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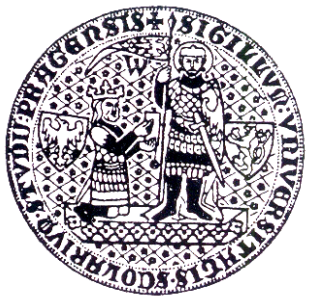

Nuclear receptors in *Caenorhabditis elegans*: NHR-40 regulates embryonic and larval development

PhD thesis

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CONTENTS

1 ABBREVIATIONS

aa	amino acid
ACEDB	a *Caenorhabditis elegans* database
AF-1	activation function 1 domain
AF-2	activation function 2 domain
bmd	body morphology defect
bp	base pair
BWM	body wall muscles
cDNA	complementary deoxyribonucleic acid
COUP	chicken ovalbumin upstream promoter transcription factor
DBD	DNA binding domain
DL	dorsal left
DNA	deoxyribonucleic acid
dpy	short, fat, "dumpy" phenotype of worms
DR	dorsal right
DR	direct repeats
ER	everted repeats
EST	expressed sequence tag
gDNA	genomic deoxyribonucleic acid
GFP	green fluorescent protein
GRE	glucocorticoid response element
HLH	helix – loop – helix motif
HNF4	hepatocyte nuclear factor 4
HRE	hormone response elements
HSE	heat shock response element
hsp	heat shock promoter
HTH	helix – turn – helix motif
IR	inverted repeats
L1-4	1^{st} – 4^{th} larval stage of *C.elegans* development
LBD	ligand binding domain
LXR	liver X receptor
LZ	leucine zipper motif
MHC	myosin heavy chain
N2	wild type strain of *C.elegans*
NGFI-B	nerve growth factor receptor-induced orphan receptor
NGM	nematode growth medium
NHR	nuclear hormone receptor
NR	nonrepeats
NR	nuclear receptor
OD	optical density
ORF	open reading frame
p	probability
PCR	polymerase chain reaction
PPAR	peroxisome proliferator activated receptor
RAR	retinoic acid receptor
RNA	ribonucleic acid
RNAi	RNA mediated interference
RT-PCR	reverse transcription – polymerase chain reaction
RXR	retinoid X receptor
SD	standard deviation
SL1, SL2	splice leader sequence 1 and 2
supnrs	supplementary nuclear receptors
TR	thyroid hormone receptor
VDR	vitamin D receptor
VL	ventral left
VR	ventral right

2 THE AIM OF THE STUDY

The aim of the study was to perform a complex biological analysis of a hypothetical nuclear receptor in a model organism, *Caenorhabditis elegans*.

We decided to study *nhr-40*, a predicted nuclear receptor that is encoded in *C. elegans* genome as a member of extremely multiplied group of genes that share sequence homology with vertebrate nuclear receptors.

Our aim was to elucidate, if the *nhr-40* is a functional gene and help to answer the intriguing question, if members of the hypothetical superfamily of NHRs in nematodes are functional genes with important biological functions.

The specific aims were to:

- Identify transcripts
- Characterize transcripts and the expression profile in specific cells or tissues and specific developmental stages
- Conduct functional studies of the gene in focus by inhibition on posttranscriptional level by RNAi, by expressing the gene ectopically, and by creating a putative dominant negative transgene
- Characterize gene functions using mutant lines
- Confirm the identified functions by rescue experiments

3 INTRODUCTION

3.1 Nuclear hormone receptors in *Caenorhabditis elegans*

C. elegans contains 284 nuclear hormone receptor (NHR) genes. This large number of genes is in contrast with the number of receptors in other species. 48 nuclear receptor (NR) genes have been identified in human and 18 NRs in *Drosophila melanogaster*. Except for DAF-12 (Motola et al., 2006), no ligand or activator of NHRs in *C. elegans* has been found till this time and all remaining NRs in *C. elegans* are called "orphan". Molecular phylogenetic analysis grouped NRs to six major NR subfamilies (Nuclear Receptor Committee, 1999). Comparative analysis of both DNA binding domain (DBD) and ligand binding domain (LBD) sequences suggested that most of the NRs in *C. elegans* arose from an extensive expansion and diversification of the HNF4 group (Robinson-Rechavi et al., 2002).

The 15 NRs from *C. elegans* are conserved. The functions of 11 genes from more then 284 predicted *C. elegans* NHRs were identified. It was shown that *nhr-23* (Kostrouchova et al., 1998) and *nhr-25* (Asahina et al., 2000, Gissendanner et al., 2000) are important for development of epidermis, *daf-12* regulates dauer formation (Antebi et al., 2000), *sex-1* affects sex determination (Carmi et al., 1998), *fax-1* and *unc-55* are important for neuronal development (Much et al., 2000; Zhou and Walthall, 1998), *nhr-67* and *nhr-85* are important for proper vulval development and function, *nhr-8* is involved in toxin resistance, *nhr-41* and *nhr-85* are important during dauer formation and *nhr-6* during ovulation (Gissendanner et al., 2004).

The remaining 269 *C. elegans* NHRs appear to be nematode specific and are referred to as supplementary nuclear receptors or supnrs (Bertrand, 2004; Robinson-Rechavi, 2005). Researchers have speculated about this large number of supnrs in nematodes. It is likely that many of the *C. elegans* supnrs evolved from an ancestral gene related to vertebrate hepatocyte nuclear factor 4 (HNF4) (Bertrand, 2004; Robinson-Rechavi, 2005). Consistent with this possibility, it was recently shown that one of the HNF4-related *C. elegans* factors, NHR-49, is a major regulator of fat metabolism and is involved in functions supported by PPARs in vertebrates (Van Gilst, 2005a; Van Gilst, 2005b). Functional analysis of the supnrs will provide the best evidence for the origin and maintenance of this unusually large family of potential ligand – binding transcription factors. One possibility is that the expansion

of this gene family was in response to exposure to a large number of xenobiotics in the environment (Lindblom, 2001).

nhr-40 represents one member of the small group of 18 receptors of *C. elegans* NHRs defined by the P box sequence CNGCKT. It was recently shown that this receptor also belongs to the more conserved group I of supplementary nuclear receptors (Robinson-Rechavi et al., 2005).

3.2 NHR structure and function

3.2.1 Transcription factors – regulation of the gene expression

Differences between the various cell types depend on the particular gene expression. The control of gene expression can be applied at all levels of the pathway leading from DNA to protein. The gene expression could be regulated in a gene – specific way at any of several sequential steps. There are six steps at which eukaryote gene expression can be controlled: (1) transcriptional control (activation and initiation of transcription), (2) RNA processing control, (3) RNA transport control, (4) translation control, (5) mRNA degradation control and (6) protein activity control (Alberts et al. 1994).

The transcription of each gene is controlled by a regulatory region of DNA near the site where transcription begins. Genes are in active state in the cells in which they are expressed. Transcription of a gene starts when RNA polymerase binds to a specific region, the promoter, at the start of the gene, and moves along the template and synthesizes RNA. This action defines a transcription unit that extends from the promoter to the terminator (Lewis, 2000). Eukaryotic differentiation is result of regulation of tissue – specific gene transcription. Regulatory transcription factors serve to provide common control of a large number of target genes.

Transcription factors require two abilities, recognition of specific target sequences located in enhancers, promoters or other regulatory elements that affect a particular target gene and binding to DNA (Alberts et al., 1994).

3.2.2 DNA – binding motifs

DNA – binding motif is the region in transcription factors which can read, recognize and bind to promoter region of target genes (Lewis, 2000). The motifs are usually short and the surface of the protein is complementary to the special surface of the double helix of DNA (Alberts et al., 1994). The proteins make a large number of contacts with DNA, involving hydrogen bonds, ionic bonds, and hydrophobic interactions. Individual interactions are very weak but more of these contacts give together highly specific and very strong interaction. There are many types of DNA – binding domains.

The first recognized motif was the helix – turn – helix motif (HTH). This motif was found in both eukaryotes and prokaryotes. It consists of two α helices and a short extended amino acid chain between them which constitutes the "turn". The more carboxyl – terminal helix can fit into the major groove of DNA. This motif is found in hundreds of DNA – binding proteins, including repressors. The second helix of the HTH motif binds to DNA via a number of hydrogen bonds and hydrophobic interactions, which occur between specific side chains and the exposed bases and thymine methyl groups within the major groove of the DNA (Brennan and Matthews, 1989). All these proteins bind to DNA as symmetric dimmers (Alberts et al. 1994).

The leucine zipper (LZ) motif is the dimerization domain. This domain is a heptad of leucine repeats that intercalate with repeats of the dimmer partner, forming a coiled coil of α helices in parallel orientation (Agre et al. 1989). One from each monomer, in one polypeptide interacts with a zipper in another polypeptide to form a dimer and to achieve strong specific binding (Alberts et al, 1994). There is a high degree of conservation among the members of this family in the dimerization domain, which is used to both homo- and heterodimerize with other members of the family, as well as in binding cognate C/EBP consensus sequences (Akiyama and Gonzales, 2003).

DNA – binding helix – loop – helix (HLH) motif is related to the leucine zipper. Over 240 HLH proteins have been identified in organisms ranging from the yeast *Saccharomyces cerevisiae* to humans (Atschley and Fitch, 1997). The solution structure of the basic HLH – leucine zipper factor Max first confirmed the existence of the HLH motif (Ferre-D'Amare et al. 1993). A helix – loop – helix motif consists of a short α helix connected by a loop to the second longer α helix. They can bind as homodimers or heterodimers as well (Alberts et al. 1994). Recent analysis of open

reading frames present in the *C. elegans* genome has revealed 24 putative HLH proteins (Ruvkun and Hobert, 1998).

An important group of DNA – binding motifs is zinc – coordinated DNA – binding motif called zinc finger. Zinc finger is the most abundant DNA – binding domain in the human genome (Moore and Ullman, 2003). Zinc finger motifs are protein domains containing typically 30 – 40 amino acid residues, whose structures are maintained by coordination of Zn^{II} ions (Witkiewicz-Kucharczyk, 2006). Each finger has a conserved ββα structure (antiparallel β strands followed by a helix) and amino acids on the surface of the α – helix contact bases in the major groove. The DNA – binding proteins have two types of structure – Cys_2/His_2 and Cys_2/Cys_2 (Berkovits and Berg, 1999; Alberts et al. 1994).

3.2.3 Nuclear hormone receptor

Nuclear hormone receptors are ligand – dependent transcription factors (Mangelsdorf et al., 1995a). They play important roles in the regulation of cell growth and differentiation and afford connection between signaling molecules, hormones, and transcriptional response (Beato et al., 1995; Mangelsdorf, 1995a; Mangelsdorf et al., 1995b; Thummel, 1995). NHRs influence embryonic development and adult homeostasis as hormone – activated transcriptional regulators and have key regulatory functions in a wide range of biological processes (Beato et al., 1995; Van Gilst et al. 2002). NHRs were not found in plants. They are present in *Tripedalia cystophora*, a cubomeduzan jellyfish, which has a retinoid receptor, the jRXR with remarkable homology to vertebrate retinoic acid X receptor (RXR) (Kostrouch et al., 1998). Recently a ligand binding domain combined of heterodimeric transcription factor Oaf1/Pip2 was discovered in yeast *Saccharomyces cerevisiae* (Phelps et al., 2006).

3.2.4 Structure of nuclear hormone receptors

NHRs have conserved structure that consists of 6 domains. The A/B domain localized at N - terminus is not conserved, is variable in sequence and in size of the region, and contains the AF-1 (activation function 1) domain. The AF-1 domain is one of two domains responsible for dimerization. The DNA binding domain (DBD), known also as C domain, is conserved and determines the superfamily of nuclear receptors. The DBD targets receptors to specific DNA sequences known as hormone

response elements (HREs) (Evans et al., 1988; Beato et al., 1991; Mangelsdorf et al., 1995a). The DBD consists of two highly conserved zinc fingers (Berg, 1989). The structure is based on a sequence that can be described by the formula:

Cys-X_2-Cys-X_{13}-Cys-X_2-Cys-$X_{(15\ or\ 17)}$-Cys-$X_{(5-6)}$-Cys-$X_{(9-13)}$Cys-X_2-Cys-X_4-Cys-X_3GM.

