial cells, plasmid DNA was purified, and the quality was checked. Other necessary disposable hardware was prepared and proceeded for injection

First, the received DNA (on a filter paper) was dissolved by incubating the paper flake for 30 min in 100 µL nuclease free water. Meanwhile, competent E. coli cells were melted and 100 µl of them were incubated together with 10 µL of DNA solution for another 30 min on ice. Heatshock was carried out for 90 s at 42 °C with subsequent cooling on ice for 1 min. The cells were then suspended in 900 µL of LB media and incubated for 40 min at 37 °C on shaking platform. Then, 10 µL and 100 µL, respectively, of cells were plated on a LB-medium plate each with CBR and further incubated overnight at 37 °C. Only transformed bacterial cells survive as each plasmid DNA contained antibiotic resistances against Ampicillin/Carbenicillin. The next day, one colony of two of these plates was selected and

grow in 50 mL LB media containing carbenicillin over night. Glycerol bacterial stock was made for each clone by adding glycerol with the final concentration of 15 % and the tube was put at -80 °C freezer.

The remaining bacteria cells are ready to purify plasmid. On that purpose, the grown bacteria was poured into a 50 mL disposable plastic vial and centrifuged at 4000 rpm (3000 g) for 50 min at 25 °C. The supernatant was poured away. The pellet was then resuspended in 500 µL of cell resuspension solution and the plasmid DNA was isolated using Promega's PureYield Plasmid Midiprep System (Cat Number A2495). DNA was eluted in 500 µL of nuclease free water. The DNA concentration was measured on a "GeneQuant pro" spectrophotometer.

2.5 volume of 100 % cold EtOH was added to precipitate DNA and placed at -20 °C. After minimum of overnight incubation at -20 °C, the sample was centrifuged for 30 min at 13000 rpm at 4 °C and the supernatant was discarded. Then, the pellet was washed with 500 µL of 70 % EtOH followed by centrifuging for 5 min. Finally, all the EtOH was removed without touching the pellet which was dried on air for 3 min. The pellet was then dissolved in 50-100 µL of water (concentration dependent; ~30% lost; 500-2000 ng/µL required). In the end, the DNA concentration was measured again and purity was checked.

As the measuring of DNA concentration delivers only information about the concentration of nucleic acids in solution, samples were checked on an agarose gel to confirm the size of the plasmid DNA.

Important above anything else, the DNA mix for injection had to be prepared. As the ideal final concentration of DNA for injection is 100 ng/µL, the DNA concentration had to be adjusted accordingly. The injection solution contained a mixture of dominant transformation maker *rol-6 (su1006)* plasmid, the plasmid of our interest (together making up 100 ng/µL), 10 x injection buffers, and nuclease free water to make 20 µL injection solution. For all four injections a 1:1 DNA ratio was used. Mixed DNA solution was filtered by means of Millipore filter, centrifuged 5 min at 13 000 rpm.

As microinjections require not only prepared DNA and a microscope but also the pulling of injection needles and making of agarose pads, more preparations were necessary before injecting. To make agarose injection pads, 40 mL of 2 % agarose solution were prepared with 24 x 60 mm cover glass slides. The room was cooled below 22 °C and an oven was heated to 100 °C. 70 µL of liquid agarose was dropped on a cover glass and immediately flattened with another cover glass. After solidifying the agarose for a few minutes, the upper glass was removed and this repeated until a baking tray was full. The baking tray was put to the 100 °C

oven for 20 min. After removal, the pads were boxed and ready for use. Experience showed that they are best to be used within 2 months after making.

The second disposable hardware for injection was injection needles. To pull these, a needle puller from Narishige was used. The one step program was used at heat level 52.3 with two heavy weights or level 54 with one heavy and one light weight. As it is crucial that the needles are completely clean and clear of all contaminations, they had to be pulled just before injection.

