Analysis of NHR-25 function on yolk expression in *C. elegans*

BSc. Thesis

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Annotation:

In this work the NHR-25 function on yolk expression in *Caenorhabditis elegans* was studied.

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1. Abstract

Nuclear receptors are key regulators in animal development and play roles in various biological processes. SF-1 (steroidogenic factor 1) is one of the conserved nuclear receptors and involved in somatic sex determination in mammals in co-operation with DM domain protein namely DMRT1. Mutations in either SF-1 or in DMRT1 cause XY sex reversal or feminization in mammals. The DM domain protein in *C. elegans* is MAB-3 and it is responsible for sex determination in the worm as mutations in the gene causes ectopic yolk production in males. NHR-25 is the homolog of SF-1 and it is an essential gene for the worm development. However the involvement of NHR-25 in sex determination has not been studied so far. To test this possibility, female/hermaphrodite specific yolk gene *vit-2::gfp* was introduced in *him-5* background and the yolk expression was monitored in *nhr-25* RNAi-treated worms both in males and in hermaphrodites. The sex transformation visualized by yolk production did not occur when NHR-25 was down-regulated. Therefore unlike in mammals, NHR-25 may not co-operate with DM domain protein in the worm sex determination. However, the yolk expressions are affected in nhr-25 RNAi-treated hermaphrodites suggesting NHR-25 may play an important role in synthesis and/or transport of yolk protein in hermaphrodites.

2. Aim of project

Sex determination is a diverse developmental process among phyla. Interestingly DM domain genes, members of *Drosophila* doublesex and *C. elegans mab-3* (male abnormal) genes are highly conserved and play roles in sexual development in both invertebrates and vertebrates. DMRT1 (*doublesex* and *mab-3* related transcription factor1) in mammals cooperates with SF-1 (steroidogenic factor 1) for somatic sex determination and mutations in these genes cause XY sex reversal or feminization. A homolog of SF-1 in *C. elegans* called NHR-25 is a key regulator in various tissues and is important in many aspects in worm development. However the involvement of NHR-25 in the worm sex determination has not been elucidated. The aim of this study is to test whether NHR-25 has a role in sex determination similar to MAB-3. A mutation in *mab-3* causes somatic sex transformation and mutant male worms produce yolk protein, which is normally restricted to the female/hermaphrodite. Therefore the yolk production, using a vitellogenin reporter gene*, vit-2::gfp*, was monitored in *nhr-25* knock-down (RNAi treated) worms.

3. Introduction

3.1. C. *elegans*

The nematode *Caenorhabditis elegans* is a eukaryotic multicellular organism that is widely utilized as a model organism or a system to study fundamental questions in developmental biology, neurobiology and behavioural biology. One of the biggest advantages of the model organism is that a sophisticated genetic approach that can be applied to address biological questions. For example, Sydney Brenner, John E. Sulston and H. Robert Horvitz broadly studied genetic regulation of apoptosis in *C. elegans*. Apoptosis is a conserved process in many other organisms and it appeared to be regulated by same set of genes that was identified in the worm.

Another advantage of *C. elegans* is that it's easy to culture on petri dishes in a laboratory; it is a small round-worm of about 1 mm length as an adult, non-parasitic free-living soil nematode and feeding on bacteria and fungi. In laboratory conditions it is grown on lawn of *E. coli* bacteria. It has a very fast life cycle (Fig. 3.1).

C. elegans genome was one of the first multicellular organism sequenced entirely. Its genome is relatively small 9.7×10^7 base pairs or 97 megabases while human genome is over 3 billion base pairs.