The D domain, functionally known as a hinge region is very variable in both length and primary sequence. The hinge region is flexible. This allows some receptors to bind as dimers on multiple types of HREs (Giguere et al., 1999). The ligand binding domain (LBD) is a conserved domain positioned at the C-terminal half of the receptor. This domain is responsible for recognition and binding of ligands and for dimerization of receptors. The binding of ligands to LBD induces conformation changes and bring the receptor to a transcriptionally active state (Mangelsdorf et al., 1995b). This region also includes homo- and heterodimerization interface, hormone dependent transcriptional activation functions, and (in some cases) hormone reversible transcriptional repression (Mangelsdorf et al., 1995a). All ligand activated receptors have a carboxy – terminal activation function termed AF-2 (Bourget et al. 1995). At C – terminus, there is a variable region known as F (Figure 1).

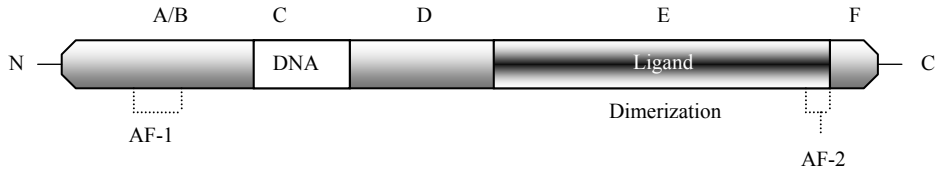

Figure 1. Schematic representation of a typical nuclear receptor

3.2.5 Hormone response elements

Response element is a short sequence in a target gene promoter that is recognized by regulatory transcription factors. The examples are the HSE (heat shock response element), HRE (hormone response element), GRE (glucocorticoid response element) etc. The response element may be located in promoters or in enhancers. The HSE is usually found in a promoter while the GRE in an enhancer. It is supposed that all response elements function by the same general principle.

The specific palindromic DNA sequences called hormone response elements (HREs) are recognized by DBDs of NHRs. HREs are configured as direct repeats

(DR), inverted repeats (IR), everted repeats (ER) or non-repeats (NR) and are spaced by the given number of nucleotides (Mangelsdorf et al., 1995b, Durand et al., 1992). Recognition of HREs is unique.

The receptors for glucocorticoids, mineralocorticoids, progesterone and androgens recognize the same DNA sequence (AGAACA as a half-site) whiles the estrogen receptor and nonsteroid nuclear receptors (RAR, RXR, TR) recognize AGGTCA (Beato et al., 1995). Hormone response elements for the vitamin D receptor (VDR), thyroid hormone receptor (TR) and retinoic acid receptor (RAR) are composed of direct repeats spaced by 3, 4, or 5 nucleotides (DR3, DR4, or DR5, respectively) (Umesono et al., 1991). In relation to these known DR there is a variety of complex HREs. All receptors bind to either DRs or palindromic HREs as either homodimers or heterodimers (Mangelsdorf et al., 1995b).

Steroid receptors and chicken ovalbumin upstream promoter transcription factor (COUP) can bind DNA as homodimers.

RXR, identified by Mangelsdorf (Mangelsdorf et al., 1990), can make partner for other receptors such are TR, RAR, VDR, liver X receptor (LXR), peroxisome proliferator-activated receptor (PPAR), the nerve growth factor receptor-induced orphan receptor (NGFI-B), COUP (Mangelsdorf et al., 1995a). RXR can additionally bind DNA either as a homodimer or as a heterodimer with RAR (Mangelsdor et al., 1991).

3.2.6 Ligands

NHRs may be activated by ligands, small hydrophobic molecules that include steroid hormones, thyroid hormone, Vitamin D, retinoids, farnesoids and related molecules. These molecules can relatively efficiently cross the cell membrane by diffusion. Binding of specific ligands induce conformational changes of receptors with functional consequences (Mangelsdorf et al., 1995a).

Discovery of radiolabeled ligands allowed the identification of binding proteins that were shown to translocate from the cytoplasm to the nucleus (Jensen et al., 1966; Mangelsdorf et al., 1995b). In the 1970s steroids were shown to be targeted to their responsive tissues by the presence of specific high affinity receptor proteins.

In 1985 Yamamoto postulated that the binding of hormone to its receptor induces an allosteric change that enables the hormone-receptor complex to bind to high affinity sites in chromatin and modulate transcription (Yamamoto et al., 1985).

The glucocorticoid and estrogen receptors were cloned in 1985 (Hollenberg et al., 1985; Miesfeld et al., 1986; Green et al., 1986). In 1987 the receptor for the vitamin A metabolite known as retinoic acid was identified (Petkovich et al., 1987). A crystal structure of RXRα LBD was described by Bourguet (Bourguet et al., 1995).

3.2.7 Orphan receptors

The nuclear hormone receptor family includes members with no known ligand. They are called orphan receptors. Recently, natural and synthetic ligands have been identified for several orphan receptors; xenobiotics (steroids, antibiotics) for PXR (Klewer et al., 2002), prostaglandin derivates, fatty acids and leukotriens for PPAR (Desvergne and Wahli, 1999; Emery et al., 2001).

Several subfamilies of orphan receptors have been found in metazoan organisms (Baniahmad et al., 1993, Giguere et al. 1995).

3.3 *Caenorhabditis elegans* as a powerful tool of developmental biology

3.3.1 *Caenorhabditis elegans* as a model organism

Caenorhabditis elegans is an invertebrate model organism. Molecular biologist Sydney Brenner worked with this organism to study the genetics and the function of neuronal system during the ´60s of last century. Today 1 mm tiny nematode has a prominent role in a modern biology. *C. elegans* belongs to Rhabditidae. It is a small transparent multicellular organism. All cells can be seen and followed during development in the light microscope. *C. elegans* develops from embryo (559 cells at hatching) through four larval stages L1, L2, L3, L4 to adult stage during 3 days at room temperature. All four larval stages are punctuated by molts. It exists in two genders, hermaphrodite and male although the incidence of males is 1:500 (Figure 2). Hermaphrodites differ from males in the number of somatic cells (in adult stage a hermaphrodite has 959 cells and male has 1,031 cells) and also in morphology (Sulston and Horvitz, 1977).

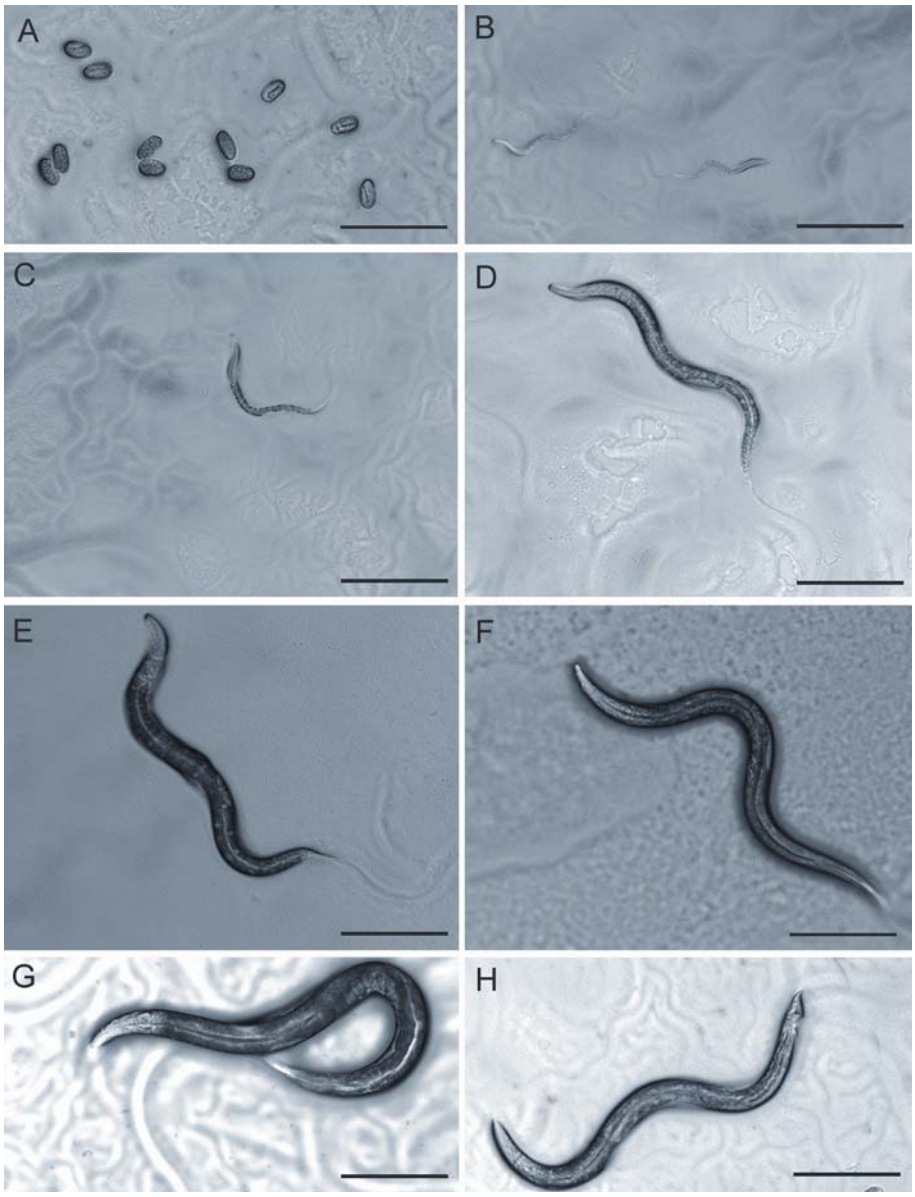

Figure 2. Developmental stages of *C. elegans*

A. Developing embryos

B. Larva in L1 stage

C. Larva in L2 stage

D. Hermaphrodite in L3 stage

E. Hermaphrodite in L4 stage

F. Young adult hermaphrodite

G. Adult hermaphrodite with eggs

H. Adult Male

Scale – 200 μm.

3.3.2 The genome

The genome of *C. elegans* was completely sequenced and published in 1998 (The *C. elegans* Sequencing Consortium, 1998). All data about genes, cosmids, cDNAs, ESTs, repeats and other information are collected and open to public in "a *C. elegans* database" ACEDB, in Wormbase www.wormbase.org., on *Caenorhabditis elegans www* server (http://elegans.swmed.edu/) and others.

The genome consists of 97 million base pairs of DNA. It is approximately 30 times smaller then the human genome. There are predicted approx. 19,000 genes (The *C. elegans* Sequencing Consortium, 1998). More then 40 percent of the predicted protein products have homologues in other organisms, including yeast and human. The number of genes is about three times more than was found in yeast and is about one-third of that predicted in human. The genome of *C. elegans* is divided into 5 pairs of autosomes and one pair of sex chromosome, XX for hermaphrodites and X0 for males. Males arise by non-disjunction.

Intensive studies of many *C. elegans* genes led to reveal both similarities and differences of functions between species such as yeast, mouse, human, etc. Such comprehensive studies can give us answers for questions from many disciplines of biology.

Computer analyses compare the DNA from *C. elegans* with that of other organisms. Understanding the regulatory pathways in worm can help to elucidate related mechanisms in other organisms including humans.

3.3.3 The genetics

Two approaches can be used to find the gene functions – forward and reverse genetics. Forward genetic is based on the identification of mutation and the function of the gene according to the phenotype. Reverse genetic is based on the inactivation of the gene (or an appropriate mRNA or protein) and subsequent identification of the phenotype and the function.

3.3.4 Culturing

Caenorhabditis elegans is easy to culture under the laboratory conditions. Worms fed on bacteria *Escherichia coli* (*E. coli*) strain OP50 on Petri dishes with

nematode growth medium (NGM). The wild-type strain is *C. elegans* variety Bristol, strain N2 (Brenner et al., 1974). Essential compound of NGM is cholesterol that worm has to take from environment. The life cycle of worms depends on culturing temperature. The worm can develop from a fertilized embryo to adult stage during 3.5 days at 20°C. The time of development is longer if the temperature is lower. When worms do not have appropriate conditions for the life they can switch the life cycle to stage-specific program dauer formation in the third larval stage of development.

3.3.5 Anatomy

Observation of animals under differential interference contrast optics in the light microscope allowed elucidation of embryonic and postembryonic cell lineage development that generates fixed number of somatic cells (Sulston and Horvitz, 1977).

The worms are covered with transparent cuticle, which is composed of collagens. The collagens are secreted by polarized epidermal cells. Till today there are predicted more then 180 collagen genes in *C. elegans*. Many of epidermal cells are multinucleate, arising by cell fusions during development. Epidermal cells of *C. elegans* can be divided into four general groups: (1) the main body syncitium, (2) the seam cells, (3) the epidermal cells of the head and tail, and (4) interfacial epidermal cells. The seam cells are arranged as rows of ten cells that run along each lateral line. The seam cells fuse in adult stage. In L1, dauer and adult stage seams form specialized cuticle structure – the alae.