Finally, it comes to the microinjection. As a last step of preparation, a few adults were transferred to a clear plate without any bacteria and any contamination which served as a pool for worms that were to be injected immediately afterwards. When the injection microscope and the pressurizer (Eppendorf Transjector 5246; Pi = 980 P_a; P_c = 30 Pa) were turned on and the needle was mounted (filled with 1,5-2 µL of plasmid DNA; angle of 30 ° to the injection pad, needle tip centered) and one to three well-fed adult worms were mounted on an agarose pad. Working quickly, the worms were covered under a drop of mineral oil and the DNA was injected into one or both gonads, of the worm. For this, the worm had to be centered and the adjustment of the optics proved to be crucial. In case of successful injection, a unique movement similar to the filling of a balloon could be seen.

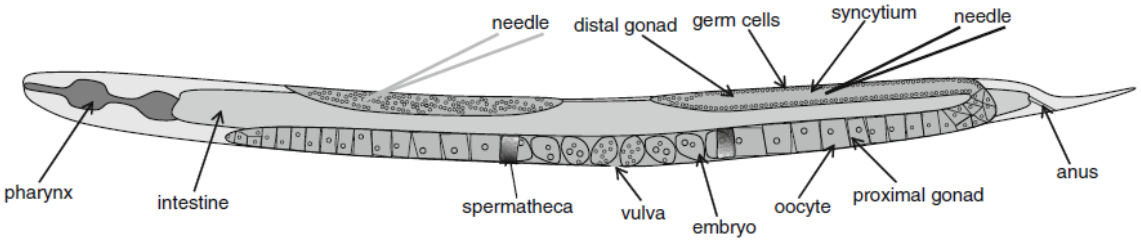

Figure 3: Schematic visualization of *C. elegans* anatomy and the way of injection

Afterwards, a drop of S basal was used to recover the injected animals and a worms were transferred back to a fresh plate with OP50. About 12 h later, the animals were picked again and separated on fresh plates again, 3 worms per plate. This was done to remove progeny that was already fertilized at the time of injection as they were improbable to have taken up any injected DNA in the gonad and to remove dead injected mothers as well. This step facilitated work immensely.

Upon checking the worms phenotype every day for both markers, transformed worms were picked and put to a new plate. Checking was continued for four to eight days. Depending on success or failing of the experiment, another injection could be done.

In the case of success, the independent lines were monitored for 5 generations and transferred every 4 days to new plates. Then, phenotypic analysis was followed.

2.4 Phenotypic Analysis

For phenotypic and transgene expression analyses of stable lines, at least five generations after injection were needed. A plate of worms was then harvested or picked with a worm picker (only taking a certain developmental stage) and put to a special, freshly made agarose pad with anesthetic solution. Working with a confocal microscope, observation was carried out and pictures were taken. Unexpected phenotypes were easily visible with higher magnification. From the analysis, many worms were necessary to observe as each has mosaic expression.

2.5 Microscopy

During the work with the worms a number of binocular microscopes were used. Routine work such as transfer of worms and regular phenotype checking was carried out with an Olympus SZX12 microscope with fluorescent housing attachment. For microinjections Olympus IX70 microscope with 40 x DIC objectives was used combined with a Leica MZ 16 stereo microscope for mounting worms on agarose pads. For final phenotype analysis, Olympus Fluoview FV1000 was used. For the imaging, an anaesthetic was used containing 5-10 mM levamisole on 5 % agarose pads.

2.6 Solutions and Abbreviations

RNGM (3 g NaCl, 7.5 g Bacto Peptone, 17 g agarose, 5 mg cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM potassium-phosphate pH 6, per litre; autoclaved during preparation, except cholesterol)

S Basal (5.85 g NaCl, 1 g K_2HPO_4, 6 g KH_2PO_4, 1 ml cholesterol (5 mg/ml in ethanol), 500 mg gelatin, H_2O per litre. Sterilize by autoclaving.)