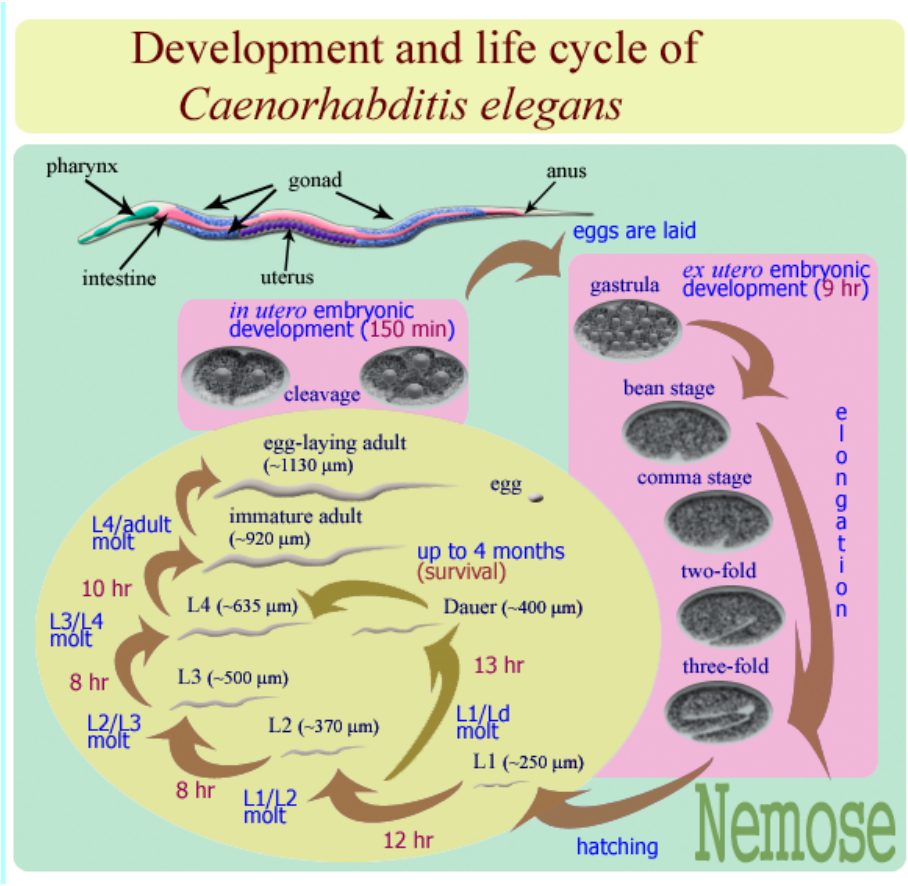

Fig. 3.1: The life cycle and morphology of *C. elegans* (http://www.geochembio.com/IMG/C-elegans-s.png)

3.2. Nuclear receptor NHR-25

The development of *C. elegans* involves many complicated and complex processes such as cell fusion, migration, and differentiation. NHR-25 is one of the key regulators for the worm development and has roles in embryogenesis, epidermal differentiation, cell fate decisions, moulting, vulva morphogenesis and fat metabolism (Asahina et al. 2000, Chen et al. 2004, Silhankova et al. 2005, Asahina et al. 2006, Mullaney et a. 2010). NHR-25 is a member of nuclear receptor family and it is a transcription factor. Homologs of NHR-25 are Ftz-F1 in *Drosophila* and SF-1/LRH-1 in mammals. The mutation in SF-1 causes somatic sex transformation from male to female (Achermann et al. 2001) and therefore SF-1 plays a role in sex determination. Homologs are involved in additional various biological processes such as segmentation and metamorphosis in

Drosophila, and regulation of steroidogenic tissue development and cholesterol homeostasis in mammals.

3.3. Sex determination in *C. elegans*

There are two natural sexes of *C. elegans*: XX hermaphrodite and the XO male. Hermaphrodites can reproduce both by self-fertilization and by mating with males. Mated hermaphrodite produces progenies of about half XX and half XO while the majority of self-fertilizing progeny is XX (Fig. 3.2). XO male occurs approximately in 1/500 chance in nature. The number of males in a population can be greatly enhanced by *him* (high incidence of males) mutations (Hodgkin et al., 1979).

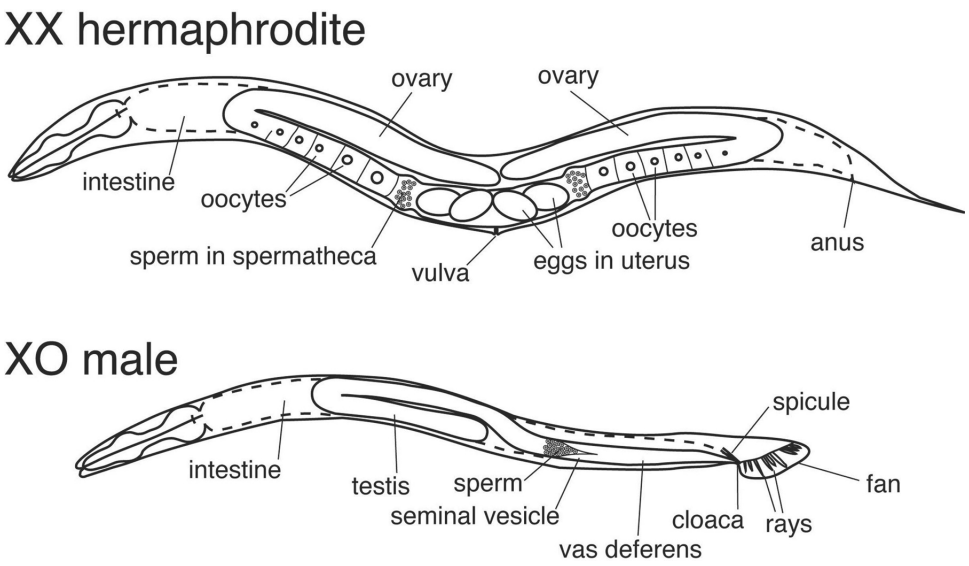

Fig. 3.2: Morphology of hermaphrodite and male. (http://www.wormbook.org/chapters/www_somaticsexdeterm/somaticsexdetfig1.jpg)

In *C. elegans*, *tra-1* gene encodes the worm transformer which is involved in sex-determination (Hodgkin and Brenner, 1977; Hodgkin, 1987). The significant impact of *tra-1* gene on somatic sex determination is proven by experiment where loss of function of *tra-1* causes sex reversal (Hodgkin 1980, 1987). The sex determination pathway is shown in Fig. 3.3. TRA-1 is a zinc finger transcription factor and regulates downstream gene *mab-3*, the DM (Doublesex and MAB-3) domain-containing protein. Sex specific transcription such as production of yolk protein in the intestine is directly regulated by *mab-3* gene and mutation in *mab-3* causes ectopic yolk production in the male. Interestingly, *Drosophila double sex*

(dsx) regulates yolk protein transcription and male sensory organ differentiation. The similarity between *mab-3* and *dsx* suggests the mechanism of sex determination might be evolutionarily conserved (Woelsung Yi et al. 2000). Mammalian homolog of *dsx* DMRT1 (doublesex and *mab-3* related transcription factor 1) is also involved in the somatic sex determination and mutation in this gene causes XY sex reversal (Raymond et al. 2000) similar to mutations in SF-1.