Four longitudinal rows of body wall muscle cells are under epidermis. In contrast to vertebrate they do not form syncitium, but are separated and mononucleated. Muscles are separated from the underlying epidermis and nervous tissue by a thin (approximately 20 nm) basal lamina. The myofilament lattice of the body wall muscles forms an obliquely striated array. The structural unit is analogous to the sarcomere of a vertebrate muscle. Thick filaments contain myosin and overlap with two sets of thin filaments containing actin (Francis et al., 1985).

Body wall muscle cells are granddaughters of AB lineage (1), MS lineage (28), C lineage (32) and D lineage (20). They are organized to four longitudinal strips placed sub- ventrally and sub-dorsally. After hatching at L1 stage, 81 of the 95 cells are present. 21 cells are in dorsal right (DR) and dorsal left (DL) quadrants, 20

in ventral right (VR) quadrant and 19 in ventral left (VL) quadrant. M blast cell produces 14 additional body wall muscle cells post – embryonically so that the adult has 95 body wall muscle cells. 24 cells are organized in DL, DR and VR quadrants and 23 in remaining (VL) quadrant (Krause, 1995). Single sarcomere muscles are the second general type of muscle tissue in *C. elegans*. The pharynx has a total of 20 muscle cells. Several of them are multinucleated. The third type of muscle cells are intestinal and the sex muscles.

C. elegans is fed on bacteria *Escherichia coli*, strain OP50. Bacteria eaten by mouth proceed to muscular pharynx with a grinder. Crushed bacteria are pumped to the intestine. Intestine is formed by 20 large epithelial cells which form a tube. The tissue ends by rectum and anus. Intestinal cells become bi-nucleated and polyploid during postembryonic development. At the beginning of the lethargus of the first molt, most of the intestinal cells divide without accompanied cell divisions giving rise to 20 intestinal cells with a total of 30 – 34 nuclei (Hedgecock and White, 1985).

The nervous system in *C. elegans* has 302 neurons in the adult stage and has a unique combination of properties, such as morphology, connectivity and position. The strict classification of neurons into sensory receptors, interneurons or motorneurons is not generally possible. Many individual neurons combine two or more of these functions. Neuronal support cells form the group of specialized epithelial cells, which is composed of glial cells, amphids, cephalic sensila cells, phasmids, inner labial and outer labial sensilla, deirids, socket and sheath cells.

Body wall muscle cells in the head are innervated by motor neurons from the nerve ring, the next four muscle cells in each quadrant (neck muscles) receive dual inervation from motor neurons of the nerve ring and ventral nerve cord and body wall muscles from the body are innervated by ventral cord motor neurons (White, 1986).

The *C. elegans* reproductive system consists of a symmetrically arranged bilobed gonad. Each lobe is U-shaped and is composed of a distal ovary, proximal oviduct and spermatheca. Germ line nuclei are produced in ovarial syncitium. The nuclei move from the distal tip proximally. They are mitotic, than progress through meiotic prophase and reach diakinesis in the oviduct prior to fertilization. Individual nuclei become enclosed by membranes to form oocytes, which enlarge and mature as they pass down the oviduct. The oviduct in each lobe terminates at a spermatheca carrying about 150 sperms. The spermathecae are connected with a common uterus,

which contains fertilized eggs in early stages of embryogenesis. Together with vulva, uterine and vulval muscles, uterus is an important part of egg-laying apparatus. Fertilized eggs passed out by egg-laying organ into the environment (Wood, 1988).

4 MATERIAL AND METHODS

4.1 Strains

The *C. elegans* Bristol N2 strain was used whenever not specifically stated and was maintained as described (Brenner, 1974). The strain RB840, that has a deletion in *nhr-40(ok667)* gene, was obtained from *C. elegans* Gene Knockout Consortium. The *C. elegans* RRF3 strain was used for some RNAi experiments. This *C. elegans* strain is more sensitive for RNAi than N2 strain. SU93-*jcls 1*[*ajm-1::gfp*; *unc-29(+)*; *rol-6(su1006)*] expressing AJM::GFP transgene (Mohler et al., 1998) and JR667-*unc-119 (e2498 ::Tcl); wls51* [*unc-119(+);scm::gfp*] expressing the transgene in nuclei of seam cells were obtained from the *Caenorhabditis* Genetic Center. The PD7963 (*hlh-1::gfp*) integrated line was a kind gift from Dr. M.Krause (Krause et al., 1994).

4.2 Preparation of growth media

Nematode growth medium (NGM)

1 l of NGM consists of bactoagar (17 g), bactopepton (2.5 g), NaCl (3 g) and cholesterol (1 ml at concentration 5 mg/ml in ethanol). 1 M $CaCl_2$ (1 ml), 1 M $MgSO_4$ (1 ml) and 1 M KH_2PO_4 (25 ml) are added after autoclaving.

Peptone medium

1 l of peptone medium consists of NaCl (1.2 g), peptone (10 g), bactoagar (25 g) and cholesterol (1 ml at concentration 5 mg/ml in ethanol). 1 M $MgSO_4$ (1 ml) and 1 M KH_2PO_4 (25 ml) are added after autoclaving.

LB medium

1 l of LB medium consists of trypton (10 g), yeast extract (5 g), NaCl (10 g) and 1 M NaOH (1 ml).

LB agar

15 g of bactoagar is added to LB medium and autoclaved.

M9 buffer

1 l of M9 buffer contains 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 5 g of NaCl and 0.5 g of $MgSO_4$ .7 H_2O.

Aqua for injection Biotika, Slovenská Ľupča, SR

Water used for molecular biology procedures (dilution of primers, mRNA, gDNA, mixtures for microinjection, reaction mixtures for ligation, restriction, PCR, cloning and etc.).

4.3 Isolation of nucleic acids and PCR

4.3.1 Isolation of genomic DNA

N2 worms and RB840 mutated worms containing the *nhr-40(ok667)* allele with intronic deletion were harvested from 2% agarose plates and washed with water 3-5x including 15 min rocking step to clear OP50 from their gut. Worms were frozen in water at –80°C. DNA lysis buffer (0.1 M Tris pH 8.3, 50 mM EDTA, 200 mM NaCl), 250 µl of proteinase K (20 mg/ml) (Promega, Medison, Wi) and 0.5 ml of 10% SDS were added to worms to total volume 5ml. The mixture was gently mixed and heated at 65°C for 30 min. After two phenol-chloroform extractions aqueous layer without any interface was transferred to a new tube. To the aqueous layer 1/10 volume of 3 M sodium acetate and 2 volumes of cold 99% ethanol were added. The solution was slowly mixed and gDNA came out of it. DNA was wound out using a glass pipette, air dried and then resuspended in 3 ml of water. DNase-free RNase A (ICN Biomedicals Inc., Ohio) was added to resuspended gDNA at a final concentration of 10 µg/ml. gDNA was incubated at 37°C for 30 min. After that gDNA was purified by phenol-chloroform extraction and precipitated with sodium acetate and ethanol as before. Peleted gDNA was air dried and resuspended in 20 µl of water (Biotika).

4.3.2 Isolation of mRNA

N2 worms and mutated strain RB840 were grown on 2% agarose plates. Mix population and separated stages of worms were washed and frozen at –80°C. Frozen pellet was resuspended in 0.5 ml of resuspension buffer (0.5% SDS, 5% 2-mercaptoethanol, 10 mM EDTA, 10 mM Tris-HCl pH 7.5) with freshly added 12.5 µl of proteinase K (20 µg/ml). After incubation at 55°C for 1 hour phenol-chloroform extraction and ethanol precipitation were done. Pellet of RNA was dissolved in 20 µl of water and kept at 4°C for next step - DNase treatment. After DNase treatment

(RQ1 RNase – free DNase, Promega) phenol-chloroform purification and ethanol precipitation the pellet of RNA was dissolved in water.

4.3.3 Reverse transcription

1 µg of total RNA was used for reverse transcription. 1 µg of RNA was mixed together with random hexamers (50 ng/µl, Promega), poly-T-primer or the gene specific primer and incubated at 65°C for 5 min and then immediately cooled on wet ice. 4 µl of 5x first strand buffer (Gibco, Invitrogen), 1 µl of enzyme Super Script™ Reverse polymerase II (Invitrogen), 1 µl of 10mM dNTPs (Takara, Japan), 1 µl of 0.1M DTT (Gibco) and 1 µl of RNasin® Ribonuclease Inhibitor (Promega) were added to total volume 20 µl and incubated 10 min at 25°C, 50 min at 42°C and 15 min at 70°C. The first strand cDNA was stored at –20°C. 2 µl of cDNA were used for PCR.

4.3.4 Polymerase chain reaction (PCR)

All PCR reactions were done with regard to the products, templates and primers under specific conditions. PCR reactions were done by recombinant Taq DNA polymerase from Invitrogen, BIOTAQ™ DNA polymerase from Bioline and BIO-X-ACT™ Short DNA Polymerase for PCR products up to 2000 bp. All PCR reactions were performed on MJ Research PTC-100® and PTC-200® Peltier thermal cyclers (Bio-Rad, Ca).

4.3.5 Polymerase chain reaction from individual worm

A single worm was transferred to a 2.5 µl drop of lysis buffer (50 mM KCl, 10 mM TRIS-HCl pH 8.3, 2.5 mM $MgCl_2$, 0.45% Nonidet P40, 0.45% Tween 20, 0.01% gelatin, 200 µg/ml proteinase K) in the cap of 0.5 µl PCR tube. Proteinase K was added to appropriate volume of lysis buffer shortly before lysis. PCR tubes with lysis buffer and worms were spin to move the drop to the bottom of the tube. Samples were frozen for at least 10 min at -70°C. Then tubes were heated 1 hour at 60°C, 15 min at 95°C and chilled to 4°C. The lysates were used as the templates for PCR with appropriate primers and conditions.

4.3.6 Semiquantitative PCR

cDNA was prepared from 5 μg of total RNA in a 20 μl reaction using reverse transcription reaction with Super Script™ Reverse polymerase II and random hexamers. The PCR products, withdrawed in exponential phase of PCR, were loaded to the agarose gel. Primers used for *nhr-40a* and *nhr-40b* isoforms were: #4580 (CGGGGTACCGTACCCCCGCTGATGTATAGGATT) and #4581 (CATGCCATGGCGGATTTTGTCTCGATCATGTTGG). Primers used for *nhr-40d* were: #6036 GAGATACGGTGCGGTATCGATC and #4581.

Primers #4684 (TTCCAAGCGCCGCTGCGCATTGTCTC) and #4685 (CAGAATTTCCAGCACTCGAGGAGCGGA) were used for amplification of *ama-1* gene, used as internal standard.

4.3.7 Quantitative PCR

Quantitative PCR was performed as described (Sun et al., 2004) with slight modifications. cDNA was prepared from 5 μg of total RNA in a 20 μl reaction using reverse transcription reaction with Super Script™ Reverse polymerase II and random hexamers as described above. The amplicons of selected regions of *nhr-40* isoforms were amplified using PCR, eluted from agarose using electrophoresis and semi-permeable membrane. The amount of DNA was determined spectrophotometrically. The purified DNA was used for determination of standard curves of each amplified region. Real-time PCR was performed in PTC200 DNA Engine® thermal cycler equipped with ALS0296 96-well sample block (Bio-Rad) and the DyNAmo™ HS SYBR® Green qPCR Kit (Finnzymes, Finland), which contains a hot start version of a modified *Thermus brockians* DNA polymerase for prevention of extension of nonspecifically bound primers during reaction setup. The characterization of amplification including number of copies in samples was calculated using the computer program Opticon Monitor™ Version 3.0. Each sample was analyzed at least by two separate analyses made in duplicates. The number of detected copies was normalized according to *ama-1* expression. Primers used for amplification of *nhr-40* isoforms *a* and *b* were #6145 (GGTTCTGCGGGATTCGCGTTTTCG) and #6147 (GCCTTGGGATCCATTCCCATATCG and for isoform *nhr-40d* #6146 GCGGTATCGATCATGCTTGTCCCT and #6147.

Primers #4684 and #4685 were used for amplification of *ama-1* gene.

4.4 Preparation of transgenic lines of *C. elegans*

Transgenic lines were performed by microinjection of DNA in final concentration 100 μg/ml (50 μg/ml of construct or PCR product + 50 μg/ml of pRF4) to the ovarial syncytium of young adult hermaphrodites of *C. elegans*. pRF4 plasmid, *rol-6* (*su1006)* was used as the marker. pRF4 plasmid vector carries the mutation of *rol-6*, one of *C. elegans* collagen genes. Transgenic animals roll (rotate) around their longitudinal axis to the right. Transgenic animals were easily detected and picked out to separate NGM plate with *E. coli* OP50.

