University of South Bohemia České Budějovice
Faculty of Biological Sciences
Department of Molecular Biology and Biochemistry

Bc. Thesis

The transcriptional regulation by the nuclear receptor NHR-25 in *Caenorhabditis elegans*

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I hereby declare that I did all the work, summarized in this Bc. thesis, on my own or on the basis of consultation with my supervisor.

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Aim of the study:

NHR-25 is a one of few conserved nuclear receptors in *C. elegans* and its family is involved in many developmental processes not only in the worm but also in flies, fish, mice and humans. Yet, the cellular mechanism of the action of this gene family is poorly understood. In *C. elegans*, it is likely to function as a transcription factor but its direct target gene has not been identified to date. In this study, I utilized the defined NHR-25 binding (target) sequence and GFP as a marker to visualize a possibility that NHR-25 regulates transcription of other gene(s) *in vivo*. I have also tried the "candidate approach" of two genes utilizing existing transgenic worm strains carrying *promoter::GFP* fusion transgenes expressed in epithelial cells to see if those genes can be regulated by NHR-25.

Contents:

1. Introduction

1.1 *Caenorhabditis elegans*

Caenorhabditis elegans is a small worm, a member of family *Rhabditidae*, a large and diverse group of nematodes. While some of the related species are pathogenic to or parasitic on animals and plants, *C. elegans* is non-parasitic, soil living nematode and it is advantageous for rearing in laboratory conditions.

C. elegans is one of the widely used model organisms in Developmental biology and in Genetics. This small, 1 mm long transparent worm has traits important as an experimental animal: it is easy to rear feeding on *Escherichia coli* on agar or agarose plates, can be stored as a frozen stock, has short life cycle, and it has small genome in six chromosomes and two sexes. The worms are usually kept at 20°C.

Hermaphrodites are self-fertilizing and can have about 300 of offspring during their reproductive period. Hermaphrodites can not fertilize other hermaphrodites but males can mate with hermaphrodites. The males are quite rare in nature but occur spontaneously or by heatshock in the hermaphrodite population by X-chromosome non-disjunction. This can happen with frequency about 1 male per 500 animals.

The development takes 2.5 days at 25°C (3.5 in 20°C) and contains embryonic stage, four larval stages from L1 to L4 (Fig. 1.1) and the adult. Each post-embryonic stage is progressed through molting. In unpreferable conditions like starvation and high density, worms go through the stage of so called dauer-larva in their development; L2 does not develop into regular L3, but into dauer, which is resistant to starvation and outer stress. The development resumes after improving the conditions to L4 and then continues to be the adult.

The worm's genome is 97 Mb with the total number of coding genes 19,000. *C. elegans* has 6 haploid chromosomes, five of them the autosomes (I, II, III, IV, V) and one sex chromosome, X. The sequence of the whole genome became available in 1997 and it was the first model organism to be fully sequenced.

The first discovery with *C. elegans* in the main role as a model organism was the genetic regulation of apoptosis analyzed by Sydney Brenner, John E. Sulston and H. Robert Horvitz. This process is conserved in other organisms and it can be

activated by many different stimulations such as extracellular growth factors, steroid hormones or viral infection. The morphological changes of cells that die in apoptotic way in *C. elegans*, are similar to the changes in mammalian cells.

The worm has the constant number of body cells. At hatching, the larva of L1 stage has 558 cells in hermaphrodite and 560 in male. In postembryonic development, cells proliferate but 131 cells of totally 1090 die with apoptosis to produce the 959 somatic cells in the adult hermaphrodite and 1031 cells in the adult male. The neurons and epidermal cells die mostly, less the muscle cells. The apoptosis occurs also in the embryonic stage as well as in the adult (the germ cells).

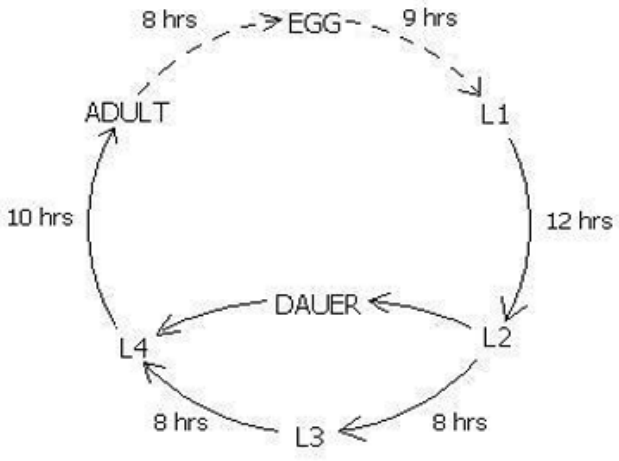

Fig. 1.1 The life cycle of *Caenorhabditis elegans* under laboratory conditions (25°C).

1.2 Transcription factors

Transcription factors are proteins necessary for one of the basic processes in the cell – the transcription. The general transcription factors help to position the RNA polymerase correctly at the promoter of a gene, then to separate the strands of DNA and at last to release RNA polymerase from the promoter. They are called "general" because the same set of proteins works on any promoters used by RNA polymerase II (RNAP II). The eukaryotic promoter for RNAP II is characteristic with the presence of TATA box, which binds the proteins like TBP (TATA binding protein) and TAF (TBP-associated factors). Other transcription factors bind on the DNA at enhancer region and they bind in sequence specific manner. Those so called "binding site" are defined for each transcription factor and could be different length. Binding sites could

be monomeric or dimeric. A transcription factor sits on its binding site that leads to a conformational change in DNA structure and help to recruit the RNA polymerase complex (Fig. 1.2). Also the number of binding sites could be important e.g. more binding sites can enhance the effect of the factor.

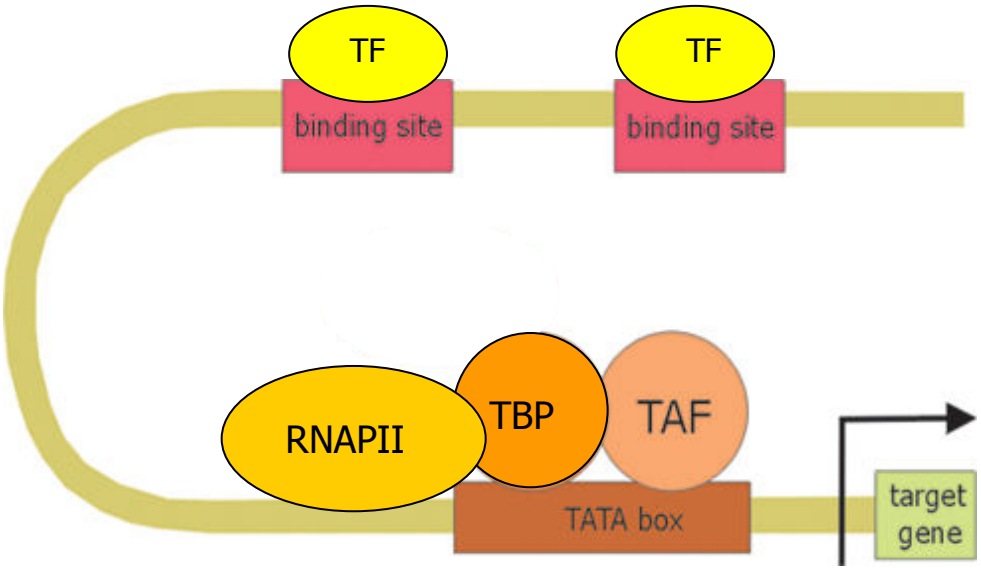

Fig. 1.2 A simple scheme of the transcription machinery on a target promoter and enhancer

1.3 NHR-25

Nuclear receptors play crucial role in the development of all metazoans, because they regulate the transcription as transcription factors. The number of them present in *C. elegans* is unusually high (284) and only very few of them are conserved with other organisms (about fifteen). NHR-25 is one of them. It is conserved with FTZ-F1 in *Drosophila*, which is involved in the control of metamorphosis, the cuticle synthesis, the molting and the embryogenesis. The homologues in mammals are SF-1/LRH-1 responsible for the gonadal and adrenal gland development and the steroidogenesis. It is also shown that members of this family work as a monomer binding protein.

NHR-25 has been shown to be required in the embryogenesis, molting and gonadal differentiation in *C. elegans* (Asahina et al., 2000; Gissendanner and Sluder, 2000; Asahina et al., 2006). Also it has an important role in epidermal differentiation (Chen et al., 2001; Šilhánková et al., 2005).