10x Injection buffer (20 % PEG, 200 mM K- phosphate, 30 mM K-citrate)

LB medium (Luria-Bertani medium)

CBR (carbenicillin)

Freezing solution (5.8 g NaCl, 50 mL 1 M KH_2PO_4 pH 6, 240 mL glycerol, 710 mL ddH_2O, per litre; sterilized by autoclaving and subsequently mixed with 300 µL 1 M $MgSO_4$)

Bleaching solution (285 µL SAVO, 180 µL 4 M NaOH, 535 µL H_2O)

3. Results

3.1 Introduction

DNA clones were provided by Dr. Jordan Ward (UCSF, USA). All constructs were in Gateway vector modified with *C. elegans* polycistronic gpd gene followed by mCherry coding sequence. Construct contains *Promoter_Myc::Gene_gpd intercistronic region_mCherry*. Before injections, the DNA obtained was amplified by the use of bacterial competent cells. Then, the plasmids amplified were purified and checked the quality and quantity by photo spectrometer and agarose gel electrophoresis. By microinjection, the plasmids were introduced to the worm gonads which lead in some cases to uptake into the worms offspring.

The four constructs used in this study were part of a bigger group of constructs which were to be injected for NHR-25 and SMO-1 functional studies. These proteins are expressed under three different promoters to analyse the gene function in three different tissues, vulval cells, hyp7 and the seam cells. The first step of the functional analyses *in vivo* is to make the transgenic *C. elegans* strains and because this was the first case to use these promoters in the polycistronic mCherry vector, the expression pattern was analysed.

3.2 pJW524 [P_*wrt-2*::*Myc*::*NHR-25*_ *polycistronic mCherry*]

Construct:

The construct pJW524 was designed in a way such that *wrt-2* promoter precedes Myc-tag and NHR-25 which are then translated into a single protein. Additionally, mCherry was driven by the same promoter which should lead to its expression in the seam cells.

Figure 4: Gene map of pJW524

Injection: The preparation of transgenic worms carrying pJW524 was the first injection done and posed the fewest problems. Out of only 4 injected animals, all of them single gonad injections, as many as two independent lines were created successfully (HL113, HL114). This was achieved in a single trial.

The DNA-solution injected contained plasmid with the *rol-6 (su1006)* gene as a transformation marker.

DNA concentrations: 50 ng/µl pJW524 + 50 ng/ µl pRF4[*rol-6 (su1006)*]

Expression:

As it shown in Figure 5, mCherry fluorescence was found only in seam cells. After reaching adult stage, degradation of mCherry expression was observed. Note, that mCherry fluorescence was visible also outside seam cells as small dots, later (Figure 5C).

The Rol-phenotype was expected to be seen due to the *rol-6 (su1006)* co-injection but it proved to be unreliable criterion for selection of transgenic worms as it expressed rather rarely (less than 1 out of 10 m Cherry-positive transgenic animals).