4.5 Preparation of synchronized population of worms

Gravid hermaphrodites were washed and collected from NGM, peptone or 2% agarose plates to 14 ml centrifugation tube by water. Worms were washed at least 3x by water to clean them from bacteria OP50. 7 ml of water was added to collected worms. Bleaching reaction was started by adding of 1 ml of 5 M NaOH and 1 ml of bleach solution (Savo, Bochemie, Czech Republic). Worms were shaking for 6 min. After that only eggs should be visible in the solution under microscope. Volume was divided into two 14 ml tubes and sterile water was added to dilute bleach solution and stop the reaction. Released embryos were spinned and washed 3-5x. 12 ml of 0.5x PBS was added to collected embryos (10 x PBS: 80 g of NaCl, 2 g of KCl, 2 g of KH_2PO_4, 29 g of Na_2HPO_4 x 12 H_2O per 1 liter pH 7.4). Embryos were incubated in rocking cuvette overnight. The second day the hatched larvae (synchronized in L1 stage) were spin at 1100 rpm for 10 min.

4.6 Cloning

PCR products were cloned to vectors using several approaches that are described in appropriate chapters. Chemical transformation of different competent cells – DH5α (Takara, Japan), TOP10F (Invitrogen), XL1blue (gift from Fatima Cvrčková, Faculty of Natural Sciences, Prague) for cloning and HT115 for feeding experiments (gift from Andy Fire, NIH) was done using heat shock 41°C per 1 min.

4.7 Identification of *nhr-40* splicing isoforms

We cloned and identified 3 splicing isoforms of *nhr-40* gene. Total RNA was used as the template for preparation cDNA as described above. The beginning of *nhr-40* cDNA gene was identified by PCR with

SL1 (GGTTTAATTACCCAAGTTTGAG) primer or SL2 (GGTTTTAACCCAGTTACTCAAG) primer as sense primers and #4581 as antisense primer that is placed in the end of the 4th exon. 2 µl of cDNA were used for PCR (35 cycles: 94°C for 30 sec, 55°C for 40 sec, 72°C for 1 min). Different PCR products were purified from the agarose gel by sterile filtration paper and membrane (Dialysis tubing, ¾ in Diameter, molecular weight exclusion limit is 12,000 – 14,000 daltons, Invitrogen). Separated PCR products were purified by phenol-chloroform extraction, precipitated by ethanol and cloned to pCR®4-TOPO® vector using TA cloning kit (Invitrogen). Insert in clone #6019 was identified as the 5´end of isoform *nhr-40a* or *nhr-40b* corresponding to these isoforms that are regulated by promoter 1. Insert in clone #6018 was identified as the 5´end of isoform *nhr-40c* or *nhr-40d* corresponding to these that are regulated by promoter 2. Sequencing revealed the sequences of 5´UTR regions and we designed primers #4580 and #6036 corresponding to 5´UTR of appropriate isoforms *nhr-40a/b* or *nhr-40c/d*, respectively.

nhr-40a isoform was identified after sequencing of clone #6053. This clone contains 762 bp PCR product prepared by PCR (35 cycles: 94°C for 30 sec, 57°C for 40 sec, 72°C for 1 min) with primers #4580 and #6048 (CCATTCCCTCAGCTTCTCCACAG) cloned to pCR®II vector of pCR®-TOPO® TA cloning kit. #6048 is backward primer placed in the 7th exon.

Identification of isoform *nhr-40b* was done by sequencing of clone #6070. This clone consists of 796 bp PCR product made by PCR (35 cycles: 94°C for 30 sec, 56°C for 40 sec, 72°C for 1 min) with primers SL1 and #6048 cloned to pCR®II vector.

Identification of isoform *nhr-40d* was performed by sequencing of clone #6072. This clone was prepared by cloning of 748 bp PCR product amplified by PCR (35 cycles: 94°C for 30 sec, 57°C for 40 sec, 72°C for 1 min) primers #6036 (GAGATACGGTGCGGTATCGATC) and #6048 to pCR®4-TOPO® vector.

We did not identify *nhr-40c* isoform.

4.8 RNA mediated interference (RNAi)

The phenomenon of RNAi was first discovered in the nematode worm *C. elegans* as a response to double-stranded RNA (dsRNA), which resulted in sequence – specific gene silencing (Fire et al. 1998; Hannon, 2002). RNAi is one of reverse genetic method. Contrary to forward genetic, where function of the gene is identified according its mutant phenotype in reverse genetic, a gene is identified first through its sequence and then mutations are recovered that inactivate or alter its function (Hope, 1999). It was found that dsRNA injected to the germline led to the epigenetic inactivation of the gene (Fire et al., 1998). Mechanism for the inactivation of genes via ds RNA was named RNA – mediated interference.

Mechanism of RNAi in the cell is partially known. dsRNA is cut to ~22 bp small interfering RNA (siRNAs) by an RNAse III-like enzyme called Dicer (two Dicer molecules with five domains). Then, the siRNAs assemble into endoribonuclease – containing complexes known as RNA – induced silencing complexes (RISCs). The siRNA strand is complementary with degraded target mRNA. RISCs recognize it and targeted mRNA is cleaved and destroy. It leads to decrease of the transcription signal on mRNA level and to inhibit gene expression (Hannon, 2002).

RNAi works for many but not for all genes and does not always reveal the null phenotype (Hope, 1999).

dsRNAi can be injected to ovarial syncitium, can be introduced by feeding or soaking.

4.8.1 RNA mediated interference of *nhr-40*

The constructs for *nhr-40* RNAi were prepared by PCR amplifications of cDNA with gene specific primers, which was cloned into either pAMP vector (Gibco, Invitrogen) for microinjection or L4440 vector (a kind gift from Dr. A. Fire, Standford University) for feeding experiments.

Clone *nhr-40 #4528* was prepared by PCR amplification (35 cycles: 94°C for 20 sec, 57°C for 30 sec, 72°C for 1 min 30 s) of a 921 bp fragment from EST clone yk213d2.5 with primers #4500 (CAUCAUCAUCAUCGTGGTATGCAGCGATTTTGCGAG) and #4415 (CUACUACUACUATTCCCAAGTATTCGCTGGCTTATT). These primers cover the *nhr-40* coding region from the beginning of exon 2 to the end of exon 9. ssRNAs

were prepared from linearized DNA by in vitro transcription reactions using SP6 DNA dependent RNA polymerase (linearization by SmaI) and T7 DNA dependent RNA polymerase (linearization by XbaI) (Promega). Both sense and antisense RNAs were mixed together at 68°C for 10 min and then cool down at room temperature for 20 min. dsRNA was purified by phenol-chloroform extraction and precipitated by ethanol. The pellet of dsRNA was diluted in sterile water to an appropriate concentration 2 – 3 μg/μl.

Clone *nhr-40* #4534 was prepared by PCR (35 cycles: 94°C for 20 sec, 57°C for 40 sec, 72°C for 1 min) amplification of a 736 bp fragment from EST clone yk213d2.5 with primers #4417 (CUACUACUACUAATATGGGAATGGATCCCAAGG) and #4415. These primers correspond to the region from exons 5 to 9 of *nhr-40* that encode a part of the putative ligand binding domain.

#4528 and #4534 dsRNAs were used for soaking and microinjection. Microinjection were done into gonad of adult hermaphrodites as recommended (Mello and Fire, 1995) and embryos of microinjected animals were collected in 10 h to 12 h intervals, incubated for 12 h and scored.

Clone *nhr-40* #4576 was prepared by PCR amplification (35 cycles: 94°C for 20 sec, 57°C for 30 sec, 72°C for 1 min) of cDNA yk213d2.5 using primers #4544 (CATGCCATGGCATGCGTGGTATGCAGCGATTTTGCGAG) and #4542 (CGGGGTACCCCGATTCCAAGTATTCGCTGGCTTATTG). In the 4th exon is an unique PstI restriction site. The PCR product was digested by PstI and KpnI restriction enzymes, gel purified and ligated to L4440 vector using T4 ligase (Gibco, Invitrogen). The PCR product corresponds to a part of the ligand binding domain (4. – 9. exon).

Clone *nhr-40* #4577 was prepared by PCR amplification (35 cycles: 94°C 30 sec, 59°C 40 sec, 72°C 50 sec) of 921 bp fragment with primers #4544 and #4542. yk213d2.5 served as the template. PCR product was purified and digested by NcoI and KpnI and cloned to L4440 vector using T4 ligase. Insert of #4577 construct corresponds to the 2. – 9. exon of cDNA.

Both *nhr-40* #4576 and #4577 were transformed to HT115 *E. coli* competent cells suitable for RNAi feeding experiments. Vector L4440 transformed to HT115 *E.coli* was used as the (negative) control.

4.8.2 RNA mediated interference of *nhr-1, nhr-47, hlh-1, cyp-4*

nhr-1 RNAi construct was made by cloning of 561 bp PCR fragment (35 cycles: 94°C for 40 sec, 59°C for 50 sec and 72°C for 1 min) with primers
#6065 (GCTCTAGATCGCGAGATGCGCATCTGCATCGA)
#6066 (CCGGTACCCTTGACAAATGGATCCACTTCCCAG). PCR product was amplified from mixed cDNA from N2 strain, gel purified and cloned to the pCR®4-TOPO®. Construct was digested by PstI and NotI (restriction sites are present in multiple cloning site of pCR®4-TOPO® vector) separately to prepare linearized templates for in vitro transcription reaction. ssRNAs were prepared using T7 DNA dependent RNA polymerase (from template digested by PstI) and T3 DNA dependent RNA polymerase (from template digested by NotI). dsRNA was made by annealing of both ssRNAs during incubation at 55°C for 10 min and subsequent cooling down at room temperature. dsRNA was purified by phenol-chloroform, precipitated by ethanol and pellet was dissolved in water in approximate concentration 2 – 3 μg/μl.

Fragment of *nhr-47* for RNAi was prepared by PCR (35 cycles: 94°C for 30 sec, 59°C for 40 sec and 72°C for 1 min) amplification of 1,040 bp long part of ligand binding domain. Product was amplified from cDNA prepared from all stages of N2 strain in our laboratory using primers #6061 (TCTCTAGAGAGGCAACGTTTGATAGCTCTATAC) and #6062 (GGGGTACCCGACCGAGCGATGTTTCCTTCACTAG). PCR product was cloned using TOPO® TA cloning kit to pCR®4-TOPO® vector immediately after PCR without gel purification. Construct was sequenced to confirm the sequence. Construct was digested by KpnI and XbaI separately. ssRNAs were prepared using T3 DNA dependent RNA polymerase (from DNA digested by KpnI) and T7 DNA dependent RNA polymerase (from DNA digested by XbaI). dsRNA was made by annealing of both ssRNAs by incubation at 65°C for 10 min and then cool down at room temperature. dsRNA was purified by phenol-chloroform, precipitated by ethanol and pellet was dissolved in water in approximate concentration 2 – 3 μg/μl.

PCR fragment of *hlh-1* for RNAi was done by amplification (35 cycles: 94°C for 20 sec, 58°C for 30 sec, 72°C for 1 min) of 552 bp DNA fragment with primers #6080 (ATGAACACGGAAACCTCAACTC) and # 6081 (CGGACACGTTCTTTGCTTCAC). The amplified fragment corresponds to the region from exon 1 to 3 of *hlh-1* gene. cDNA prepared from all stages of N2 strain

was used as the template. PCR product was cloned immediately after amplification to pCR®4-TOPO® vector using TOPO® TA cloning kit. The construct was sequenced. ssRNAs were prepared using T7 DNA dependent RNA polymerase (from template digested by PstI) and T3 DNA dependent RNA polymerase (from template digested by NotI). Both sense and antisense RNA were mixed together and annealed during incubation at 65°C for 10 min and then cool down for 20 min at room temperature. dsRNA was purified by phenol-chloroform extraction, precipitated by ethanol and pellet was dissolved in water in approximate concentration 2 – 3 μg/μl.

cyp-4 RNAi construct was prepared by PCR amplification (35 cycles: 94°C for 20 sec, 58°C for 30 sec, 72°C for 1 min) of 762 bp fragment with primers #6078 (ATGGGAAAAAAGCAACATCAGAAAG) and #6079 (TACAGTGGATGTGAATCCAGC). PCR product was amplified from mixed N2 cDNA and subsequently cloned into pCR®4-TOPO® vector. Cloning and dsRNA preparation was done as described in the case of *hlh-1* RNAi.