1.4 RNA interference

The great discovery of RNA interference (RNAi) by double stranded RNA (dsRNA) was achieved by C. C. Mello and A. Fire (Fire at al., 1998) and has been a big breakthrough in biology. The principle seems to be very old and widely used by many organisms to face the threat of inserting transposones and retroviruses into their genome. This posttranscriptional mechanism, when dsRNA is introduced into the organism, small interfering RNAs are generated by Dicer, leads to the degradation of specific homologous mRNAs by RISC complex and consequently to the down-regulation of the expression of the targeted gene – so the gene is silenced (so called "gene knock down"). RNAi mechanism was first investigated in *C. elegans*, then it was also found in *Drosophila* (Kennerdell and Carthew, 1998), mouse (Wianny and Zernicka-Goetz, 2000), plants (Waterhouse et al., 1998) and many others. The RNAi has been employed in investigation of gene function and now it emerges for usage in medicine.

The RNAi in *C. elegans* can be accomplished by four different ways: by injection when the dsRNA is injected into the gonad arm of the worm or the body cavity, by soaking when the worms are submerged in the solution of dsRNA; by feeding when they are fed on bacteria expressing dsRNA and by transformation of a vector containing palindrome sequence of a targeted gene. The unique feature of the RNAi in *C. elegans* is the systemic effect of RNAi. The dsRNA can be aplicated into any tissue of the body and the animal will be affected, because dsRNA can spread among the cells independently on the cell type (just not in the neurons). This surprising behavior is caused by transmembrane protein SID-1, which is responsible for the income of dsRNA inside of the cell.

1.5 The *C. elegans* apical junctions (CeAJ)

C. elegans is very convenient organism for studying epithelial adhesion. Epithelia are crucial in organizing the body of the animals and especially important in embryos. Genome analysis of *C. elegans* unveiled that many of the adhesion proteins present in it are common in other organisms (Cox et al., 2004, Hutter et al., 2000, Michaux

et al., 2001). What is more, *C. elegans* produces a number of intermediate filaments (Karabinos et al., 2001, Hapiak et al., 2003, Woo et al., 2004), which are possible to study in similar way to the vertebrates.

There are several types of major adhesion complexes in *C. elegans*: Muscle dense bodies containing many proteins found at focal contacts in vertebrates, including integrins and their associated proteins (e.g. integrin-like kinase, ILK); an adherens junction subdomain containing the cadherin/catenin complex (CCC) composed of HMR-1/E-cadherin, HMP-2/β-catenin, JAC-1/p120-catenin and VAB-9/claudin; a junctional domain containing the *C. elegans* homologue of Discs large and its binding partner AJM-1; and an apical region which contains transmembrane proteins of the Crumbs family Crumbs/Stardust/Disc Lost complex, the *C. elegans* PAR-3/PAR-6/atypical protein kinase C (aPKC) complex, and proteins stabilizating cytoskeletal attachments to the apical membrane. What is surprising is that in cuticular epithelia (epidermis, parts of pharynx and anus containing cuticle), the components of tight junction in vertebrates PAR/aPKC and Crumbs complexes are lacking (Bossinger et al., 2001).

1.5.1 The DLG-1/AJM-1 complex

DLG-1/Discs large and AJM-1 are present in the basal domain of the CeAJ (Fig. 1.3). They colocalize to form the electron-dense junctional region (McMahon et al., 2001, Bossinger et al., 2001, Koppen et al., 2001, Firestein and Rongo, 2001) . DLG-1 is orthologue of *Drosophila* tumor supresor Discs-large, and is also a member of the membrane-associated guanylate kinase (MAGUK) family. DLG-1 is essential for the proper localization of AJM-1 and is likely to be a regulator of AJM-1 (Koppen et al., 2001). Recent research suggests the reciprocal role of AJM-1 in regulation of localization of DLG-1 in the intestine (Segbert et al., 2004).

The function of DLG-1/AJM-1 complex is probably connected with other types of adhesion complexes and could be partially redundant with the CCC. These pathways could be crossed through some of the transmembrane proteins and interactions with DLG-1. Candidate proteins are *C. elegans* claudins, like homologue claudin CLC-1, colocalized with AJM-1 in the pharynx; or LAD-1, containing PDZ

binding motif at its C terminus (Chen et al., 2001), which can possibly interact with the PDZ motifs on DLG-1.

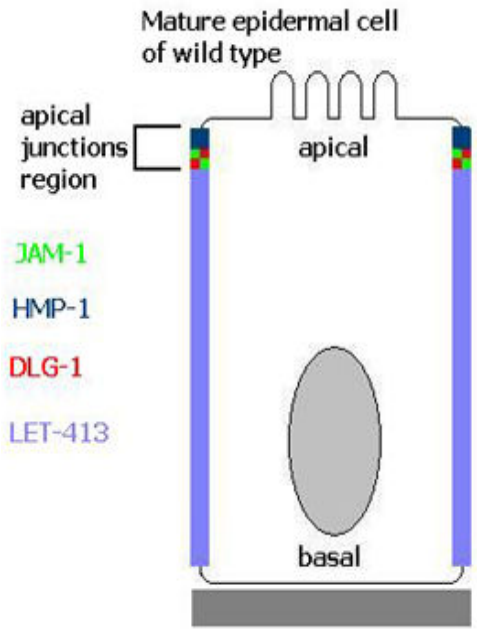

Fig. 1.3 The mature epidermal cell of wild type worm with localization of adhesion proteins. Green – JAM-1 (=AJM-1), dark blue – HMP-1, red – DLG-1, violet – LET-413

2. Materials and methods

2.1 Culture of *C. elegans*

The worms were cultured on nematode growth medium (NGM) plates fed on *E. coli* strain OP50 at 20°C as described (Brenner 1974).

2.2 DNA clones and DNA amplification for cloning

DNA clones (pOLDO-luciferase) containing two copies of either NHR-25 binding sites and ROR binding sites (gift of M. Van Gilst, Fred Hutchinson Cancer Research Center, Seattle, WA) were used to subclone into a GFP vector, pPD107.97 (Fig. 2.1, gift of A. Fire, Stanford University, USA), developed for *C. elegans* transformation. This GFP vector contains a minimal promoter from *myo-2* gene. DNAs were amplified either by transforming bacteria by heatshock or recovering from a frozen stock then plasmid DNAs were isolated and purified with QIAprep Spin Miniprep Kit (QIAGEN).

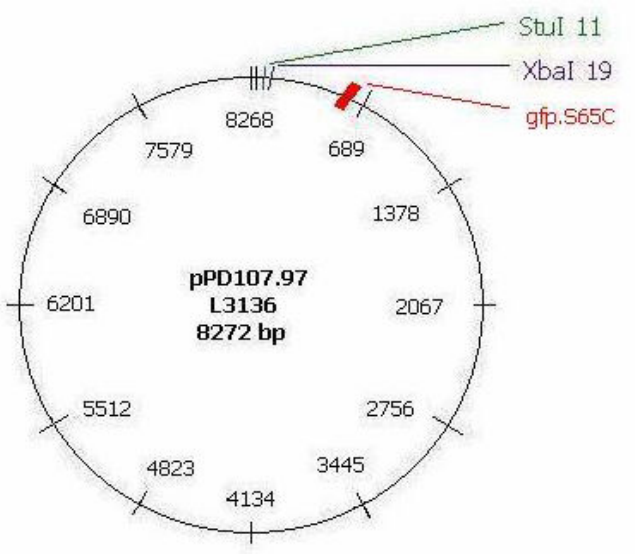

Fig. 2.1 The GFP vector (pPD107.97), restriction sites for restriction endonucleases StuI and Xba I and the location of GFP are indicated.

2.3 Sequencing

Sequencing of DNA was performed using Big Dye terminator system kit (Perkin Elmer). Reaction mix was prepared according to the instruction of manufacturer with a small modification. Briefly, 4 µl of Big Dye, 4µl of 2.5x sequencing buffer and either 0.5 µl of the luciferase primer or 3.2 µl of 1 µM M13 reverse primer was premixed and then 1µg of DNA was added. The total volume of the reaction was set to 20µl. The sequencing reaction was purified with G50 AutoSeq column (Amersham) then dried by the vacuum centrifuge (Labnet Dyna Vap) for 20-30 min. The sequencing was performed on an ABI 310 automatic sequencer. Obtained sequences were analyzed with a program Chromas Lite and further processed using DNASTAR (EditSeq, MegAling, MapDraw).

2.4 Cloning

Directional cloning using XbaI (3' overhang) and StuI (blunt) was performed. The double digests of the vector containing two copies of NHR-25 binding site creates the fragment of 275 bp with the binding sites. The GFP vector pPD107.97 was digested with same set of enzymes and linealized. Single cut with each of the enzymes alone was also performed to see if each enzyme works. The reactions were done in total of 20 µl volume with 2 µl of 10x M buffer, 2 µl of BSA (Takara), 0.5 µl of XbaI and StuI (both Takara), and 500 ng of DNA.