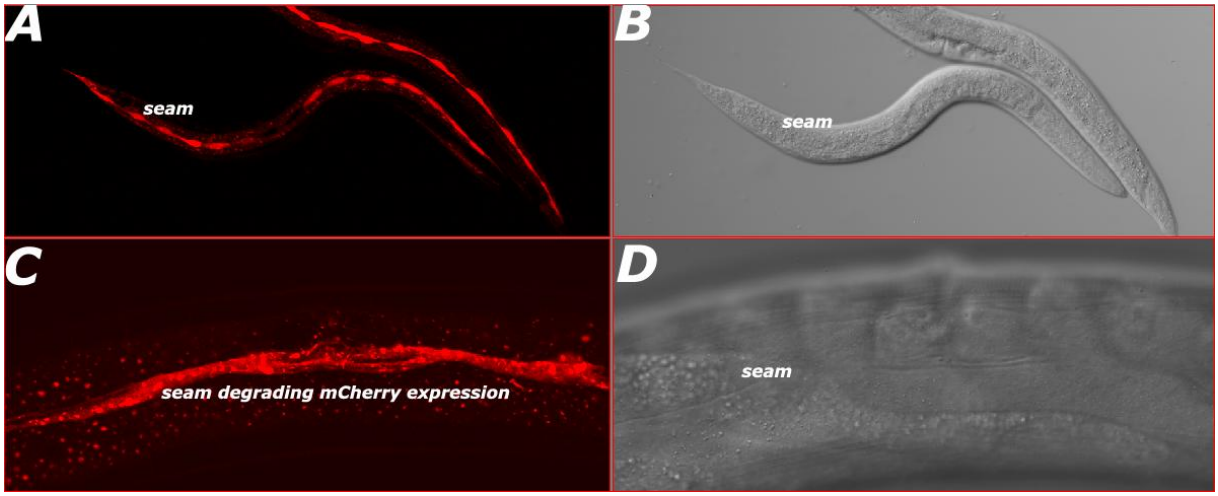

Figure 5: Expression-pattern of pJW524. A and B show the same worms at larval stage showing fluorescence (mCherry expression) in the seam cells (A) and DIC (B). C and D show the expression in the adult syncytial seam cell and DIC of the same worm respectively. Note that granulose spots were seen during the degradation of mCherry in hypodermal syncytia.

Observation/Discussion:

As *wrt-2* is known to be expressed predominantly in the seam cells throughout development, so we hoped to express *NHR-25* in this cell type[9]. We confirmed that, by confocal microscopy, the transgene expression was limited to seam cells. The expression of *rol-6 (su1006)* was rarely observed, indicating that a higher amount of NHR-25 or transgenic proteins in the seam cells may influence the effect of the su1006 dominant mutation. HL113 was used for the expression analysis.

3.3 pJW526 [P_*grl-21::Myc::NHR-25*_ *polycistronic mCherry*]

Construct:

The construct pJW526 contained *grl-21*promoter together with Myc-tag and *nhr-25* gene in order to express NHR-25 in hypodermal cells.

Figure 6: Gene map of pJW526

Injection:

In the case of microinjections of pJW526, two trials and a total of 12 worms were needed to create transgenic animals. From these, a total of three independent lines were kept. However, as one line did lose it's fluorescence during the selection process, only two lines are established as stocks (HL111, HL112).

The DNA-solution injected contained plasmid with the *rol-6 (su1006)* gene as a transformation marker.

DNA concentrations: 50 ng/µl pJW526 + 50 ng/ µl pRF4[*rol-6 (su1006)*]

Expression:

The promoter used is known as to be specific for arcade cells and hypodermis. However, in our case, also weak fluorescence in the vulva was observed (Fig. 7E). Confocal pictures shown in Figure 7 do show strong expression of mCherry in the hypodermis. The frequency of worms with Rol-phenotype was less frequent than the expression of mCherry (but more than one third of all mCherry positive animals). HL111 was used for expression analysis.