4.8.3 Soaking

Worms at different stages were placed to the 10 μl of dsRNA in the lid of eppendorf tube overnight. Next day worms were transferred on NGM plates with OP50. Animals and their progeny were followed for possible phenotypical changes.

4.8.4 Feeding

Constructs #4576 and #4577 were transformed to *E. coli* HT115 strain by heat shock. HT115 has ability to produce dsRNA by transcription of transformed construct containing L4440 vector. One colony was inoculated to 10 ml of LB with ampicillin (100 μg/ml) and incubated at 37°C overnight. Next day the culture was diluted with fresh LB with ampicilin to optical density (OD) 0.4 at 600 nm. IPTG (isopropyl – β – D – thigalactopyranoside, ICN) was added in final concentration 0.4 mM and culture was induced for 4 h at 37°C. Such prepared culture served as a food for worms. For feeding experiments we used NGM with ampicillin (100 μg/ml) and IPTG (0.4 mM).

4.9. Overexpression of *nhr-40* and overexpression of *nhr-40* AF-2

4.9.1 *nhr-40* overexpression constructs

Overexpression is method based on increase of number of copies of the gene. Gene is cloned under regulation of heat shock promoter. After heat shock the transcription of study gene is increased. It can lead to competition of DNA binding proteins, saturation of cofactors or other synergic proteins during regulation of transcription of target genes.

The clone #6076, which corresponds to transcript *nhr-40b* (1,394 bp) was prepared by PCR (35 cycles: 94°C for 40 sec, 59°C for 50 sec and 72°C for 1 min) using primers #4580 (CGGGGTACCGTACCCCCGCTGATGTAT) and #6038 (CATGCCATGGGGAAGATCTACAAGCCAACGATAATG) and cDNA from mixed population of the worms as a template. PCR product was cloned to pCR®4-TOPO® vector. After confirmation by sequencing, the insert was re-cloned into the heat shock promoter vector pPD49.83.

A second clone #6077 that corresponds to transcript *nhr-40d* (1,395 bp), was prepared by PCR (35 cycles: 94°C 20 sec, 58°C 40 sec, 72°C 2 min) with primers #6073 (CGGGGTACCGAGATACGGTGCGGTATCGATC) and #6038 and from mixed N2 cDNA. The PCR product was cloned to pCR®4-TOPO® vector. The insert was sequenced and re-cloned into the heat shock promoter vector pPD49.83. Expression from transgenic animals harboring #6076 or #6077 was induced by heating of gravid hermaphrodites at 31°C for 2 h. As control, N2 worms were heated under the same conditions.

4.9.2 *nhr-40* expression construct #6010 for dominant negative experiments

Dominant negative experiment is based on the fact where a mutant gene eliminates the activity of its normal counterparts in the cell. Gene or its part is cloned to the vector with heat shock promoter. The construct ensures the production of mRNA according the cloned incomplete template after the heat shock. We prepared dominant negative mutated construct that contains the sequence of *nhr-40* gene without predicted AF-2 transactivation domain. NHR-40, which is transcribed according incomplete template, has fully function DBD, but partially function LBD. It can lead to saturation of HRE in the target genes of NHR-40 but non-functional activation of their transcription.

The clone *nhr-40* #6010 which corresponds to *nhr-40a* was prepared by PCR (1,033bp) (35cycles: 94°C 40 sec, 57°C 50 sec, 72°C 1 min 10 sec) using primers #4580 and #4540 (CATGCCATGGCATGTTACCAAGTATTCGCTGG). yk213d2.5 served as the template. 1,033bp long PCR product consists of 5´UTR, DBD and part of LBD till the exon 9. PCR product was purified and digested with appropriate enzymes KpnI and NcoI. Vector pPD49.78 with heat shock promoter was digested with the same enzymes. Insert was cloned using T4 ligase. Expression of transgenic animals harboring #6010 was induced by heating of gravid hermaphrodites at 31°C for 2 h. As control, N2 worms were heated under the same conditions.

4.10 *nhr-40* green fluorescent protein (GFP) reporter gene

The inserts for constructs #4586, #6011, #6059, #6007 were prepared by PCR amplification of the first promoter region using primers with SphI and BamHI restriction sites. The scheme of all GFP constructs is on Figure 8. Conditions for amplification of #4586 PCR fragment were 35 cycles: 94°C 30 sec, 55°C 40 sec, 72°C 3 min. Conditions for amplification of #6011 PCR fragment were 35 cycles: 94°C 1 min, 57°C 1 min, 72°C 1 min 30 sec. Conditions for amplification of #6059 PCR fragment were 39 cycles: 94°C 40 sec, 58°C 50 sec, 72°C 1 min. Conditions for amplification of #6007 PCR fragment were 35 cycles: 94°C 1 min, 57°C 1 min, 72°C 1 min 30 sec. N2 gDNA served as the template. The digested PCR products were cloned in-frame to the corresponding restriction sites in the digested GFP vector pPD95.67 containing a nuclear localization sequence (NLS). The gDNA region for construct #4586 was amplified by PCR with primers #4579 (ACATGCATGCTCCCAATCGGAAGAAGTTGTCACATC) and #4575 (CGGGATCCGGGCCCAACTCACTGTTATTCGGCAT), #6011 with primers #4591 (ACATGCATGCATGATTGATGGGACAAGGTAA) and #4575, #6059 with primers #6050 (ACATGCATGCCAATATTCATAATGCGCTTTCTATC) and #4575 and #6007 with primers #4592 (ACATGCATGCACGGAAGTAACACTTAAAAGC) and #4575.

nhr-40 fragments for constructs #6022, #6023, #4523, #6058 were generated by PCR amplification of the downstream promoter region using primers with PstI and BamHI restriction sites. Conditions for amplification of #6022 and #6023 were 35cycles: 94°C 1 min, 59°C 2 min, 72°C 5 min. Conditions for amplification of

#4523 were 35 cycles: 94°C 40 sec, 57°C 50 sec, 72°C 3 min. Conditions for amplification of #6058 were 40 cycles: 94°C 50 sec, 58°C 1 min, 72°C 2 min. Conditions for amplification of #6058 were 35cycles: 94°C 30 sec, 57°C 40 sec, 72°C 2 min. The digested PCR products were cloned in-frame into the corresponding restriction sites in the GFP vector pPD95.73 also containing a nuclear localization sequence (NLS). The construct #6022 was prepared with primers #4593 (AAAACTGCAGGGCAATATGGCAAGCATTTCAC) and #4502 (CGCGGATCCACGCAAAGTGTTCCTTCCGGGAT), #6023 with primers #4594 (AAAACTGCAGTTGGGGTGGCTGGAAGGAAGTT) and #4502 BamHI, #4523 with primers #4501 (AAAACTGCAGCAGTCACTATCCAGGAAGTATTCT) and #4502, #6058 with primers #6049 (AACTGCAGCGTCTTCTTGGACGGACTCGCCGAC) and #4502.

The construct #6093 was generated by PCR using the same conditions for amplification and set of primers as #4523 (#4501 and #4502) but from gDNA isolated from the RB840 strain containing the *nhr-40(ok667)* intronic deletion. This construct contains a 1,821 bp fragment cloned into the GFP vector pPD95.73.

4.11 Study of RB840 deletion strain

4.11.1 RB840 deletion strain – *nhr-40(ok667)*

To confirm that RB840 strain obtained from *C. elegans* Gene Knockout Consortium harbor the deletion in *nhr-40* gene we prepared gDNA from RB840 strain. We run three overlapping PCRs from this template to determine predicted region of deletion. The first PCR was prepared with primers #6041 (TTCCATCTTTCTTCGTTCCG) and #6043 (CAAGTTGGACATTTGGGTCTTATC). The second PCR with primers #4595 and #4502 and the third with primers #6044 (GTACGCATAAAACTACAACGGCCA) and #6042 (TCGTCGACTTCTTTCCGTTT). PCRs were done under these conditions: 35 cycles 94°C 40 sec, 58°C 50 sec, 72°C 1 min 30 sec. PCR products were sequenced and the deletion of 840 bp was confirmed.

4.11.2 Backcross of mutated strain RB840

This procedure is based on the mating of wild type males (5AA, X0) and mutated hermaphrodites (5AA, XX) to eliminate any secondary mutations from its

genetic background. For this purpose we prepared wild type N2 males by heat shock of L4 N2 hermaphrodites at 30°C for 5 hours. After that they laid the progeny with higher incidence of males. Males were transferred to the NGM plates adapted for mating. Reproduction by mating produces a high frequency of males, in part, because male sperm contains an equal frequency of null-X and X-bearing gametes and because male-derived sperm out-compete hermaphrodite sperm in the fertilization of oocytes (http://www.wormatlas.org/maleHandbook/GenIntroMalePartI.htm). We placed one RB840 mutated hermaphrodite at L4 stage and ten N2 males at the same developmental stage on one mating plate. Their F1 progeny consists of heterozygotes. Their progeny is segregates homozygotes of RB840 mutation (25%), heterozygotes (50%) and homozygotes for wild type allele (25%). Hermaphrodite progeny was spread up one animal per one NGM plate and let to lay eggs. 2 days old adult hermaphrodites were used for 1st single worm PCR to find heterozygotes. Progeny from heterozygote hermaphrodite (20 worms) was spread up one animal per one NGM plate again. 2nd single worm PCR was performed. We looked for homozygotes for mutation. We used N2 worms as controls, RB840 original worm as control of mutated homozygotes and a mix of one N2 and one RB840 worm as control for heterozygotes. Single worm PCRs were done with primers #4595 and #4502. PCR was done under these conditions 15 cycles: 94°C 20 sec, 57°C 30 sec, 72°C 1 min 30 sec and 15 cycles: 94°C 20 sec, 56°C 30 sec, 72°C 1 min 30 sec. PCR product of wild type allele has the size 1,403 bp and mutated allele 569 bp. The electrophoretic analysis of the PCR reactions was used to distinguish homozygotes and heterozygotes.

4.11.3 Rescue of *nhr-40(ok667)*

4.11.3.1 Rescue by gDNA

Genomic DNA to rescue the mutant *nhr-40(ok667)* was made by PCR amplification of overlapping fragments from wild type gDNA using specific primers (Maryon et al., 1996; Maryon et al., 1998; Mercer et al., 2003; Watanabe et al., 2005). Fragment A: 2,860 bp, primers #4579 and #4575; fragment B1: 3,184 bp, primers #4592 and #6099 (GGGATTCAATACAAGGACGTGACTTTC); fragment B2: 3,088 bp, primers #6098 (GAGGAGCCTAGTCAACAAGGTAGATC) and #6043; fragment C: 3,665 bp, primers #6049 and #4581; fragment D: 4,468 bp,

primers #6068 (AAAACTGCAGAAGTTGTCGGCTGAAAAAATGC) and #6069 (TTTTCTGCAGGTATTTCGAGATGTGAATACTTTG) (Figure 24). PCR products were gel purified, mixed together at the final concentration 500 ng/µl and combined with 50 ng/µl of pRF4 as a marker prior to injection into animals.

4.11.3.2 Rescue by tissue specific constructs *myo-3::nhr-40d*

cDNA rescue of *nhr-40(ok667)* with tissue-specific promoters were done as follows:

The promoter region of *myo-3* (2,279 bp) was amplified by PCR with primers #6100 (GGGCCCGTGCCATAGTTTTACATTCCAC) and #6101 (CCGCGGTCTAGATGGATCTAGTGGTCGT) and cloned to pCR®4-TOPO® vector. A PCR product corresponding to cDNA of *nhr-40d* was prepared with primers #6103 (GCTCTAGAGAGATACGGTGCGGTATCGATC) and #6104 (GCTCTAGAGGAAGATCTACAAGCCCACGATAATG) and was cloned under the *myo-3* promoter using unique XbaI restriction site. Constructs *myo-3::nhr-40d* was sequenced.

4.11.3.3 Rescue by tissue specific fused PCR product *nhr-23::nhr-40d*

To drive expression of *nhr-40* in epidermal cells, the *nhr-23* promoter (1.6 kb) (Kostrouchova et al., 1998) was amplified from gDNA using PCR and fused with the full – length cDNA of *nhr-40*. Promoter of *nhr-23* was amplified by PCR with primers #4955 (ATTTCACGGTCACTCTGCCATTGCCCC) and #7092 (TCATTGACABBCGAGGGACAAGCAGCCAAGCGCTCTGATGTGCCTTGAC) cDNA of *nhr-40d* isoform was amplified with primers #7091 (CAAGGCACATCAGAGCGCTTGGCATGCTTGTCCCTCCCGTGTCAATGA) and #6038. The final PCR product used for rescue was prepared by 3 rd PCR reaction with primers #4955 and # 6038. The sequence of the construct was confirmed by direct sequencing.