Before loading to the electrophoresis wells in the gel, the samples were put into 70°C water bath for 15 min. The enzymes are denatured in this temperature and stop working. 1Kb Plus DNA Ladder (Invitrogen) for distinguishing the linear DNA (cut fragments) and Supercoiled DNA Ladder (Invitrogen) for distinguishing the circular DNA (uncut fragments) were added to the outer wells of the gel. 0.8 µl of 10x DNA Loading Buffer was added in each sample and run the electrophoresis in TAE buffer. Fragments of 275 bp (insert) and 8,260 bp (vector) were cut out from the gel of 4% or 0.8 % low-melting agarose respectively. Gels were melted and ligation was performed in the gel using T4 ligase (Takara) at 15 °C overnight. The ligation reaction was melted and TCM was added and then transformed into *E. coli*

DH5α cells by heatshock. Colonies were picked to grow in 5 ml LB medium and plasmids were isolated using QIAprep Spin Miniprep Kit (QIAGEN). The correct clones were further confirmed by sequencing.

2.5 Preparation of DNA for the worm transformation

The DNA for injection was prepared with QIAGEN Plasmid Midi Kit according to the instruction of the manufacturer. Total concentration of 100 ng DNA per 1 µl was prepared with 80 ng of 2xTGA::GFP, 20 ng of *rol-6* in 1x injection buffer. The *rol-6(su1006)* mutation is routinely used as a dominant visible marker for transformation of *C. elegans* (Mello and Fire, 1995). Just before injection, the DNA mix was filtered through Ultrafree-MC column (0.1 µm, Millipore).

2.6 Transformation of the worms

The DNA transformation with microinjecting was first discovered in 1982 by Kimble et al. This is the germline transformation, when the syncitial gonad is injected with needle (Stinchcomb et al., 1985, Mello et al., 1991). The effect of transformation can be analyzed in F1 progeny and later generations.

The wild type N2 worms from synchronized cultures were used for transformation. They were kept on NGM plates with OP50 in 20°C to reach the stage of young adult. Injection needle (GDC-1, Narishige) was pulled by a needle puller (Narishige) and about 1.5 µl of purified and freshly centrifuged DNA was placed at the tip of the injection needle.

The worm was put from the original NGM OP50 plate to one new NGM plate without bacteria to clean its body from bacteria and placed on the agarose pad with a drop of mineral oil (SIGMA). The worm is fixed on the agarose. The pad was placed under the injection microscope (Olympus) with the objectives 60x, DIC and the gonadal arm and the tip of the needle were aligned on the same focal plane. The gonad was injected as quickly as possible, because the animal gets dry during the time of staying on the pad in the oil. After taking away the pad from the microscope, the worm was removed from the agarose with a drop of S-basal with gelatin. The

injected animal was put on a new NGM OP50 plate and next day separated each one on new NGM OP50 plates.

2.7 RNAi

2.7.1 Strains of *C. elegans* used in RNAi experiments

For RNAi of *nhr-25*, two strains were used in this study. In both strains, GFP (green fluorescent protein, originally from jellyfish, Chalfie et al., 1994) are driven by a promoter and these transgenes are integrated into their genome. Both of them express GFP in the cells of epidermis where NHR-25 functions.

Strain carrying *dlg-1::gfp*

The *dlg-1* coding sequence was cloned with 7 kb promoter sequence up to the preceding gene C25F6.3 into GFP containing vector pPD95.75.

dlg-1::gfp carrying strain was first described in McMahon et al., 2001. DLG-1::GFP is expressed from embryogenesis to the adulthood at the epithelial adherence junction. The gene is homologue of *Drosophila lethal discs large,* a tumor supresor, and plays important role in the assembly of *C. elegans* apical junctions (CeAJ). The DLG-1 is a member of the membrane-associated guanylate kinase (MAGUK) family. It colocalises with the junctional protein AJM-1 and apical to laterally localized LET-413 (Fig. 1.3). The strain was kindly provided by Dr. Zwartkruis (Utrecht, the Netherlands).

Strain carrying *pxf-1::gfp*

The second strain, *pxf-1::gfp* (PDZ-domain containing exchange factor) (Pellis-van Berkel et al., 2005), was kindly provided by Dr. Zwartkruis (Utrecht, the Netherlands) and shows also the green fluorescent pattern in seam cells in the epidermis.

The construct was prepared from T14G10.2 gene fragment harboring 2.4 kb upstream region of the pxf-1 gene inserted into pPD95.75 GFP vector (Pellis-van Berkel et al., 2005).

PXF-1 activates Rap1 and Rap2. Rap1 is a member of the family of Ras-like GTPases. These GTPases are key regulators of differentiation, cell division, vesicle transport, nuclear assembly and control of the cytoskeleton. In *C. elegans* genome 56 members of the major Ras GTPase subfamily are encoded – this include Ras/Ral/Rap family, the Rho family, the Rab family, Ran, and the Arf/Sar family. Ras/Rap family members control cell fate specification and differentiation.

Ras-like GTPases switch between an active GTP-bound state and an inactive GDP-bound conformation. Activation of them is mediated by guanine nucleotide exchange factors (GEFs). They induce the dissociation of GDP to allow the binding of GTP. The Ras-like small GTPases form a family of proteins, including Ras, Rap1, Rap2, R-ras, TC21, Ral, Rheb and M-ras.

C. elegans has three Rap-like genes. *rap-1* and *rap-2* are very similar to vertebrate *Rap-1b* and *Rap-1a*, *rap-3* is more different, but most similar to *Rap-1b*. Their function in *C. elegans* involves the function and morphogenesis of epidermal cells. Rap1 regulates integrins associated with the actin cytoskeleton, but not integrins connected with intermediate filaments. Also cadherins are regulated by Rap1. *rap-1* mutants are defective in epidermal morphogenesis and function, including formation of adherens junctions, secretion of cuticle and basement membrane and epidermal cell integrity and viability. *rap-2* mutations on their own are wild-type, but they enhance the effects of *rap-1*. *rap-1* and *rap-2* also possibly act redundantly and can partially balance each other's loss (Berkel et al., 2005).

Similarly, mutations in *pxf-1*, which encodes a Rap GEF similar to PDZ-GEF/RA GEF, cause a phenotype comparable to *rap-1; rap-2* double mutants (Berkel et al., 2005). Further more, constitutively-active RAP-1(G12V) can partly rescue the *pxf-1* mutant phenotype. This indicates, that PXF-1 and RAP-1 act together to control epidermal morphogenesis and function.

2.7.2 Preparing the bacteria for RNAi

The transformed bacterial cells from frozen stock were streaked on LB plates containing antibiotics (carbenicillin, tetracyclin) and incubated overnight in 37°C. There were two kinds of cells; the transformed with a plasmid containing *nhr-25* in a double T7 vector (pPD129.36, gift of A. Fire) and transformed with the vector only as a control. Next day one colony from each plate was picked and grew in 5 ml LB medium with antibiotics (100 µg/ml CRB, 12.5 µg/ml TET) and incubated overnight at 37°C again. Third day of the procedure, 500 µl of overnight culture were put in 20 ml LB (100 µg/ml CRB, 12.5 µg/ml TET), incubated for 2-4 hours in 37°C until OD_{600} reached 0.4. The dsRNA was induced by adding IPTG (0.4 mM) and then incubated for 4 hours in 37°C. Then the induction was spiked with additional antibiotics (100 µg/ml CRB, 12.5 µg/ml TET) and IPTG (0,8 mM final concentration). Before using those cells for experiments, the cells were resuspended then 1 ml of the liquid culture was centrifuged, the pellet resuspended again with pipetting and plated on RNAi plates (30 µl per 1 small plate). For RNAi, special plates were used to grow worms; NGM plates with antibiotics (50 µg/ml CRB, 12.5 µg/ml TET) and IPTG (0.4 mM).

2.8 Microscopy

For routine work and checking the worms, stereo-microscope Olympus SZX12 was used. For checking the phenotype of RNAi affected worms and to see detail GFP expression pattern in transgenic worms, the fluorescent microscope Zeiss Axioplan2 equipped with DIC was used. Images are captured by the software Analysis and processed using Adobe Photoshop.

The collected worms in certain stage for observing were anestetised with the mixture of 0.2 % tricaine and 0.02% tetramisole and put on 5% agar pads. Then they were checked in Nomarski view and fluorescence in magnifications 20x, 40x or 63x.