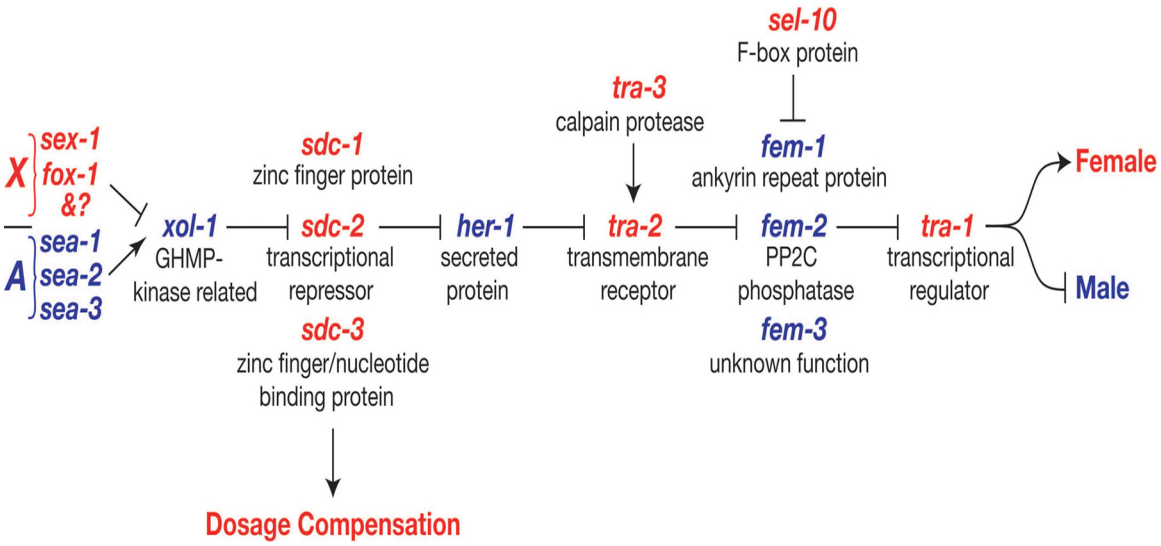

Fig. 3.3: Sex determination pathway (http://www.wormbook.org/chapters/www_somaticsexdeterm/somaticsexdetfig2.jpg)

4. Materials and methods

4.1. Worm culture and list of worm strains

Worms were cultured on nematode growth medium (NGM) plates, fed on E. coli (OP50) at 20 °C. NGM plates were prepared as following: 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 L.

List of worm strains used: DH1033 (*vit-2::gfp* transgenic worm line), DR466 (*him-5*) obtained from Caenorhabditis Genetic Center (USA). By crossing DH1033 hermaphrodites and DR466 males, a new strain HL90 was generated (*him-5; vit-2::gfp*, see Results and Discussion).

4.2. Feeding RNAi on worms

RNAi is a technique widely used to reduce (or eliminate) the function of a gene. The effect of this reduction of function can be followed by observing the development and phenotype of the worm.

For the preparation of the bacteria for feeding RNAi, the bacteria carrying RNAi construct were recovered from frozen stocks by spreading over Luria-Bertani (LB) medium containing antibiotics: carbenicillin (CRB) and tetracyclin (TET). Subsequently the plates were incubated at 37°C overnight. A single colony was further cultured in 5 mL LB with antibiotics mentioned above overnight at 37°C. Then 500 µL of the culture was inoculated in 20 mL LB (100 µg/ml CRB, 12.5 µg/ml TET) and incubated for 2-4 hours at 37°C. When the OD_{600} reached 0.6, IPTG (isoprophylthio-β-D-galactoside, 0.4 mM) was added to induce the dsRNA and followed by incubation for 4 hours at 37°C. The culture was spiked by adding antibiotics (100 µg/ml CRB, 12.5 µg/ml TET) and IPTG (0.8 mM final concentration)and stored at 4°C . Before each experiment, 1 ml of the liquid culture was centrifuged, the pellet was resuspended and put on RNAi plates (30 µl / 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). Synchronized

embryos by bleaching (SAVO, 4M NaOH, H2O) were place onto RNAi plates with RNAi bacteria.

4.3. Microscopy

The Olympus SZX12 microscope with fluorescent attachment was used for picking and monitoring the worms. For selecting *vit-2::gfp* transgenic worms, GFP filter was used. When observing RNAi treated worms and to study the detail GFP expression pattern in transgenic worms, Olympus FluoView® 1000 confocal microscope was used. The worms were anesthetised with the mixture of 0.2 % tricaine and 0.02% tetramisole and placed on 5% agar pads with a thin platinum wire.

5. Results and discussion

5.1. Genetic cross of C. elegans

DH1033 strain carrying *vit-2::gfp* transgene is a stock strain of hermaphrodites. The natural occurrence of male is very low (less than 0.2%) and it was not convenient for the further study. Therefore, these hermaphrodites were mated with males of DR499: *him-5 (e1490)* strain, which has a mutation in *him-5* gene that causes the male occurrence about 30%.

Parents: In one NGM plate with a bacteria spot, 3 hermaphrodites of young adults (DH1033) and 5 males of young adults (DR499) were put together.