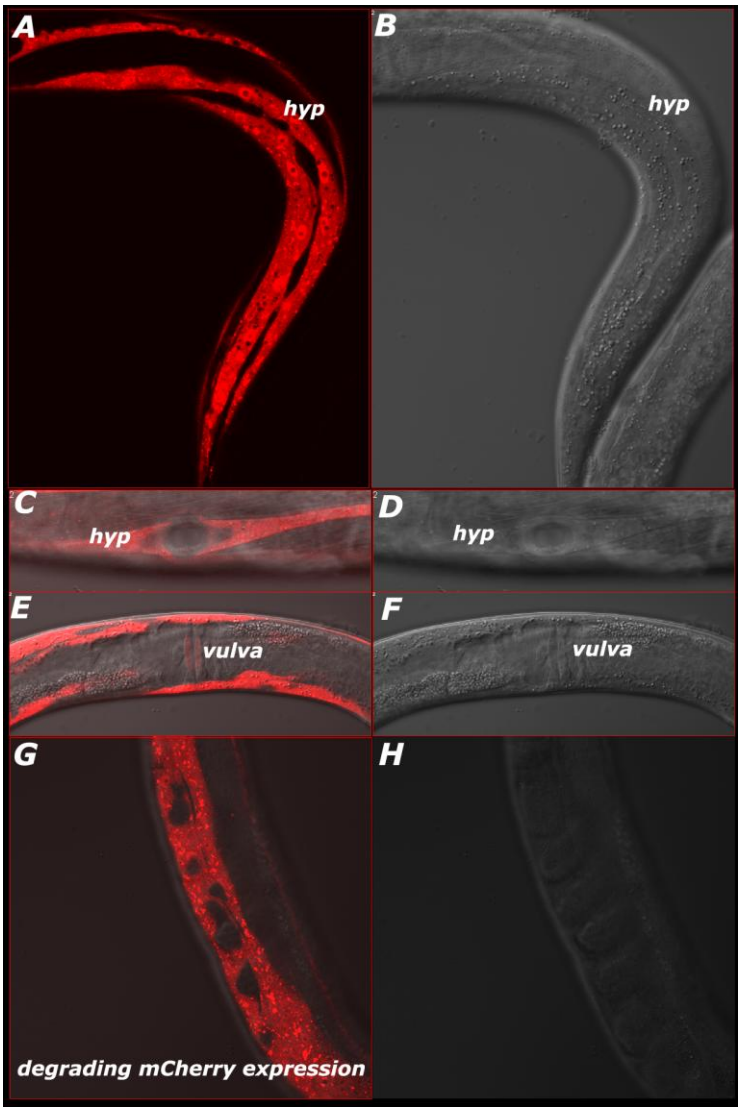

Figure 7: Expression pattern of pJW526. The pairs A and B, C and D, E and F, and G and H alsways show the same worm using a filter for mCherry fluorescence or DIC, respectivelly. A- D show the expression in the hyp7. E shows a weak expression in the vulva and G shows the degrading process of the mCherry fluorescence (granulose dots).

Observation/Discussion:

The gene *grl-21* is known for tissue specific expression in arcade cells and hypodermis [8]. When observing the expression of the transgene by means of fluorescence, it was clear that the expression was not only limited to hypodermis and arcade cells but was also present, weakly, in the vulva. This means that the promoter was in our case not truly tissue-specific and might come from an interaction of the increased levels in NHR-25 in the hypodermis.

As it was observed with the construct pJW524, not all the transgenic animals exhibited Rol phenotype. This confirms the observation that high expression of NHR-25 decreases the phenotype *of rol-6 (su1006),* however, not as strongly as in the case of seam cells.

3.4 pJW774 [P_*egl-17*::*Myc::NHR-25(3KR)_polycistronic mCherry*]

Construct:

In construct pJW774, *egl-17* promoter (VPC specific) was used to drive the expression of Myc-tagged NHR-25 with mutations (3 lysines mutated into arginine), and mCherry.

Figure 8: Gene map of pJW774

Injection:

In the case of the construct pJW774, a total of 23 injected animals were needed to obtain stable lines. This was done in a total of 3 attempts of injections and yielded altogether 4 independent lines (HL107-110). Here, it was the first time that DNA was injected in both gonads. Relatively many trials was needed not because of bad transformation but rather by lack of experience in detecting the weak expression fluorescence.

The DNA-solution injected contained plasmid with the *rol-6 (su1006)* gene as a transformation marker.

DNA concentrations: 50 ng/µl pJW774 + 50 ng/ µl pRF4[*rol-6 (su1006)*]

Expression:

The promoter used is known to drive expression of a growth factor in vulval precursor cells (P5-7.p) [7]. Still, we were able to observe expression of our gene in vulva (Fig. 9A, C and G), but also in the hyp7 (Fig. 9E). Judging from fluorescence, the expression of our protein of interest was rather weak by use of the *egl-17* promoter. Rol-phenotype was observed in about the same frequency as fluorescent animals. HL107 was used for expression study.