2.9 Solutions and abbreviations

LB medium – Luria-Bertani medium

IPTG – isoprophylthio-β-D-galactoside

CBR – carbenicillin

TET – tetracyclin

AMP – ampicillin

NGM plates –3 g NaCl, 2.5 g Bacto Peptone, 17 g agarose/agar, 5 mg cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM potassium-phosphate (pH. 6.0) in 1 liter

10x injection buffer – 20% polyethyleglycol, 200 mM K-phosphate, 30 mM K-citrate

TCM buffer – 10 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$

3. Results

3.1 Sequence of *nhr-25* binding sites

Since the map and detail sequence of the luciferase vector containing NHR-25 binding sites were not provided, both of those pOLDO-luciferase vectors containing either NHR-25 binding sites (*NHR-25bs::luc*) or ROR binding sites (*RORbs::luc*) were sequenced using a luciferase primer to read upstream (Fig. 3.1). Provided information of the *nhr-25* binding sequence was TCAAGGTCA and ROR binding sequence was with one base mutation TCTAGGTCA. To our surprise, the NHR-25 binding sequence in this vector was not TCA but TGA (Fig. 3.1, NHR-25bs). The ROR binding site was TCTAGGTCA, Fig. 3.1 RORbs) and used as a control.

XbaI
I

NHR-25bs **AGTCGACCTGCAGCAGATCTGAAGGTCATTAATAATGAAGGTCATGGCCGCTATAGCT**
RORbs **AGTCGACCTGCAGCAGATCTCTAGGTCATTAATAATCTAGGTCATGGCCGCTATAGCT**

NHR-25bs **TGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTACCCAACTT**
RORbs **TGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTACCCAACTT**

NHR-25bs **AATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTANTAGCGAAGAGGCCCGCA**
RORbs **AATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTANTAGCGAAGAGGCCCGCA**

NHR-25bs **CCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCNCCTGATGCGGTA**
RORbs **CCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCNCCTGATGCGGTA**

NHR-25bs **TTTTCTCCTTACGCAATCTGTGCGGTATTTCACACCGCATAGG**
RORbs **TTTTCTCCTTACGCAATCTGTGCGGTATTTCACACCGCATAGG**

|StuI

Fig. 3.1 Enhancer region sequence containing 2 copies of *nhr-25* binding sites (TGAAGGTCA) or ROR binding sites TCTAGGTCA. The sequence of the fragment (XbaI-StuI) used for subcloning is shown here.

The binding sequence TGAAGGTCA is responsible for transcriptional activation in transfected cells (Asahina et al., 2006). While AGGTCA is classic hormone response element; universal to many other nuclear receptors, TGA at its 5'- seems to be NHR-25 specific as mutations in them (TCTAGGTCA) could no longer activate a target gene transcription (Asahina et al., 2006).

3.2 Cloning and transformation of 2xNHR-25bs::GFP construct

The fragment containing the two NHR-25 binding sites (Fig. 3.1) was cloned into GFP-vector pPD107.97 (Fig. 3.2) between the restriction sites of Stu I and Xba I. The efficiency of cloning was very low and after transformation of the bacterial cells we got only two colonies but both had correct insert of NHR-25bs (TGA) into the GFP vector confirmed by sequencing. The control insert with binding sites 2x TCTAGGTCA was unsuccessful. The transformed bacteria were amplified in liquid LB medium and the DNA isolated and purified for transformation to the worms. The N2 worms at young adult stage were injected and the result of it impatiently expected. The transformation of the worms was successful as over 60 F1 worms exhibited the transformation marker *rol-6* phenotype. Rolling progenies were transferred to new plates and checked their progenies for inheritance of this transgene. After three more generations when the number of rolling worms in the brood increased, they were checked under the fluorescent microscope if the binding sites are active and they show the GFP expression *in vivo*. The expression of the GFP was seen in few of worms in different stages (L3 to adults) in special cell of the body, likely in coelomocytes (Fig. 3.3).

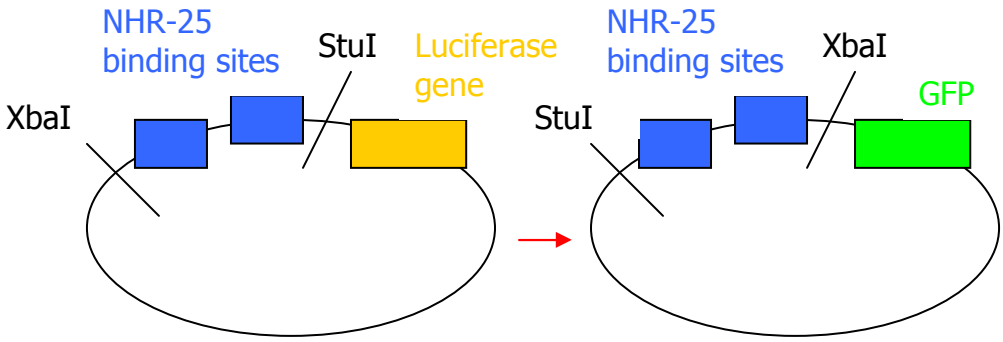

Fig. 3.2 The scheme of cloning – the fragment with two NHR-25 binding sites was cloned from luciferase vector plasmid for transfection assay in human cells into GFP vector plasmid for worm transformation.

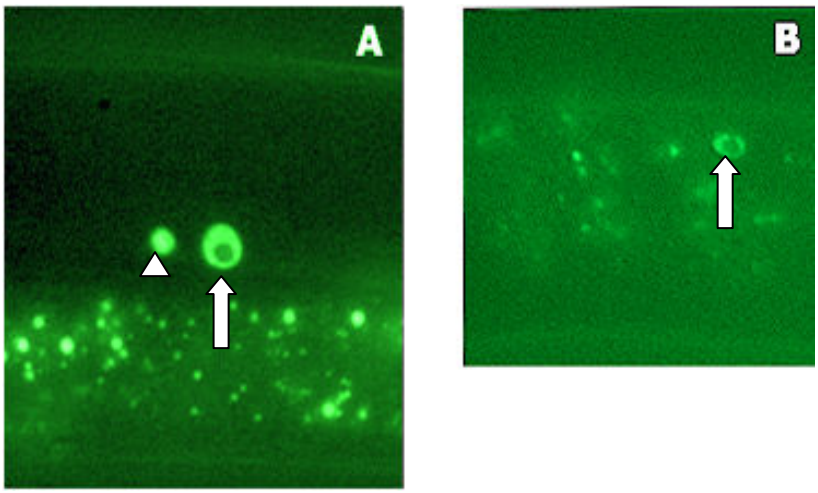

Fig. 3.3 The expression of GFP seen in transformed worms carrying 2x NHR-25 binding sites. A: coelomocyte (arrow), a droplet of GFP (arrowhead), granules at the bottom are autofluorescence of the gut, 63x, B: GFP expression in coelomocyte (arrow) in different animal, 40x

3.3 RNAi

3.3.1 NHR-25 RNAi affects the development of the cuticle

The RNAi was performed as described in Materials and methods. The worms were observed in intervals according to the stages, from L1 to adults. The *dlg-1::gfp* worms with silenced *nhr-25* had harsh molting defects, when the old, unshed cuticle attached on the body of the worm on the tail (Fig. 3.4A) or made a constriction around the vulva (Fig. 3.4B). The, *nhr-25* silenced worms carrying *pxf-1::gfp* were exhibiting also severe molting defects. The cuticle was detached on the head (Fig. 3.5A, B, Fig. 3.6A) and on the tail of the worm (Fig. 3.6B), sometimes it was

constricted near the vulva similar to the RNAi treated *dlg-1::gfp* strain. Those molting defects were never seen in control bacteria fed animals.

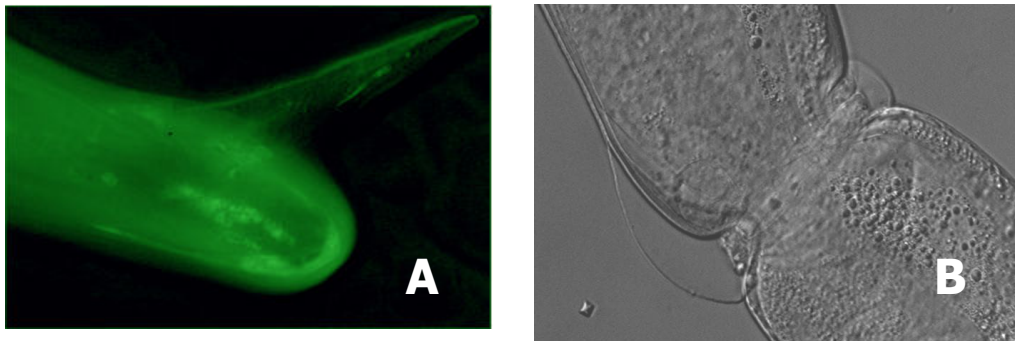

Fig. 3.4 NHR-25 RNAi on worms carrying *dlg-1::gfp*, the molting defects were observed. A: at the tail, 63x, B: at around vulva, 40x