4.12 Staining of the animals: detection of NHR-40 protein

The antibody agains NHR-40 were prepared by immunization of two rabbits with polypeptide CNRTNDLIDQLIIVGL. Test of antibodies was done using custom antibody ELISA test.

Embryos after bleach, synchronized larvae and adult animals were washed several times with water and placed on poly – L – lysine coated slides. 10 µl of sediment containing animals was placed on the slide together with the same volume of 5% paraformaldehyde diluted in 1xPBS, covered with cover glass and incubated in a wet chamber for 10 min at room temperature. Slides were put on a pre-chilled aluminum platform on dry ice and frozen for 8 min. After that cover glasses were quickly cracked out. Then the samples were placed into ice cold methanol (-20^{o}C) for 10 min and then into ice cold aceton (-20^{o}C) for next 10 min. After the fixation the glasses were air dried and primary polyclonal rabbit IgG antiNHR-40 antibodies #4527 and #4528 were applied on in dilution 1:200 in 1xTTBS (Tris Tween Buffered Saline: 100 mM Tris pH 7.5, 0.9% NaCl, 0.1% Tween 20). Slides were covered with cover glasses and kept in a wet chamber at 4^{o}C overnight. Next day, the slides were washed 3 times in 1xTTBS and secondary goat anti rabbit IgG antibody conjugated to Alexa Fluor 568 (Molecular Probes, Eugene, Or) in dilution 1:400 in 1xTTBS was applied on. Slides with cover glasses were incubated at room temperature for 2 h and then washed 3x in 1xTTBS. 5 µl of 1 mg/ml DAPI (4',6-diamidino-2-phenylindole) was added and slides were mounted with 10 µl of mounting medium (Shandon, Pittsburgh, Pa).

4.13 Staining by phalloidin

Phalloidin is a mushroom *Amanita phalloides* toxin that binds actin filaments. Phalloidin binds specifically at the interface between F – actin subunits. It is a bicyclic heptapeptide, which binds to actin filaments much more tightly than to actin monomers (Cooper, 1987). Fluorescently labeled phalloidin binds to filamentous F – actin and reveals the various muscles of the worm. Nonmuscle tissues containing F – actin are also stained, including the microvillus border of the intestine (Waterston, 1985).

L1 synchronized population of worms either N2 or RB840 were spin in 14 ml centrifugation tubes. 7 µl of sediment of larvae was placed on poly – L – lysine coated slides together with the same volume of 5% paraformaldehyde diluted in 1xPBS. Staining continued as described above. After the fixation and washing phalloidin in 1:400 and 1:200 dilutions in 1xTTBS was applied on. Slides were kept in a wet chamber at 4^{o}C overnight. Next day, the slides were washed 3 times in 1xTTBS and mounted with 10 µl of mounting medium.

4.14 Western blot

Worms from all developmental stages were washed twice in water and spin at 1000 rpm for 4 min at 4°C. The pellets were transferred to eppendorf tubes. The same amount of Tris-Glycine SDS Sample Buffer (2x concentrate) (Invitrogen) was added. SDS has a function of anionic detergent and proteins are separated according to their molecular weight (with no influence of protein charge). The samples were boiled for 5 min and than chilled. The protein concentrations in supernatants were estimated by BCA kit (Pierce, Rockford, Il). 1 μl of mercaptoethanol was added to the samples and they were boiled again. Mercaptoethanol is used to destroy S-S bonds in proteins. For Western blot analysis 30 μg of protein samples were separated by 4%/15% SDS PAGE. Proteins were blotted onto nitrocellulose membrane. Membrane was incubated overnight at 4°C in 1xT-PBS-M (phosphate buffered saline (PBS) pH 7.4, 0.1% (v/v) Tween 20, 5% low-fat milk powder (w/v)). Low-fat milk powder contains casein which occupies nitro groups in proteins. Epitops for primary antibodies are accessible. The second day the membrane was incubated in 1xT-PBS-M with primary antibody (dilution 1:1000 in 1xT-PBS) for 1 h at room temperature. The membrane was washed six times by rocking in 1xT-PBS for 10 min at room temperature and incubated with secondary antibody coupled to horseradish peroxidase (Sigma, St. Louis, Mo) diluted 1:5000 in 1xT-PBS for 40 min at room temperature. Membrane was washed four times by rocking in 1xT-PBS for 10 min at room temperature. SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used for detection of peroxidase activity. Signal was detected on medical X-ray film (FOMA, Hradec Králové, ČR).

4.15 Movement assay

The method used was modified from Wicks et al., 2000. The starving L1 animals were placed on a marked line on chemotaxis agar on a 9 cm Petri dish. A second line was made at a distance of 2 cm and was filled with OP50 bacteria. 1 ml of isoamylalcohol diluted 1:50 in ethanol was placed in the opposite side of the dish. Isoamylalcohol was used to attract worms toward the line of OP50. This assay was done on 723 RB840 L1 animal and 100 wild type N2 L1 larvae. The movement of the worms was measured in 1, 2, 5 and 24 h intervals.

4.16 Motility assay

The motility assay was performed as described Mercer et al., 2003 with slight modifications. Single wild type N2 or RB840 L1 larvae were placed in 15 µl drops of M9 buffer and left to acclimate for 1 min. Each full sinus movement was counted. Motility was determined in groups of 15 animals.

4.17 Bioinformatics

Sequences were compared by the MultAlin program (Corpet, 1998) and the phylogenic tree was prepared by using Vector NTI Suite 5.5 (Lu and Moriyama, 2004), available from Invitrogen.

4.18 Instruments

Observation of worms was provided on Olympus microscope SD30 and stereomicroscope system SZX12 (Olympus). Microinjections were done on inverted microscope Olympus IX70 equipped with micromanipulator (Narishige, Japan). Fluorescence microscopy was done on Olympus BX60 microscope equipped with CCD camera DP30BW and Nikon Eclypse E800 microscope equipped with C1 confocal head and 488 nm laser line (Nikon, Spectra-Physics Lasers). Sequencing of some constructs was done by traditional Sanger dideoxy sequencing method by ALF*express*® II DNA Analyses (Amersham Pharmacia Biotech AB, Sweden) kindly performed by Lucie Maršálková, Laboratory of Molecular Pathology, Institute of Inherited Metabolic Disorders. Sequencing of both constructs and PCR products was provided on sequencer ABI 3100-*Avant* (Applied Biosystems, USA) kindly performed by Helena Myšková, Institute of Inherited Metabolic Disorders.

INVOLVEMENT

Involvement of the defender of the Thesis in the experiments: I performed or participated in majority of experiments described in this Thesis.

5 RESULTS

5.1 Characterization of the *nhr-40* gene

The *nhr-40* gene is localized on the chromosome X, with a cosmid T03G6.2. The gene consists of 12 exons and spans 12,177 bp (Figure 3A). WormBase web shows the gene data on web site http://www.wormbase.org, releases WS150, Nov 30, 2005). EST clone yk213d2.5, which appeared to cover the entire coding region, was obtained from Dr. Y. Kohara (National Institute of Genetics, Japan). Sequencing of the cDNA clone confirmed the predicted coding region reported in WormBase and GenBank. The gene structure is typical for *C.elegans* except for the first intron that is unusually long and has 6,696 bp.

Based on expressed sequence tag (EST) three transcripts (*a*, *b* and *c*) have been described for *nhr-40* gene on WormBase. In order to determine if other isoforms of *nhr-40* exist, we prepared total RNA from embryonic, larval and adult stages of *C.elegans*. We used RT-PCR with sets of gene-specific, random hexamers and the trans-spliced lead sequences SL1 or SL2 primers (Blumenthal and Thomas, 1988; Krause and Hirch, 1987). The sequencing of clones revealed the presence of representing transcripts *nhr-40a* and *nhr-40b*. Both of them were trans-spliced to splice leader SL1 as previously reported in AceView (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/index.html). We also identified a new transcript, we called *nhr-40d.* This isoform utilizes the same first exon as transcript *nhr-40c* but differs in the length of exon 7. Isoform *nhr-40c* has the same length of exon 7 as *nhr-40a* and isoform *nhr-40d* has the same as *nhr-40b*. The difference is in 15 bp on the 5´splicing site of exon 7. The *nhr-40a* transcript has a shorter intron 6 (361 bp) and exon 7 encodes five more amino acids (FLFFS). Primary transcripts *nhr-40b* and *d* have longer intron 6 (376 bp) with a corresponding shorter exon 7. The isoforms encoded by *nhr-40a*, *b* and *nhr-40d* differ at the amino acid level at the beginning of the proteins. Isoforms NHR-40a, b start with MPNN while NHR-40d starts with MLVPPVSMIMYHELPSIKNK. We sequenced 20 cDNA clones, 10 clones represented transcript *nhr-40a*, 4 clones transcript *nhr-40b*, and 6 clones *nhr-40d*. These results demonstrated and confirmed that *nhr-40* is regulated by at least two distinct promoters (Figure 3A).

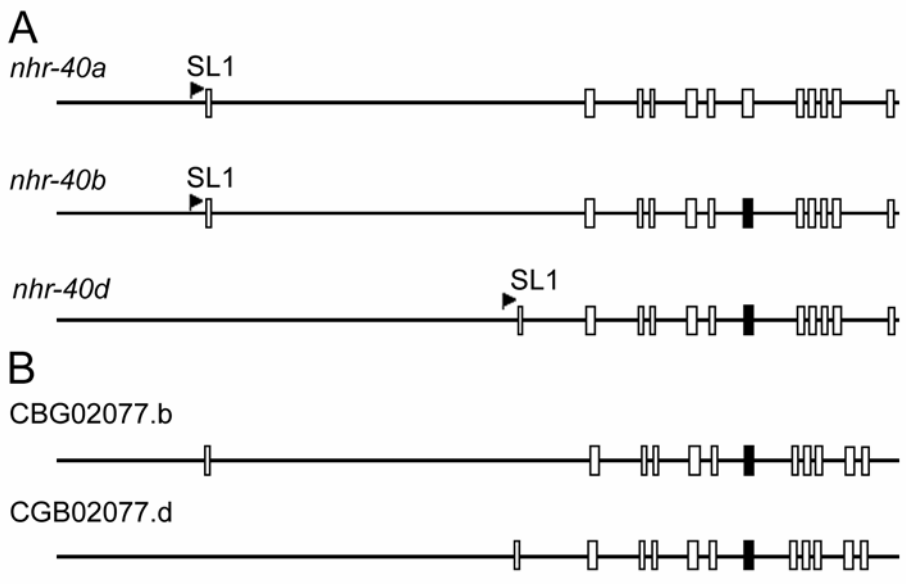

Figure 3. The *nhr-40* gene structure from *C. elegans* and *C. briggsae*

A. The scheme of the genomic organization of *C. elegans nhr-40* gene and transcripts. SL1 trans-splicing occurs at both alternate exons 1 as indicated (arrowhead). Transcripts *nhr-40a* and *nhr-40b* differ by alternative splicing of exon 7, as indicated by a black box. *nhr-40d* utilizes an alternate first exon but is otherwise identical with *nhr-40b* in exons 2 to 12.

B. The predicted genomic structure of *C. briggsae* transcripts *Cbnhr-40b* and *Cbnhr-40d* based on the conservation of coding regions with *C. elegans*.

5.2 *nhr-40* gene has an orthologue in the *C. briggsae*

A orthologue of *nhr-40* named CBG02077 is present in the *C. briggsae* genome. The gene sequence is present in the clone cb25.fpc0045 but the annotation appeared incomplete and included only the region from exon 2 to exon 11. We performed an alignment of clone cb25.fpc0045 with the *C. elegans nhr-40* gene using the MultAlin program. The alignment demonstrates that *Cbnhr-40* has a gene structure that is nearly identical to *Cenhr-40* (Figure 3B). It is of particular interest that both genes have short first exon (13 bp) and very large first intron: 6,696 bp for *C. elegans* and 5,905 bp for *C. briggsae*. A comparison of the genes flanking *nhr-40* in the two species demonstrated synteny of this chromosomal region.

5.3 NHR-40 and its homology

We performed an alignment of the DNA binding domain (DBD) amino acid sequence derived from a NHR-40 with 17 predicted *C. elegans* NHRs that are characterized by the P box sequence CNGCKT. We have found that *nhr-40* is a closest homolog to other nuclear hormone receptors in *C.elegans.*

NHR-40 is defined by the highly conserved DNA binding domain (DBD) composed of two zinc fingers more closely determined by 13 amino acids in the second zinc finger (Figure 4).