Genotype of DH1033: +/+ (V); *vit-2::gfp/vit-2::gfp* (X)

Genotype of DR499: *him-5/him-5* (V); +/+ (X)

Cross: ♀ +/+ (V); *vit-2::gfp/vit-2::gfp* (X) x *him-5/him-5* (V); +/+ (X) ♂

F1 progeny: Three days later, GFP positive F1 worms were checked under the fluorescent stereo microscope and 10 GFP positive hermaphrodites were transferred to fresh plates individually to self-fertilize for the next generation.

Expected genotype (crossed progeny): *him-5*/+ (V); vit-2::gfp/+ (X)

Expected genotype (self-fertilized progeny): +/+ (V); *vit-2::gfp/vit-2::gfp* (X)

Self-fertilization: *him-5*/+ (V); vit-2::gfp/+ (X) x *him-5*/+ (V); vit-2::gfp/+ (X)

or +/+ (V); *vit-2::gfp/vit-2::gfp* (X) x +/+ (V); *vit-2::gfp/vit-2::gfp* (X)

F2 progeny: 50 GFP positive hermaphrodites were transferred to fresh plates individually to self fertilize for the next generation. *him-5(e1490)* is a recessive allele and hermaphrodites homozygous for *him-5* should throw male progeny in the population.

Expected genotypes of both crossed and self-fertilized progeny (ratio):

(V)		(X)
him-5/him-5 (1)	;	*vit-2::gfp/vit-2::gfp* (5)
him-5/+ (2)		*vit-2::gfp/+* (2)
+/+ (5)		+/+ (1)

F3 progeny: Plates having males were selected. Only homozygous *him-5* hermaphrodite can have male progeny. If the worms were homozygous for *vit-2::gfp*, all hermaphrodite progeny are GFP positive. 20 GFP positive hermaphrodites from the male occurring plate were transferred on fresh plates individually to self-fertilize for the next generation.

Expected genotypes: him-5/him-5 (V); vit-2::gfp/vit-2::gfp (X)

him-5/him-5 (V); vit-2::gfp/+ (X)

him-5/him-5 (V); +/+ (X)

+/+ (V); vit-2::gfp/vit-2::gfp (X)

+/+ (V); vit-2::gfp/+ (X)

+/+ (V); +/+ (X)

F4 progeny: Whether all individual plates are having males and if all hermaphrodites are GFP positive are checked. Then a stock of this new strain was established and named HL90.

Expected genotype: him-5/him-5 (V); vit-2::gfp/vit-2::gfp (X)

5.2. *vit-2::gfp* expression in *nhr-25* (RNAi) worms

Worms subjected to RNAi of *nhr-25* exhibited morphological defects such as moulting defects. Vulva defects were also clear in the adult hermaphrodites, as they were not able to lay eggs. Being unable to push fertilised eggs outside because of the abnormal vulva, the fertilised embryos hatch from their eggshells in the body of hermaphrodite and start feeding on the parental tissue. This phenomenon is also known as “Bag of Worms”. Those worms were found in *nhr-25* RNAi treated worms but not with the control RNAi (bacteria carrying empty RNAi vector). Since morphological defects reported previously were observed, we were confident that the RNAi of *nhr-25* was successful.

5.2.1. NHR-25 does not alter the yolk expression in the male

RNAi was subjected to the newly established HL90 strain (*him-5; vit-2::gfp*) and males were pick to observe under confocal microscope. Vitellogenin is normally synthesized in the gut of the hermaphrodite. Gut ganules are visible with DAPI channel (Fig. 5.1B) but no GFP signal was detected (Fig. 5.1C). At least 50 male worms were analysed but only weak autofluorescent could be detected.