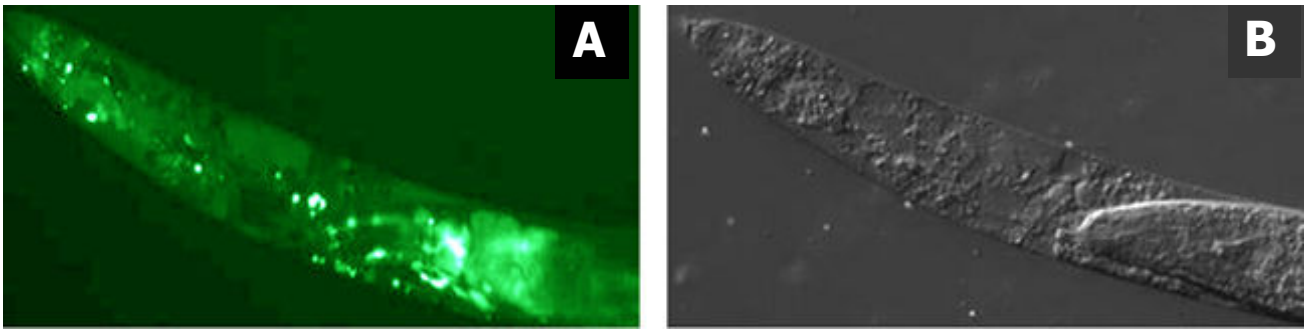

Fig. 3.5 NHR-25 RNAi on worms carrying *pxf-1::gfp*i, the molting defects were observed at the head region. A: the head of adult worm (20x); B: the head of the same adult worm (20x) with DIC

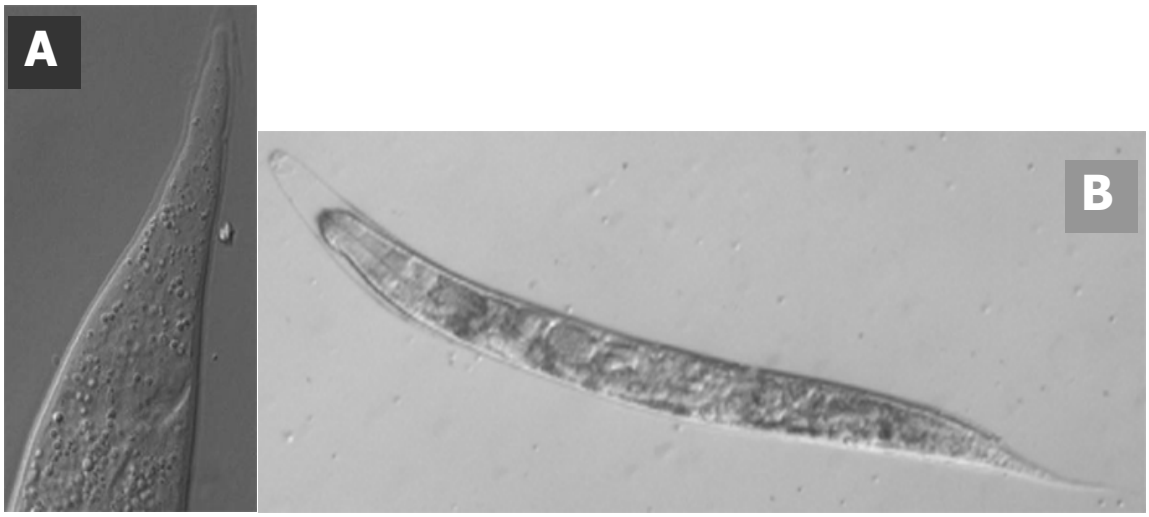

Fig. 3.6 NHR-25 RNAi on worms carrying *pxf-1::gfp* A: The molting defects at tail of the adult worm was observed (40x); B: Adult worm which could not shed old cuticle (10x)

3.3.2 NHR-25 is responsible for defects in seam cells and alae but not for *dlg-1::gfp* expression

The RNAi against *nhr-25* caused defects in seam cells and alae. Alae is lateral, longitudinal cord produced by seam cells on cuticle surface and it is present only in L1, dauer and adult stage. In wild type epithelial junction looks like double line of uninterrupted appearance close to the surface of the worm's body on each side (Fig. 3.7A, G). After treated with RNAi against *nhr-25*, there were seen terrible defects. With the strain carrying *dlg-1::gfp,* structures like maps or blossoms were observed from L1s instead of straight double green fluorescent lines (Fig. 3.7B, C, D, E, F, H, I, J, K, L). The apical junctions between seam cells were interrupted and the cells are defective, their shape was not usual. The alae in adults were again interrupted. Although strong abnormality of the seam cells was observed, the GFP expression driven by *dlg-1::gfp* promoter does not seem to be reduced.

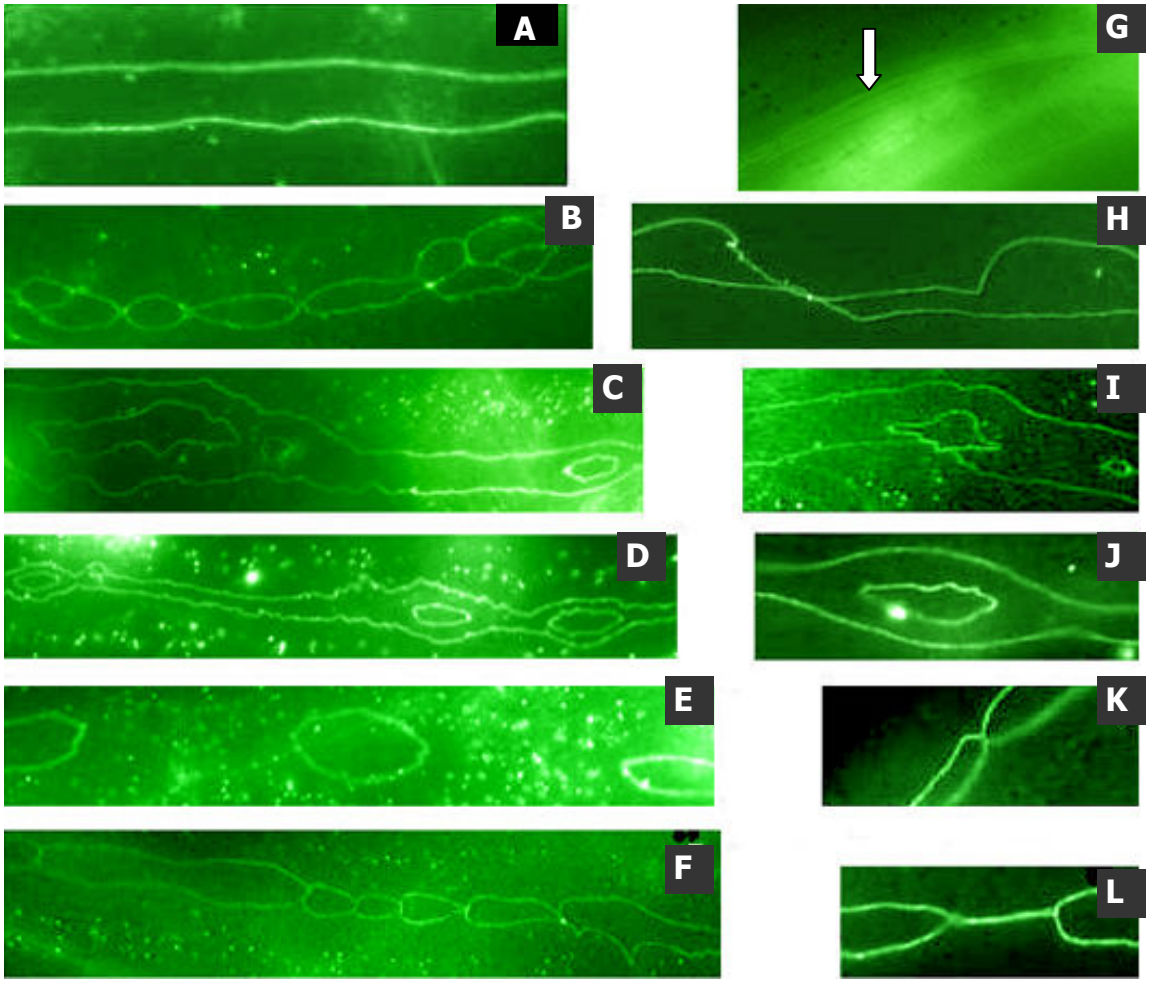

Fig. 3.7 NHR-25 RNAi on worms carrying *dlg-1::gfp* expression in the seam cell junction. A and G: wild type of seam cells and alae (arrow) in the adults, the worm was fed on control bacteria, 63x, B – F, H – L: defects in seam cells affected by NHR-25 RNAi in young adults, different types of interrupted cell-cell junction in seam cells can be observed; B, C, D, E, F – 40x, H, I, J, K, L – 63x

With *pxf-1::gfp,* again worms with defective seam cells were obtained when NHR-25 RNAi was applied. In wild type the seam cells have nice green fluorescence and very well distinguishable nuclei, all present almost all the time of life of the animal (Fig. 3.8, Fig. 3.9). In NHR-25 RNAi affected worms, the cells were loosing the fluorescence, when just after the hatching the situation was normal. During the development some of the seam cells (mostly these in the middle part of the length of the body) lost the green marker and observed as dark cells among the fluorescing ones (Fig. 3.10). The seam cells at the anterior of the body and near the tail were normal.