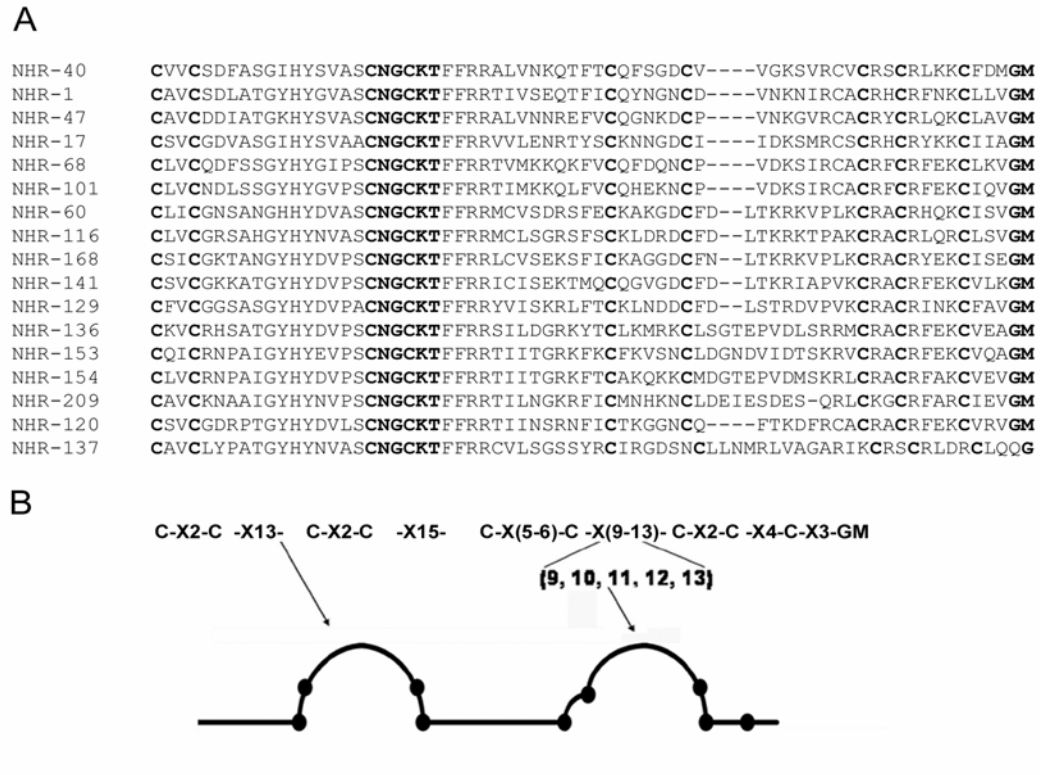

Figure 4. Sequence analysis of NHRs that are homologues to NHR-40 in *C. elegans*

A. An alignment of the DNA binding domain (DBD) amino acid sequence derived from a NHR-40 and 17 other predicted *C. elegans* NHRs that are characterized by the P box sequence CNGCKT. The P box of the DBD and the conserved cysteins are marked in bold.

B. A schematic representation of the molecular signature of the DBD with conserved cysteins and indicated number of variable intervening amino acids. Arrows point to sequences between internal cysteins of the first and second zinc fingers, where the second zinc finger consists of 9 to 13 amino acids in the subgroup supnrs-CNGCKT of *C. elegans*.

5.4 *nhr-40* expression pattern

nhr-40 expression profile was done by two approaches, semiquantitative and quantitative PCRs. For both methods we prepared at least two sets of templates. Templates were prepared by RT-PCR from total RNA isolated from embryos, synchronized larval stages L1 – L4 and adult animals.

Semiquantitative expression profile was done using *nhr-40* gene specific primers. For unique 5´ region of *nhr-40a* and *b* a primer #4580 was used, for *nhr-40d* a primer #6036 and one common backward primer #4581 was used for all transcripts. PCR reactions terminated during exponential phase. Primers for *ama-1* were added in the 5th PCR. The results revealed that all transcripts of *nhr-40* gene, that we have characterized, are expressed in all developmental stages (Figure 5).

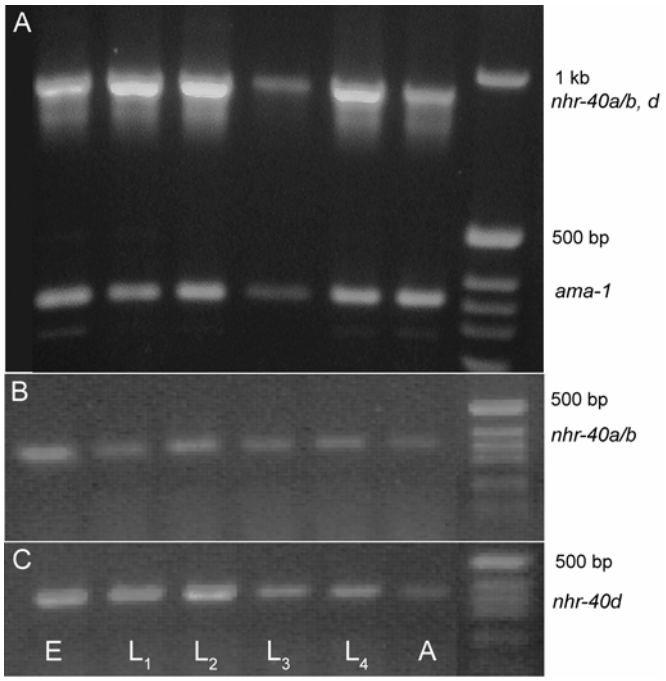

Figure 5. Expression profile of *nhr-40* during all developmental stages

A. Expression of *nhr-40* (950 bp) with comparison to *ama-1* (350 bp) using cDNA from all developmental stages of *C.elegans.*

B. Expression profile of *nhr-40a* and *nhr-40b* transcripts regulated by promoter 1. PCRs were prepared using primers #4580 and #4581.

C. Expression profile of *nhr-40d* transcript regulated by promoter 2. PCRs were prepared using #6036 and #4581 primers.

Quantitative real-time PCRs were done with *nhr-40* gene specific primers for both unique 5´ regions. Primer #6145 was used for transcripts *nhr-40a* and *b*, primer #6146 for transcript *nhr-40d* and one common antisense primer #6147 for all transcripts (Figure 6).

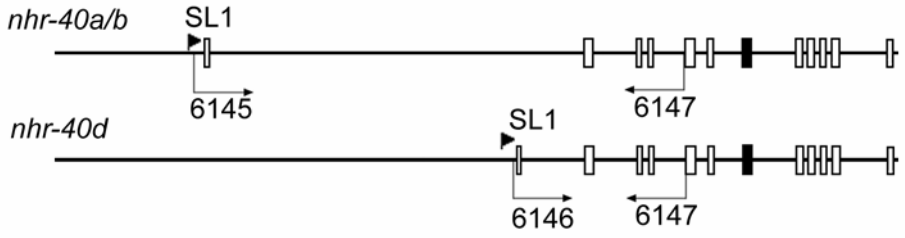

Figure 6. Schematic representation of *nhr-40* coding regions and primers used for quantitative real-time PCR

Expression levels of *nhr-40* transcripts *a*, *b* and *d* were normalized against the *ama-1* transcript encoding the large subunit of RNA Polymerase II. We found that the expression of *nhr-40* from both promoters was present throughout all development and was highest in L2 stage for all isoforms (Figure 7). Expression levels were calculated from calibration curves and normalized for *ama-1* expression levels (without introducing a calibration step for the efficiency of reverse transcription) that revealed that L2 stage animals had approximately 3-fold more *nhr-40d* than *nhr-40a* and *b*. These results suggest that a higher efficiency of transcription is transcription from promoter 2.

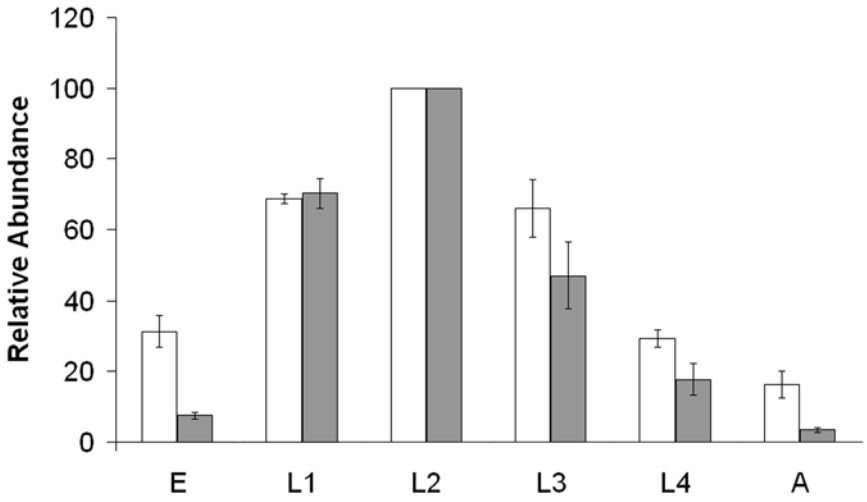

Figure 7. The expression of *nhr-40* during development.

The expression of *nhr-40* during development was assayed by quantitative real-time PCR based on cDNA prepared from total RNA extracted from all developmental stages as indicated (E – embryos, L1 – L4 – individual larval stages, A – adults). Relative levels of expression were determined from calibrated experiments that were normalized for the expression of *ama-1*. The highest expression was found in L2 larval stage. Normalization of the expression level in other stages was determined to the expression level in L2 stage. The normalized values correspond to 7,673 arbitrary units in case of transcripts *a* and *b* and 21,240 arbitrary units in case of transcript *d*. White bars represent *nhr-40a* and *nhr-40b* and dark bars *nhr-40d* isoforms. Standard deviation bars are indicated.

5.5 Expression of *nhr-40* reporter gene constructs

We studied the function of the two *nhr-40* defined promoter regions using promoterless green fluorescent protein (GFP) reporter vectors (Figure 8).

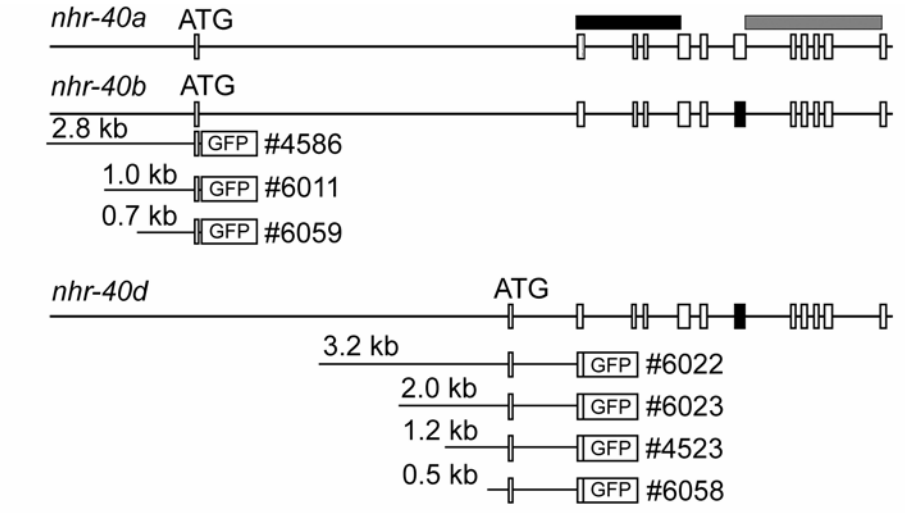

Figure 8. Schematic representation of *nhr-40* genomic regions used in gfp reporter gene constructs

The gene structure corresponding to each of three transcripts (*nhr-40a*, *nhr-40b*, *nhr-40d*) is shown. The dark bar indicates exons, which contain DNA binding domain. The gray bar indicates the region, which is coding ligand binding domain. Reporter gene constructs in which *nhr-40* sequences have been fused to green fluorescent protein (GFP) are shown and identified by the numbers to the right. Different sizes of promoter regions, which were used for these constructs, are also indicated with regard to the 1st ATG.

Denoted promoter 1 which is shared by transcripts *nhr-40a* and *nhr-40b* was studied using several reporter gene constructs. A reporter gene construct #4586 that begins at 2,833 bp upstream of the first ATG is expressed in body wall muscle cells, pharyngeal muscles, rectal gland cells, a subset of neurons in the head, in the tail and in the ventral nerve cord (Figure 9A – F). In adult stage GFP expression was detected in vulval and uterine muscles. The expression was first detected in the embryo at the 1.5 fold stage in the precursors of body wall muscle cells (Figure 9A). GFP expression continued until adulthood. At the 2 fold stage GFP expression was found also in P cells.