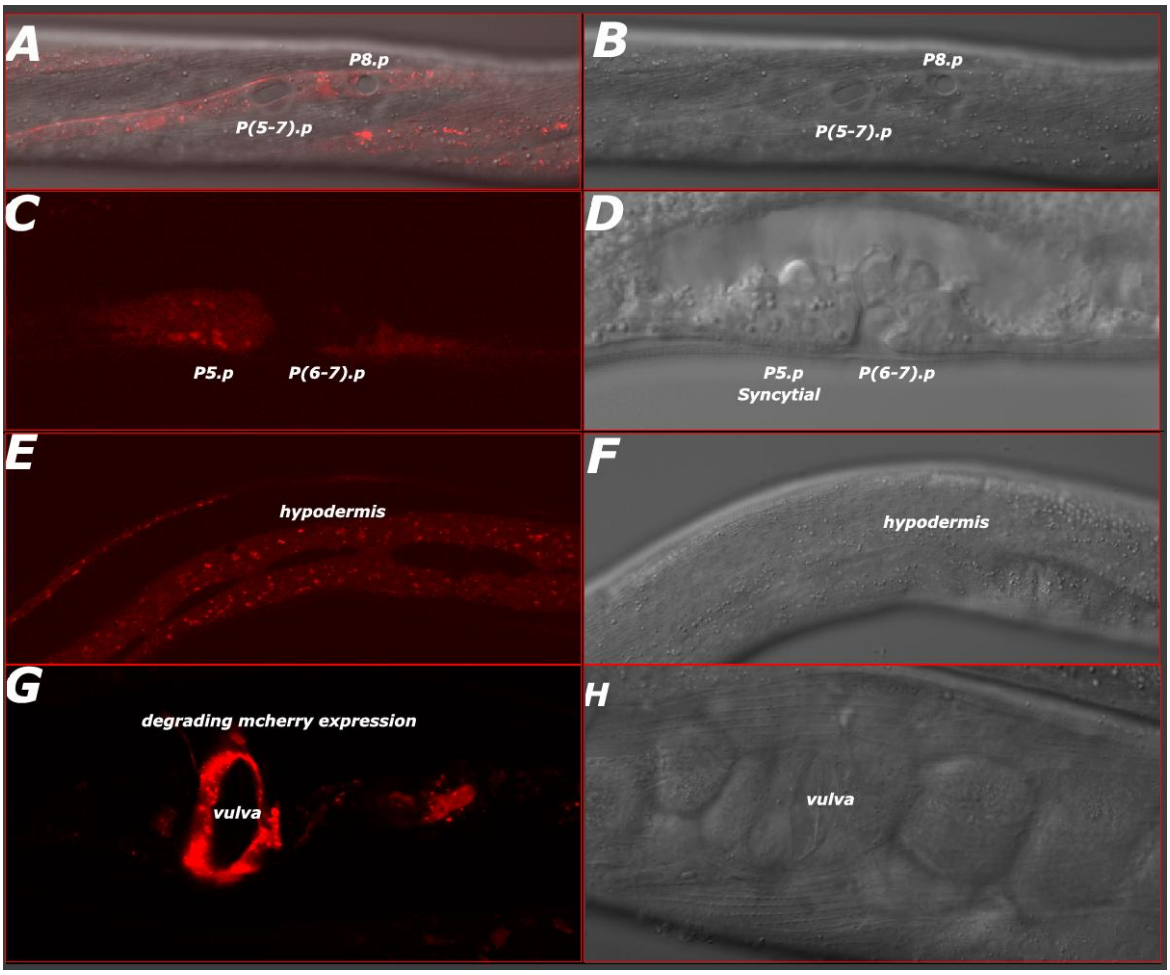

Figure 9: Expression of pJW774. The pairs A and B, C and D, E and F, and G and H always show the same animal using a filter for mCherry fluorescence or DIC, respectively. A, C, G show the expression in the vulval cells. C and D further show adoption of syncytial fate of the P5.p cell. E shows the expression in the hypodermis.