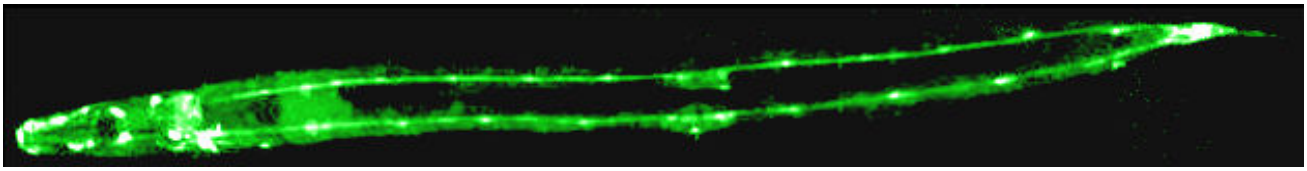

Fig. 3.8 *pxf-1::gfp* expression in the wild type worm (stage L4), two fluorescent lines of the cells on the sides are fused seam cells, 10x, fluorescence

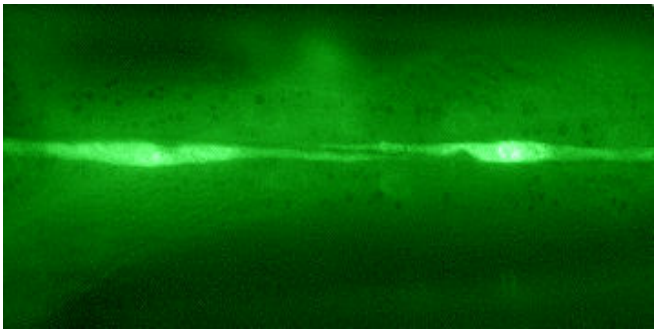

Fig. 3.9 *pxf-1::gfp* expression in the wild type worm. Well distinguishable nuclei of the seam cells in L4 can be seen , 40x

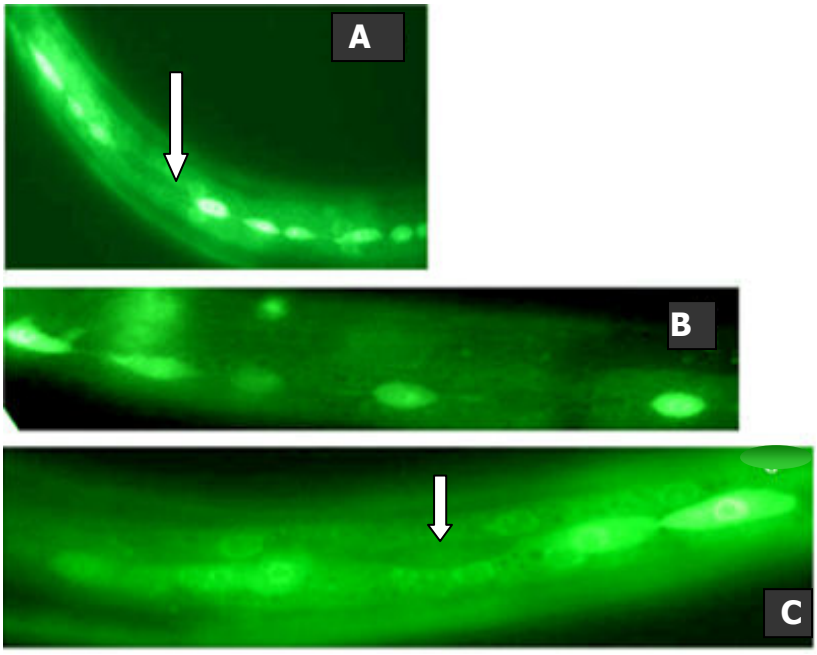

Fig. 3.10 NHR-25 RNAi on worms carrying *pxf-1::gfp* A: missing fluorescence (arrow) in the seam cells, 40x; B: different intensity of fluorescence in seam cells, 40x, C – missing fluorescence (arrow), 63x

3.3.3 Another defects enhanced by NHR-25 RNAi

The worms had more defects than these in the cuticle – they had tumorous gonad (Fig. 3.11A, B) or missing vulva and sometimes they were not able to lay eggs. After that, they died with full of young larvae (Fig. 3.11C). With *dlg-1::gfp* transgene, dumpy body shape (Fig. 3.12C) and unusual vacuoles under the epidermis (Fig. 3.12A, B) were observed.

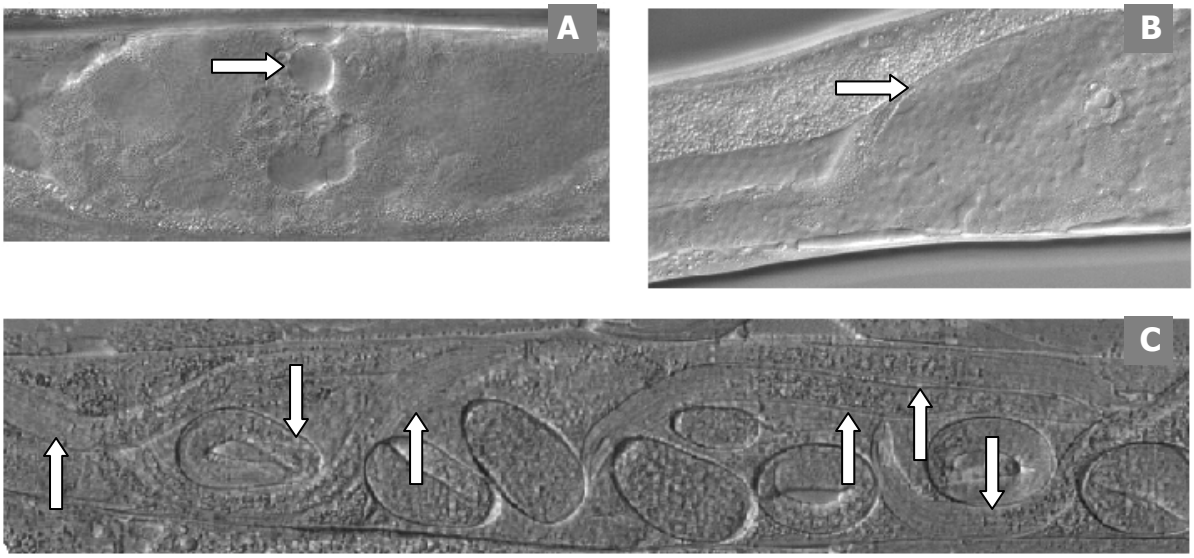

Fig. 3.11 NHR-25 RNAi on worms carrying *dlg-1::gfp* A: tumorous gonad and vacuoles (arrow); B: tumorous gonad (arrow), 40x; C: an adult vulvaless worm with full with young larvae (arrows), 20x

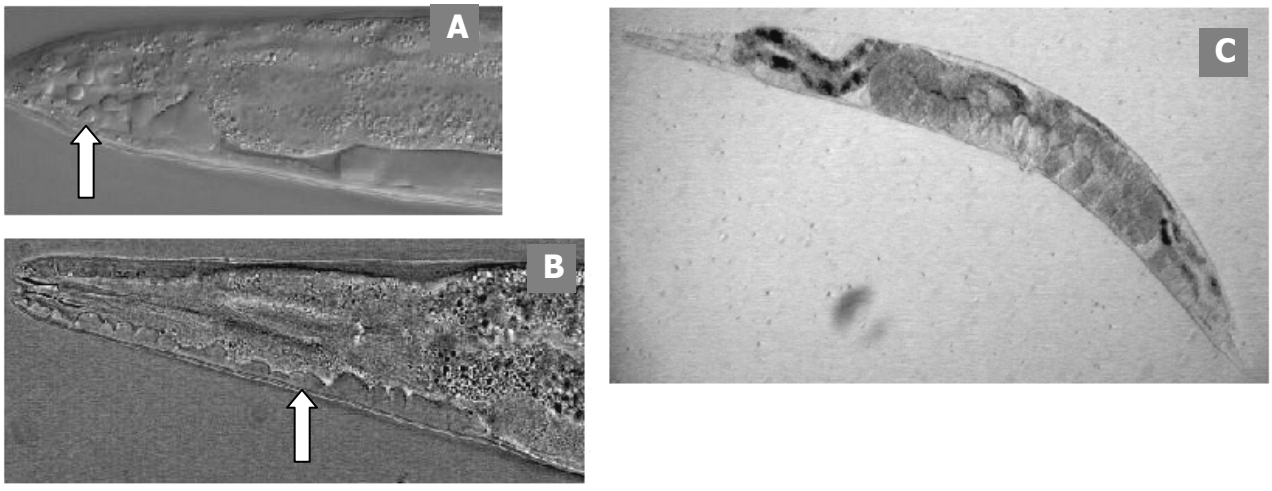

Fig. 3.12 NHR-25 RNAi on worms carrying *dlg-1::gfp*, A, B: vacuoles under the epidermis (arrow, A: the tail, 63x, B: the head, 40x), C: dumpy body shape (short and thick), 10x