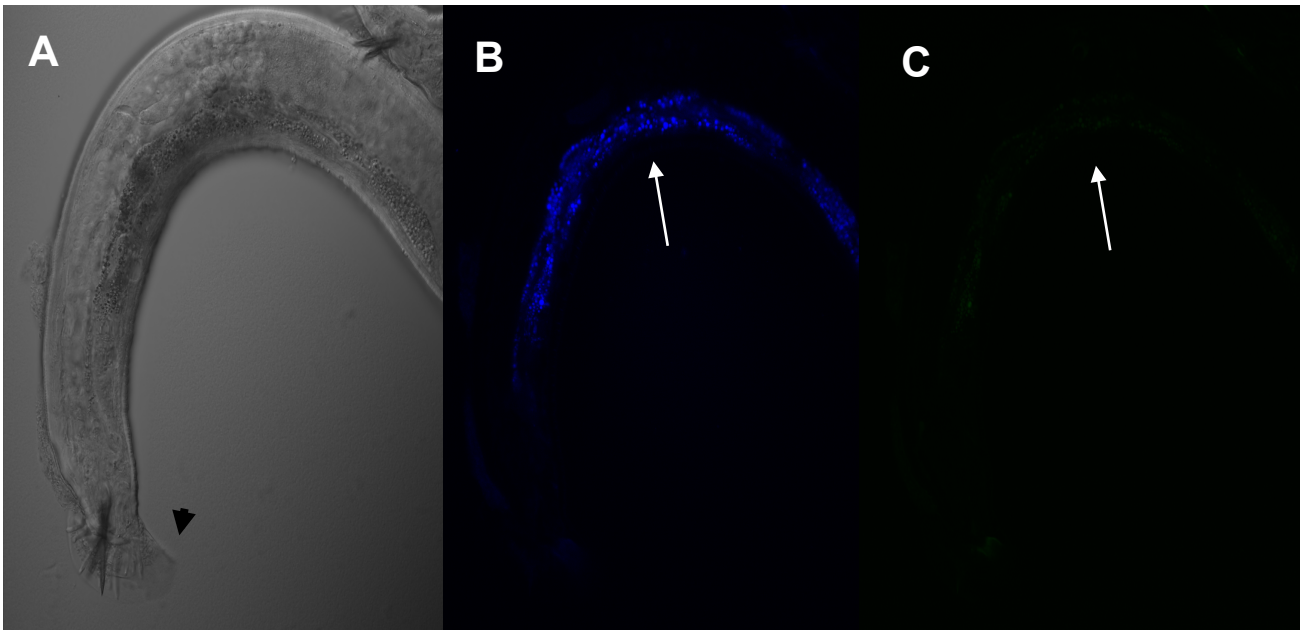

Fig. 5.1 : *vit-2::gfp* expression in *nhr-25* RNAi male worms. A: DIC image of posterior region of male worm. Black arrow head indicate tail morphology specific to males. B: Fluorescent image taken with DAPI channel showing granules in the gut. C: Fluorescent image taken with GFP channel. Only weak auto fluorescence could be detected. White arrows indicate the intestine. Images taking at 600x magnification.

Therefore the reduction of function of NHR-25 did not alter the yolk expression in the male. This result suggests that NHR-25 is not involved in somatic sex determination and the DM box gene MAB-3 and NHR-25 may not function in the same pathway unlike in mammals.

5.2.2. Yolk expression in the hermaphrodite

The yolk distribution was monitored in control and *nhr-25* RNAi treated hermaphrodites*. vit-2::gfp* is expressed in the intestine and then secreted into the body cavity. In the control animal, more or less uniform distribution of yolk protein is observed in the gut (Fig. 5.2B) while the expression was stronger in the nhr-25 RNAi treated animals. Moreover the accumulation of yolk protein was seen in the pseudocoelemic cavity (Fig. 5.2D, F). The accumulation of the yolk protein was also seen in the head region (Fig. 5.3). In the tail region, the nhr-25 RNAi significantly affects the tail morphology and increased yolk protein can be detected (Fig. 5.4). Fig. 5.5 shows that yolk is present in the cytoplams of the oocytes in control RNAi. Disorganised gonad caused by *nhr-25* RNAi and abnormal distribution of the yolk is evident. These results clearly demonstrates that the destribution of yolk protein is highly affected in the entire body of the *nhr-25* RNAi-treated animals. This suggests that NHR-25 might be involved in the synthesis and/or transport of the yolk protein in the hermaphrodites.