Observation/Discussion:

The promoter used is known to drive expression in vulval cells [7]. The pictures taken confirm that the promoter is active in the vulval cells. However, expression in the hypodermis was observed in the form of granules. Further, the formation of Multivulva (Muv)-phenotype was observed. The data suggest that the high level of non-sumoylated NHR-25 may be responsible for the ectopic expression in the hyp7 and Muv phenotype.

3.5 pJW776 [P_*wrt-2*::Myc::*SMO-1*_ *polycistronic mCherry*]

Construct:

The construct pJW776 contains genes coding for Myc-tagged SMO-1 and mCherry which are driven by *wrt-2* promoter (seam cell driver).

Figure 10: Gene map of pJW776

Injection:

In the case of pJW776 a total of 23 worms were injected in both gonads in altogether 2 trials. In total, the injections did yield 2 independent lines of worms (HL115, HL116). The main problem during the fist series of injections was probably because of the use of old injection needles.

The DNA-solution injected contained plasmid with the *rol-6 (su1006)* gene as a transformation marker.

DNA concentrations: 50 ng/µl pJW526 + 50 ng/ µl pRF4[*rol-6 (su1006)*]

Expression:

The transgenic animals showed fluorescence not only on the seam cells (Fig. 11 A and C), but reproducibly also in the vulva cells (Fig. E). Further, no Rol-phenotype animals were observed at all. HL115 was used for expression study.

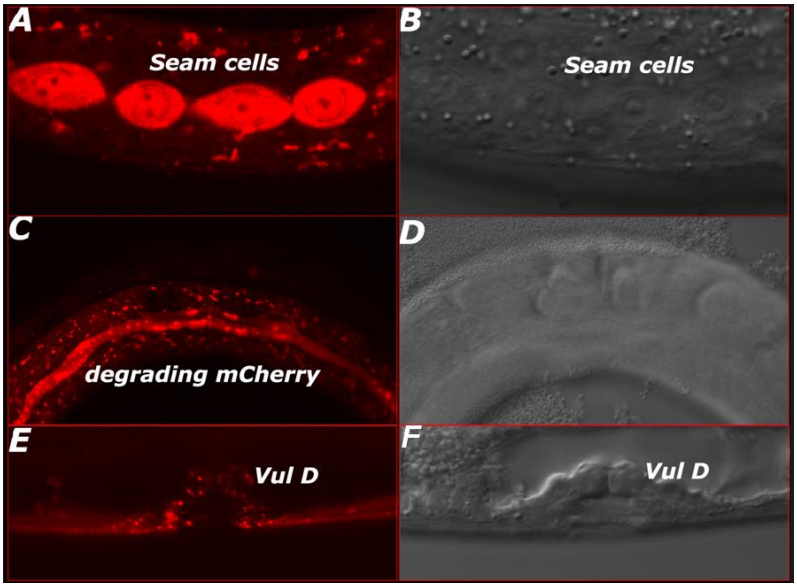

Figure 11: Expression-pattern of pJW776. The pairs A and B, C and D, E and F, and G and H show the same worm using a filter for mCherry fluorescence or DIC, respectively. A and C show the expression in the seam cells. C and D depict degraded mCherry expression in the worm. E shows the expression in Vul D.

Observation/Discussion:

Wrt-2 is known to be predominantly expressed in seam cells [9]. Thus, we hoped to express SMO-1 in this tissue. Strong mCherry expression was seen in both larval and adult seam cells. However, we observed expression in vulval cells. Because this ectopic expression was not seen in pJW524 transgenic worms (see Chapter 3.2), this effect could be SMO-1 specific. Further, we observed a total silencing of the expression of *rol-6*. This indicates that high levels of SMO-1 or transgene in the seam cells might influence the effect of *rol-6 (su1006)*.