The *pxf-1::gfp* strain had very similar problems as the *dlg-1::gfp* but more severe. Sometimes the vulva had phenotype known as "burst vulva". The worms had problems with laying eggs and the development of them was slower than in the control worms. Instead of three days it took four or nearly five and the worms died earlier than control animals. Since the youngest larval stages there were disorganized interior of the body (Fig. 3.13A, B), which sometimes maintained until the adulthood (Fig. 3.14A, B). The worm's body was often deformed with defective inside and smaller than the body of the wild type (compare Fig. 3.14 and 3.8). The pharynx was broken or not straight (Fig. 3.15A), the lumen of the gut was enlarged (Fig. 3.15B). These animals developed slower than wild type ones, and die younger, probably in connection of cuticle defects.

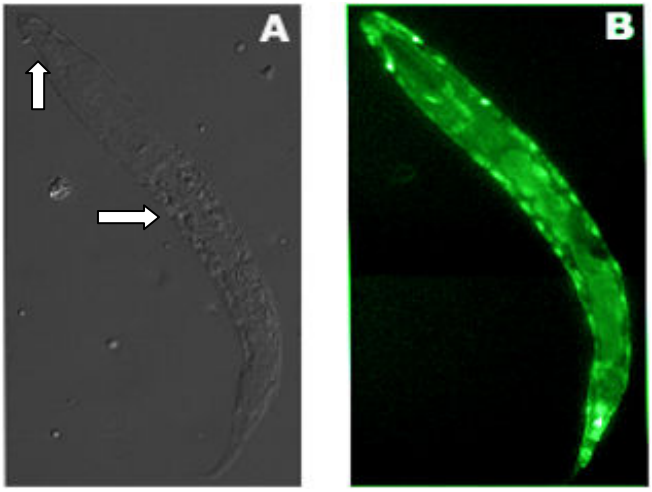

Fig. 3.13 NHR-25 RNAi on worms carrying *pxf-1::gfp* A: L1 stage with disorganized inside of the body (arrows), 20x, B: GFP expression in the same animal, 20x

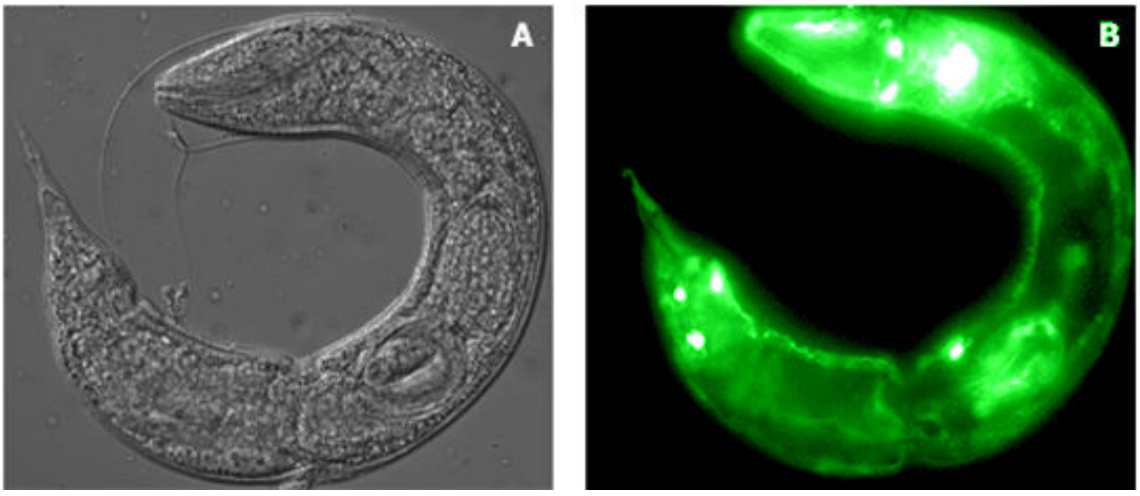

Fig. 3.14 NHR-25 RNAi on worms carrying *pxf-1::gfp* A: L4/adult worm with disorganized inside of the body and molting defect, 40x, B: GFP expression in the same worm, 40x

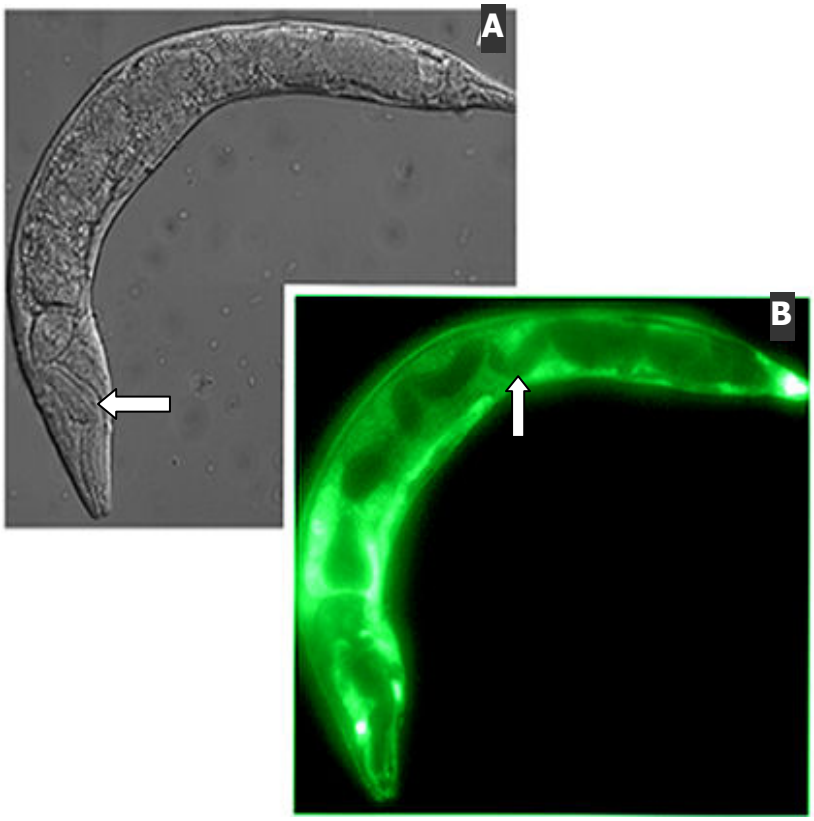

Fig. 3.15 NHR-25 RNAi on worms carrying *pxf-1::gfp* A: L1 stage with strange shape of pharynx (arrow) and enlarged lumen of the gut, 40x, B: GFP expression in the same L1 stage worm showing the enlarged lumen of the gut (arrow), 40x

4. Discussion

The objective of this work was to search for the function of nuclear receptor NHR-25 in epidermal cells of *C. elegans* in transcriptional regulation. This was achieved by two different ways – first with analysis of NHR-25 binding sites activity *in vivo* and second with RNA interference in two different worm strains carrying promoter::GFP fusion expressing in epithelial seam cells

4.1 Binding sites assay

The luciferase vector containing the two binding sites for NHR-25 was used for cloning these binding sites into GFP vector. Luciferase and its fluorescence is a tool used in human cell transfection assay, but in *C. elegans* is not possible to use it because the process of visualization of the fluorescence is by enzymatic reaction and it is lethal for the worms. However, the GFP was many times demonstrated to work in the living worms without problems.

The GFP vector has a *myo-2* minimal promoter, we cloned the fragment with binding sites in front of the minimal promoter to test the activity of the binding sites visualized by the GFP expression *in vivo*. The expression was really observed, also the transformation was successful. The activated cell type was unpredictable and it seems so, that the green positive cells are unexpectedly coelomocytes, large cells in the body cavity.