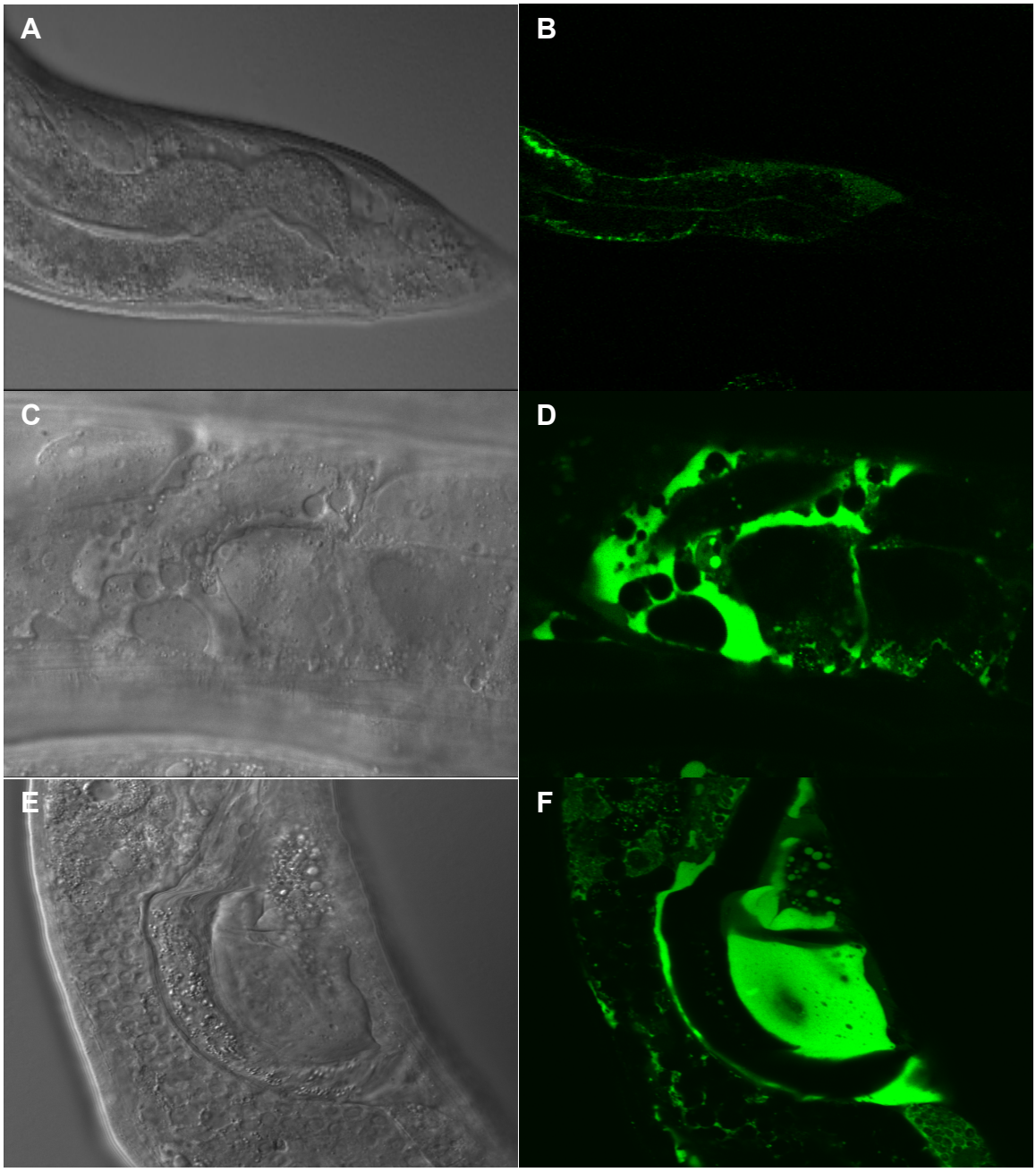

Fig. 5.2 : Strong and droplet-like expression of yolk protein in *nhr-25* RNAi worms A, B: Posterior region of control-RNAi worm. (left) DIC image, (right) Uniform distribution of yolk protein is seen in the gut. C, D, E, F: Mid-body region of the worm in *nhr-25* RNAi-treated worm. The *vit-2::gfp* expression is stronger compared to control and accumulation of the yolk protein is seen in the pseudocoelemic cavity (arrows).

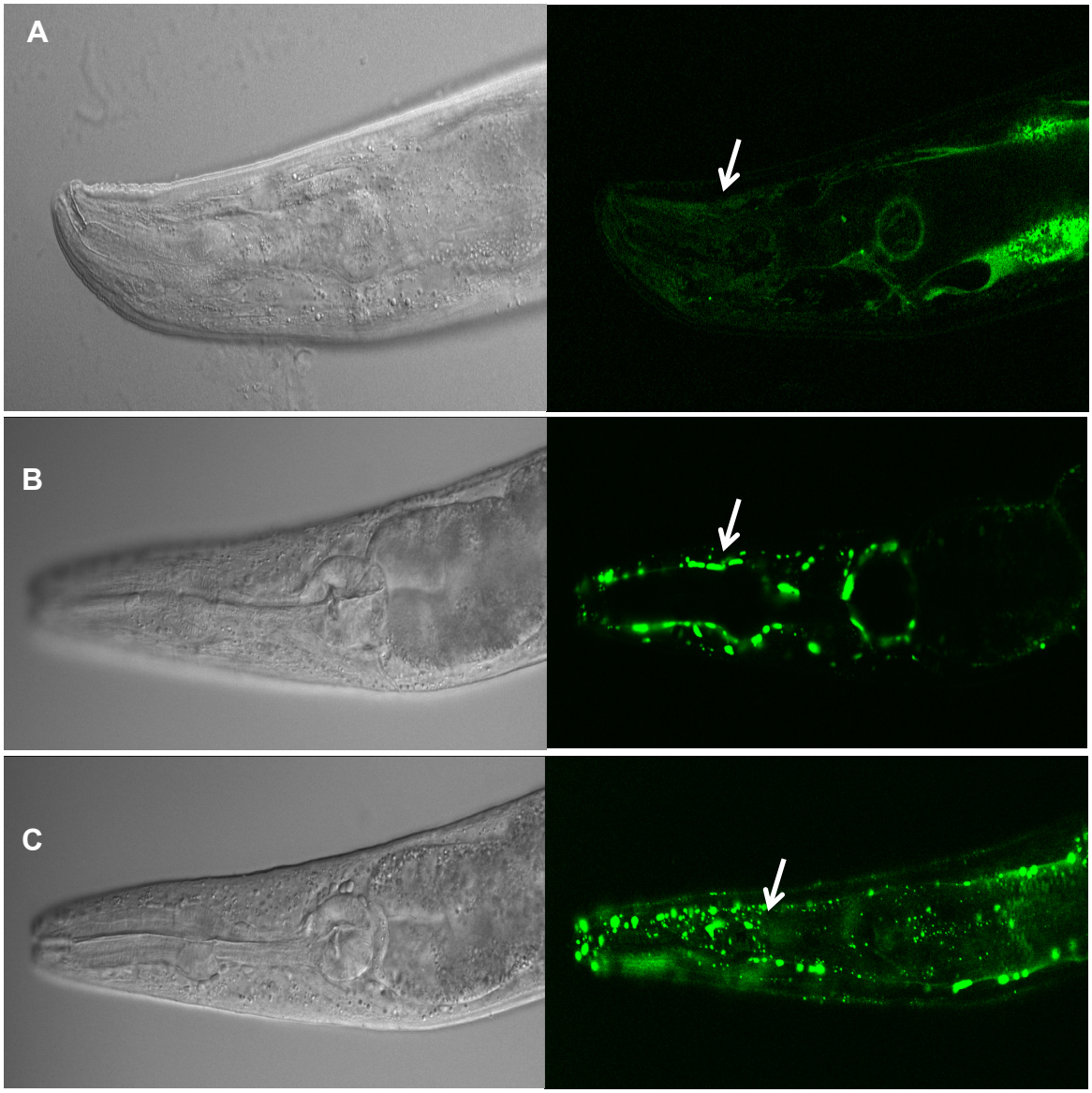

Fig. 5.3 : Yolk protein expression in the head A: Head region of control-RNAi worm. (left) DIC image, (right) weak yolk protein expression is seen. B, C: Head region of the worm in *nhr-25* RNAi-treated worm. The *vit-2::gfp* expression is higher compared to control and the accumulation of the yolk protein appears to be droplet like granules. (arrows).