4. Discussion

All the injections were very successful. Although only few animals were injected, a comparably high number of independent lines were obtained. This is probably due to a combination of factors such as, good equipment, high quality material (DNAs and needles), fresh pulling of needles each time before injection, freshly made agarose pads, and a fair share of luck and skill. For example, I encountered technical difficulty in my last attempt of injections (data not shown). There, I was supplied with old agarose pads by my colleagues and had more than 100 unsuccessful microinjections. During a repetition by a colleague, using fresh pads one successful line was achieved in about 50 injections.

The Rol phenotype appeared to be suppressed in the case *wrt-2* promoter was used (constructs pJW524 and pJW776, in this study) but not with egl-17 promoter. As those two constructs over-express different proteins, though we cannot exclude the possibility that NHR-25 and SMO-1 do cooperate and influence the Rol phenotype, it is more likely that the over-expression of transgenes in the seam cells does dilute the amount of mutated ROL-6 protein in the cell such that its impact is much lower.

In the case of pJW774, NHR-25 with sumoylation-site mutations was expressed under *egl-17* promoter and caused multivulva (Muv) phenotype as mentioned above (especially at P3.p, P4.p position in three independent lines namely HL107, HL108, JL110, Bojanala personal communication). This was not seen with a construct with wild-type NHR-25 in the same vector (Bojanala personal communication), therefore this observation implies that sumoylation of NHR-25 may play a role in preventing ectopic vulval induction in P3.p and P4.p.

In the case of the construct pJW526, a tissue-specific promoter for the hypodermis was used. However, a weak expression was found also in the vulva cells. This is an important indication that overexpression of NHR-25 may influence the transcriptional activity of *grl-21* directly or indirectly.

The two constructs using tissue-specific the driver for seam cells *wrt-2*, pJW524 and pJW776 did show different expression patterns. Where the former constructs up-regulates the expression of NHR-25 in the seam cells only, whereas the latter, driving the over-expression of SMO-1in the seam cells as well as in extra tissue such as vulva. This observation indicates that overexpression of SMO-1 may influence the transcriptional activity of other genes.

5. Conclusions

In this study, I generated ten independent transformed *C. elegans* strains for four different expression constructs. Newly developed polycistronic mCherry vector system was used to express our gene of interest and therefore, the expression pattern was analysed in the live worms. In the case of the construct pJW524, a total of two lines were obtained. The promoter *wrt-2* showed to act tissue-specifically on seam cells and a clear suppression of co-injected rol-6 was observed. For the construct pJW526, two independent worm lines were created. The promoter *grl-21* showed high expression in hyp7 cells as mentioned in literature. However, NHR-25 over-expression in hypodermis seems to influence ectopic expression in adult vulval cells. The construct pJW774, four independent lines were generated, showed weak fluorescence but its transformation and stability proved to be better than in the other constructs injected. Over-expression of mutated NHR-25, which cannot undergo sumoylation showed in some cases the development of Multivulva-phenotype as well as ectopic expression in the hyp7 cell. Further, a defect in the vulval cell fate was observed where either or both of the cells P5.p and P7.p adopted syncytial fate. The last construct, pJW776, injected successfully (two independent strains generated) and strong expression was seen in the seam cells as expected. However, there was an ectopic expression in the vulval cells. The Rol-phenotype was not observed at all.

In summary, I have generated useful tools to analyse gene function. I confirmed the promoter activity, by monitoring polycistronically transcribed mCherry, of three different drivers, wrt-2 (seam cells), grl-21 (hyp7) and egl-17 (vulval cells). The expression pattern followed as expected but I also noticed that there was unexpected expression in some cases. Data obtained here, especially the ones for ectopic behaviour of the tissue-specific promoters might be important observation for further studies.

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