We were able to see the green-positive cells, it means it is highly likely that those NHR-25 binding sites are reacting to NHR-25 in those cell types in the adult. It would be very interesting to take a closer look in other developmental stages as NHR-25 is expressed in earlier developing stages as well as other cell types. The NHR-25 function in the adult is a new aspect which was uncovered by this study. There are six coelomocytes which are oblong cells residing as 3 pairs (ventral anterior, ventral posterior and dorsal) in the pseudocoelomic cavity. Four of them are present at hatching and two are generated in the first larval stage. Because of their ability to take up a variety of molecules from the body cavity fluid these cells have been suggested to act as scavenger cells. They have quite large nucleus and are

actively endocytic. For further study, first it needs to be verified that those GFP positive cells are truly coelomocytes and if yes, it might lead to another important function of this nuclear receptor in endocytosis. It is also necessary to make a transgenic worm strain with a control construct (2x TCTAAGGTCA) to make sure the activation of this transgene is really due to NHR-25. Although I have tried to make such construct, the efficiency of the ligation was not high enough to obtain a positive clone.

Nevertheless, we have shown in this study that those binding sites are active *in vivo*, it is likely that NHR-25 can work as in the following scheme shown before as a transcription factor in *C. elegans* (Fig. 4.1).

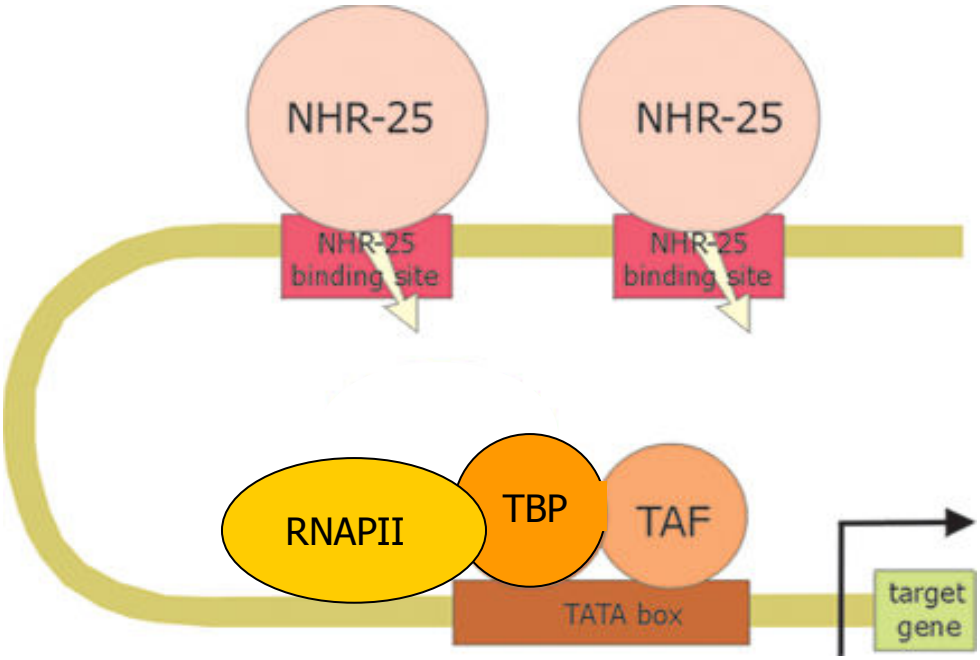

Fig. 4.1 NHR-25 may have its own target *in vivo*.

4.2 RNAi – NHR-25 is involved in development of the epidermis and causes defects in seam cells

The NHR-25 is expressed in the seam cells and it is indispensable for them to elongate and reach their neighbors, consequently to form the alae in the right way (Silhankova et al., 2005). The lateral seam cells are arranged in a row and each of the cells is connected via adherens junction to its anterior and posterior neighbor and to the hyp7 syncytium. During the development the seam-cell contacts are

interrupted with an asymmetric division and the new anterior cells fuse with the hyp7 syncytium. There stay temporary gaps between them. The posterior daughter-cells actively bridge these gaps and the development in L1 stage animals normally continues (Austin and Kenyon, 1994, Silhankova et al., 2005). The loss of *nhr-25* function disturbs the cell fates after asymmetric division. NHR-25 is responsible for the periodic restoration of mutual seam-cell contacts through whole worm's development (Silhankova et al., 2005). The NHR-25 silenced worms have usually problems in development of the epidermis, there were seen anomalies like molting defects and interrupted alae.

The genes *dlg-1* and *pxf-1* have functions connected with the epidermis, also for this reason they were chosen for trying the effect of NHR-25 RNAi on them. The defects in *dlg-1::gfp* and *pxf-1::gfp* NHR-25 silenced worms are in cooperation with known characteristics of NHR-25. There is really the connection between NHR-25 and development of the epidermis, especially the seam cells.

The DLG-1::GFP is expressed in differentiating epithelial cells and neuroblasts, where its production diminish very soon. After ventral enclosure, the protein was detected also in the epidermis, pharynx and intestine. The pharynx and intestine were not affected with NHR-25 RNAi as well as the epithelial cells connected with reproductive system (vulva, uterus, spermatheca). The biggest difference between wild type *dlg-1::gfp* worms and *nhr-25* silenced *dlg-1::gfp* worms was in the epidermis and especially in seam cells. As mentioned in Results, the fusion of these cells were quite never completed, there remained gaps and interruptions. The gaps formed in the line of seam cells in *dlg-1::gfp* animals could be caused with several different reasons. The seam cells affected with RNAi could be unable to reach their neighbors to connect with them, so the gaps stay and the alae is finally interrupted. Or, possibly the cells which do not fuse, are not the right cells, not seam cells, also their function can not be correct and the connection is full of cracks and forms window-like structure.

More defects of epidermis and cuticle were seen and the dumpy body shape, too.

Since DLG-1 expression itself does not seem to be affected by nhr-25 RNAi, it is an excellent tool to further investigate the problem of seam cell fusion and differentiation.

The PXF-1::GFP is expressed in endodermal precursor, neuronal precursor cells and epidermal cells of the embryo, then in the epidermis and gut during the whole development of the worm and neuronal cells of the head and tail and the pharynx, too. The strongest expression is in the lateral seam cells and hyp10, but also in syncytial hyp7. The protein production in pharynx wobbles during the development, when GFP is present around the time of molting, but is very weak during part of the intermolts (Pellis-van Berkel et al., 2005).

The expression of GFP was not affected by *nhr-25* RNAi in other cells than in seam cells. Changes in fluorescence in *pxf-1::gfp* expression was observed during the larval stages. The production of the protein in the cells oscilated likely in according to the time of seam cells division – in the time of dividing the cells are loosing the fluorescence and after that, they are again green and try to fuse with their neighbors. Similar oscillation was seen in wild type *pxf-1::gfp*, but there the process normally ended in fusion of seam cells and creation of long fused lines of them (Fig. 3.8). The biggest different was in the stages, although L1 worms had often normal seam cells with normal expression of the protein (but the interior of the body was disturbed), after two more stages the fluorescence became lower (independent of cell division) and by the adult stage, they were not able to make the fused line. Since there is no convincing NHR-25 binding site in this promoter, this effect might be indirect. L3 stage was observed with severe molting defects and those worms did not proceed the development to L4 stage and adulthood. The strange organisation of the gut and other inner organs were present in most of the *nhr-25* silenced worms in this strain. The dumpy body shape was also observed in these animals, so it is possible, that PXF-1 and NHR-25 together cooperate on this process of formation the body of the worm.

Based on these results, it can be said that *pxf-1* and *dlg-1* transgenes are sensitizing the NHR-25 RNAi effect and enhanced phenotypes on the epidermal cells were observed. The nuclear receptor NHR-25 must be required for cell-shape dynamics during epidermal differentiation in cooperation with other genes.

Summary

In this study, construct containing NHR-25 binding sites (2x TGAAGGTCA) was introduced into worms to test the transcriptional activity of NHR-25 *in vivo*. Cloning of the construct and the transformation of the worm with it were successful. This transgene was active in coelomocyte in the adult. This suggests a new function of NHR-25 in endocytosis. Furthermore, *nhr-25* RNAi was applied on two worm strains carrying *dlg-1::GFP* and *pxf-1::GFP*. Those genes are expressed in the epithelial cells where NHR-25 has an important role. Expression of *dlg-1::GFP* was not affected by the *nhr-25* RNAi but severe seam cell junction/fusion defects were observed. Although *pxf-1::GFP* expression seems to be affected by the *nhr-25* RNAi, the effect may not be direct. Interestingly, the defects caused by *nhr-25* RNAi were enhanced by having those transgenes especially with *pxf-1::GFP*. This sensitizing phenomenon may suggest a possibility of interaction between those genes.

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