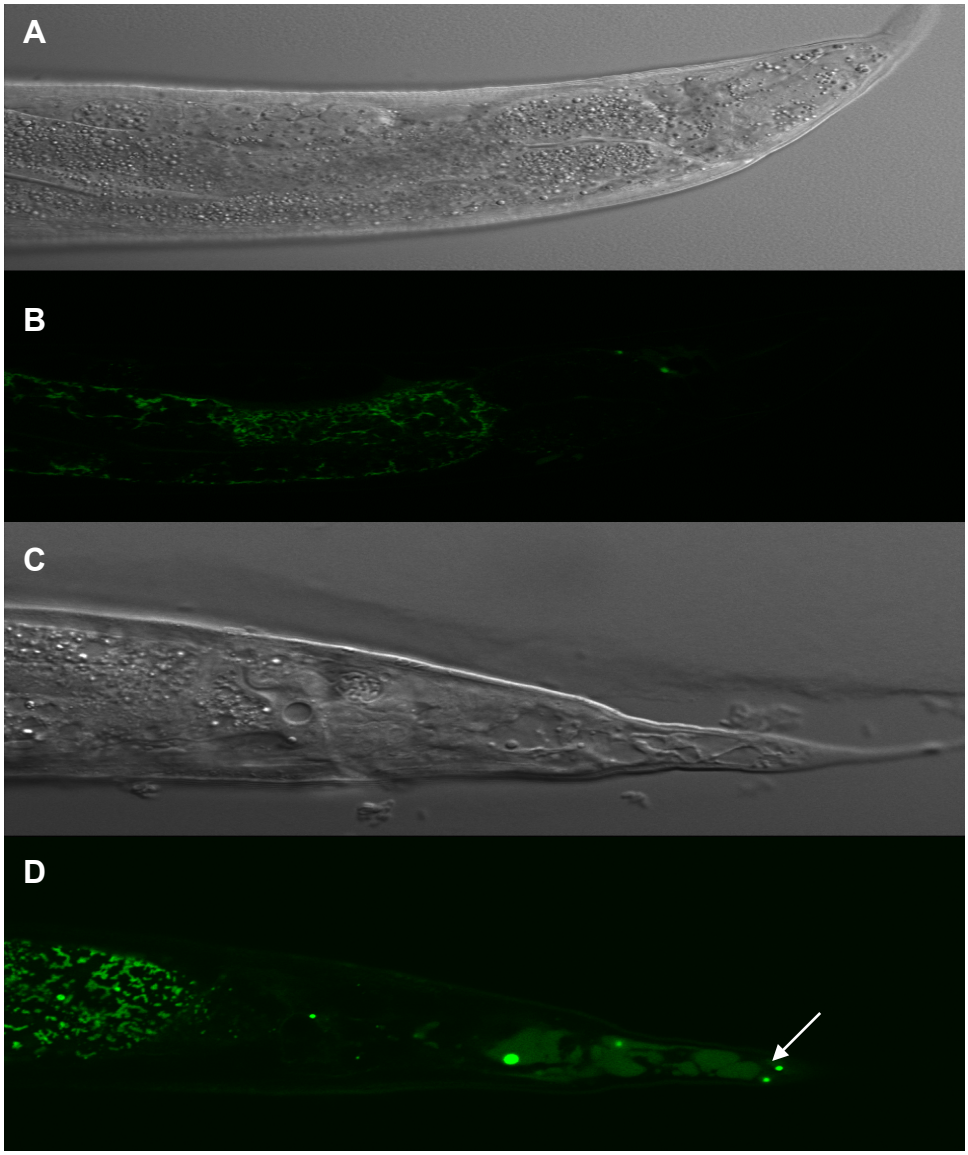

Fig. 5.4: vit-2::gfp expression in the tail region of hermaphrodites.

A, B: control RNAi showing weak yolk expression in the gut and not GFP is detected in the tail. C, D: nhr-25 RNAi exhibits visibly deformed tail with increased amount of yolk expression.

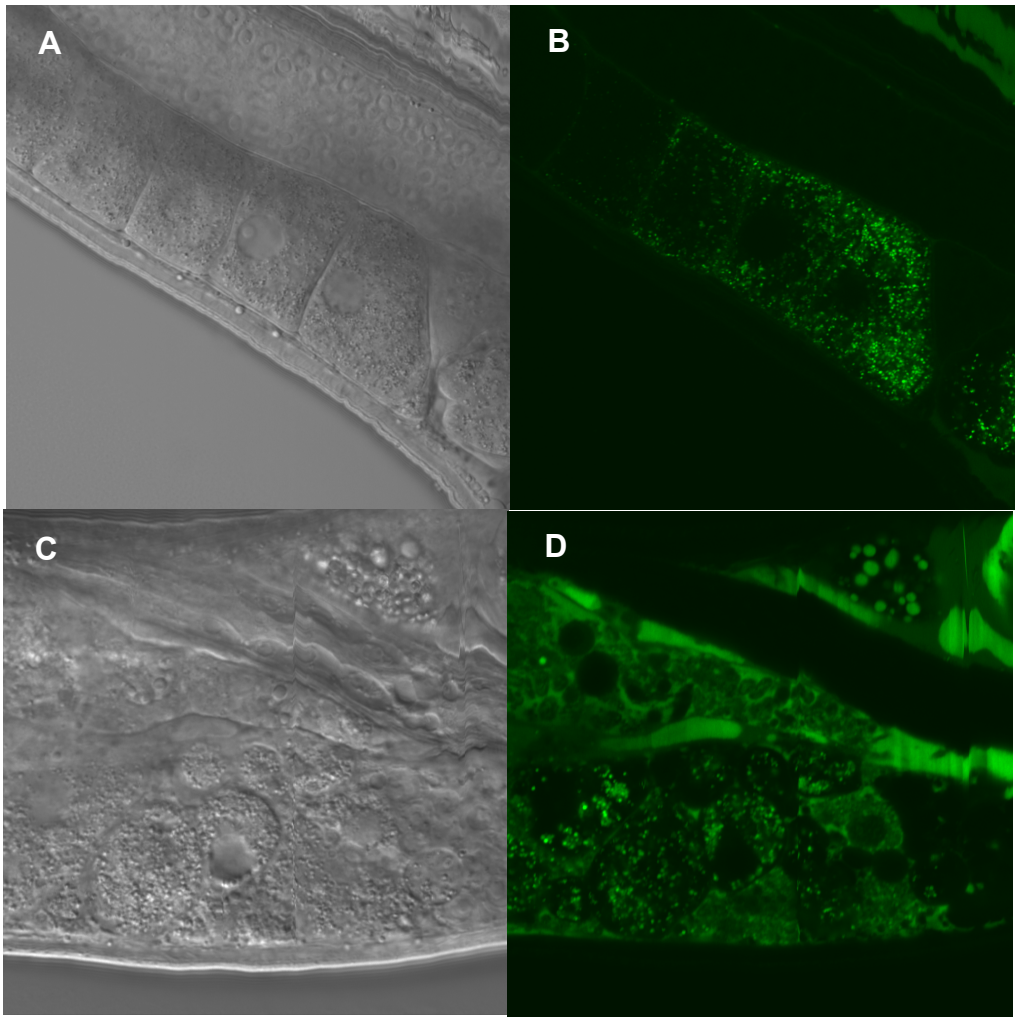

Fig. 5.5: *vit-2::gfp* expression in the gonad

A, B: Cytoplasmic expression of the yolk protein in the oocytes of control RNAi worms. C, D: Disorganised gonad caused by nhr-25 RNAi and abnormal distribution of the yolk is evident.

6. Conclusions

In this study, the involvement of NHR-25 in somatic sex determination was tested. First, a transgenic line carrying *vit-2::gfp* in *him-5* background was established by means of genetic cross. This strain was subjected to *nhr-25* RNAi to monitor yolk distribution in both males and in hermaphrodites. Although the RNAi technique was working properly, there was no ectopic expression of yolk protein in males. Therefore no sex transformation was observed using the female/hermaphrodite marker gene *vit-2*. For this reason, it is less likely that NHR-25 plays a role in the worm sex determination and the relationship with DM domain protein (e.g. *mab-3*) might be different between mammals and the nematodes. However, the yolk expression in the hermaphrodites was highly affected by *nhr-25* RNAi. Accumulation of the yolk in entire body was striking and it appears to be droplet-like structure rather than fine granules. Hence NHR-25 may play a significant role in yolk synthesis and/or transport.

There was no sex transformation in terms of yolk production in males after nhr-25 RNAi. This was not due to technical problems as RNAi worked well; the expected defects on phenotypes (such as tail morphogenesis defect) were observed. The phenotypes also showed molting defect and vulvaless adults (adults containing hatching embryo inside themselves).

In the nhr-25 RNAi treated adult hermaphrodites an abnormal distribution of yolk was observed. Yolk is secreted in cytoplasm and it is spread all over the cytoplasm, on the other hand it is absent in nucleus as it takes no function in nucleus. Within the adult hermaphrodite the yolk distribution appeared uneven all over the body, recognized as droplets of yolk (Results Fig 4.3 – Fig 4.16). Yolk distribution in embryo was uniform. Yolk was not observed in pharyngeal muscle, this muscle is part of epidermis.

7. References

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8. Additional of methods

These are the methods and their data I've obtained and studied during my project. These methods and data were not fully included in this thesis, but as they were very useful to study I've included them here.

- Handling with C. elegans worms
- Preparation of NMG, RNGM plates for C. elegans
- RNAi
- Plasmid Isolation – Plasmid DNA purification (Promega Kit)
- Worm bleaching and cleaning procedure
- Single worm PCR
- DNA transformation in bacteria
- Microscopy

9. Acknowledgements

This project would not have been possible without the guidance and the help of several individuals who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study.

First and foremost, my utmost gratitude to Masako Asahina whose sincerity and encouragement I will never forget. Masako has been my inspiration as I hurdle all the obstacles in the completion of this work.

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