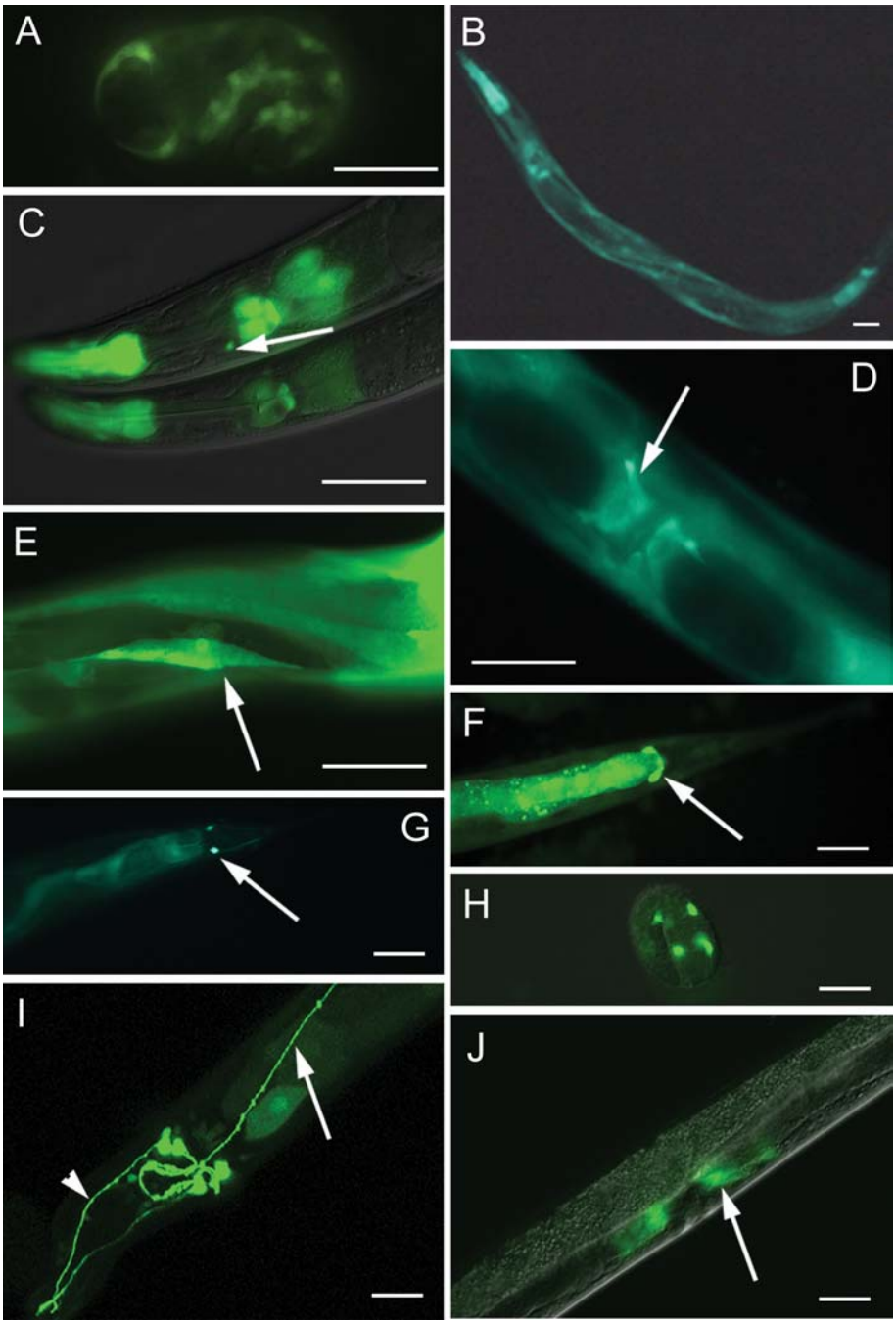

Figure 9. Expression of *nhr-40::gfp* regulated by promoter 1

Expression of reporter gene construct #4586 is first detected at the 1.5 fold stage of embryogenesis (A) and continues during larval stages to adulthood (B – D). Strong expression is observed in the pharynx and neuronal cells in the head (C, arrow) and in rectal gland cells (F, arrow). Expression of *nhr-40::gfp* #4586 in body wall muscle cells is demonstrated in figure E (arrow) and in vulva muscle cells in figure D (arrow). Expression from truncated reporter gene constructs *nhr-40::gfp* #6011 and #6059 is first detected at the 1.5 fold embryo (H) and then observed in neuronal cells in the tail (G, arrow), head neurons of ventral nerve cord (marked by arrow) and axon (marked by arrowhead) (I) and uterine muscles of young adult hermaphrodites (J). Scale – 20 μm.

The next two reporter gene constructs *nhr-40::gfp* #6011 (1,013 bp upstream of the first ATG) and *nhr-40::gfp* #6059 (682 bp upstream of the first ATG) revealed the expression of GFP in neuronal cells in the head and tail, ventral nerve cord and uterine muscle cells (Figure 9G – J). The expression of GFP in muscle cells was partially lost. A reporter gene construct *nhr-40::gfp* #6007 that begins at -385 bp relative to the start of exon 1 did not show any expression of GFP. pRF4 positive transgenic lines were tested by single worm PCRs to confirm the presence of *nhr-40::gfp* #6007 construct in all lines.

We prepared genomic reporter gene constructs for promoter 2. Promoter 2, which regulates the expression of transcript *nhr-40d*, corresponds to the genomic region of intron 1 relative to transcripts *nhr-40a* and *nhr-40b*. Reporter gene constructs *nhr-40::gfp* #6022 beginning at 3,190 bp upstream of the ATG, *nhr-40::gfp* #6023 at -2,021 bp, *nhr-40::gfp* #4523 at -1,248 bp and *nhr-40::gfp* #6058 -517 bp upstream of the ATG revealed GFP expression in body wall muscle cells, neurons in the head, nerve ring, retrovesicular ganglia, ventral and dorsal nerve cord, neurons and some epidermal cells in the tail. Intermittent expression was also observed in pharyngeal muscles. The expression from promoter 2 started also in the embryos at the 1.5 fold stage and was continuous throughout development. In late L4 and young adult hermaphrodites GFP expression was observed in specialized epithelial cells creating connection between uterus and vulva: uterine-vulva cells ut 1, 2, 3 and surrounding epithelium utse (Figure 10). Our reporter gene analysis demonstrated that the genomic regions upstream of either of the two alternate first exon sequences could function as a promoter and resulted in similar temporal and spatial patterns of expression.

Because the GFP expression patterns observed for both promoters substantially overlapped (muscle and neuronal tissue) we tested both genomic segments for presence of similar regulatory sequences upstream of the ATG of the two alternate first exons. No obvious similarity was detected. We also searched for sequence similarity between the two predicted *nhr-40* promoter regions from *C. elegans* and *C. briggsae*. We found stretches of repetitive sequences but none appeared likely binding sites for known transcription factors.

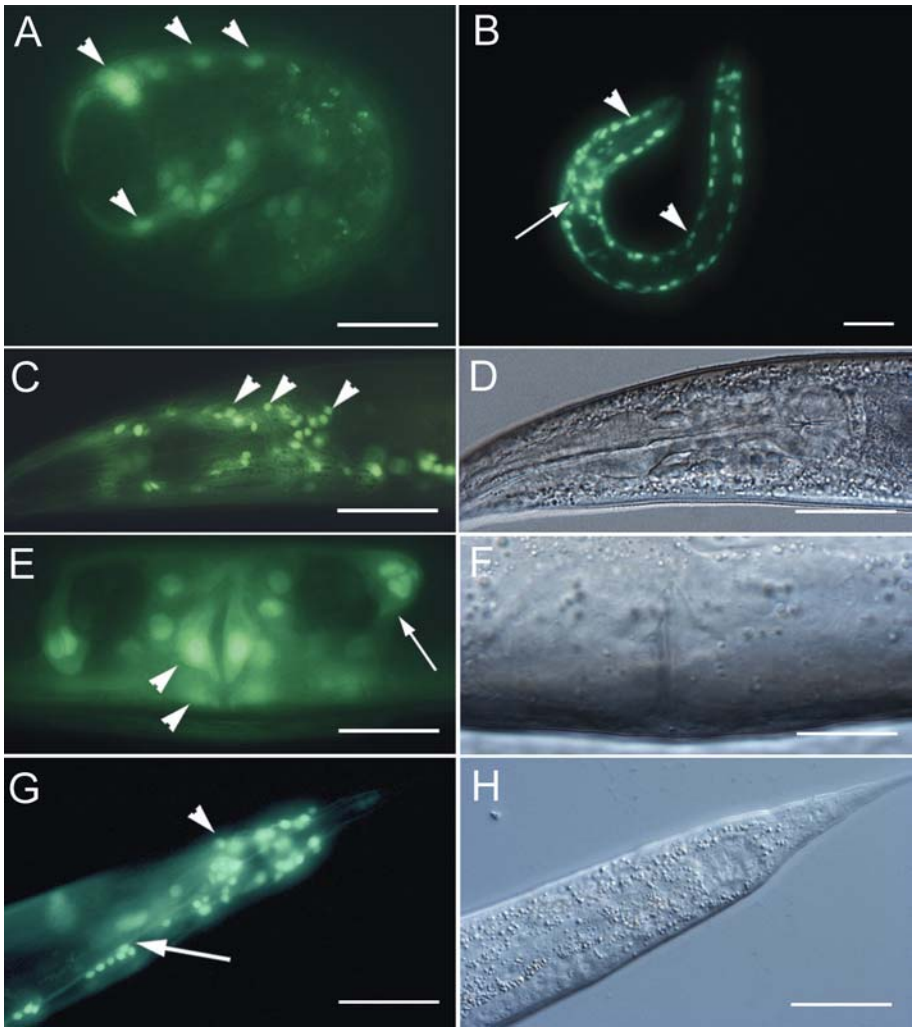

Figure 10. Expression of *nhr-40::gfp* regulated by the promoter 2

Expression of *nhr-40::gfp* regulated by the promoter 2 is similar for all reporter gene constructs used for this study (#6022, #6023, #4523, #6058). Expression is first detected at the 1.5 fold embryo in the precursors of body wall muscle cells (A, arrowhead). Later expression includes body wall muscle cells (B, arrowhead) and neurons in the head (arrow in B and arrowhead in C), ventral nerve cord (G, arrow), neuronal cells in the tail (G, arrowhead). In young adults the expression is observed in uterine-vulval cells (E, arrowhead) and epithelial cells forming utse (E, arrow). Panels D, F, H show the same animals as panels C, E, G in Nomarski optics. Scale – 20 μm.

5.5.1 *nhr-40::gfp* is expressed in P-cells but not in seam cells

To better distinguish the GFP expression driven by promoter 1 we prepared double GFP expressing line *nhr-40::gfp* #4586 on the background of *ajm-1*:: *gfp* (SU93 strain). *ajm-1* encodes a member of the apical junction molecule class AJM-1 that is required for a correct elongation and is localized to the apical borders of all *C.elegans* epithelia. *ajm-1::gfp* serves as the marker of epithelial cells. Expression occurs in the embryonic epidermis, pharynx and intestine as well as in post-embryonic epithelia including the epidermis, pharynx, intestine, hindgut, vulva, uterus and spermathecae. Observational data of *nhr-40::gfp* #4583 on the background of *ajm-1::gfp* (SU93 strain) revealed that *nhr-40::gfp* #4586 is also expressed in P-cells (P1/2, P3/4, P5/6, P7/8, P9/10, P11/12). P cells are epidermal precursors that are positioned in two parallel rows. P1-P12 ventral cells divide and the anterior daughters detach from the epithelium and generate primarily ventral cord neurons (Sulston and Horvitz, 1977). The posterior daughters of P1, P2 and P9-12 fuse with hyp7 cell at the end of the L1 stage. The posterior daughters of P3-8 divide at L3 stage to make 12 cells. The posterior daughters of P3, P4, and P8 fuse with hyp 7 cell. The remaining cells become vulva precursor cells (Sulston and Horvitz, 1977; Hedgecock, 1987). It correlates with expression of *nhr-40* which was found in ventral nerve cord and also in vulva cells during adult stage. We found that *nhr-40::gfp* is not expressed in seam cells (Figure 11).

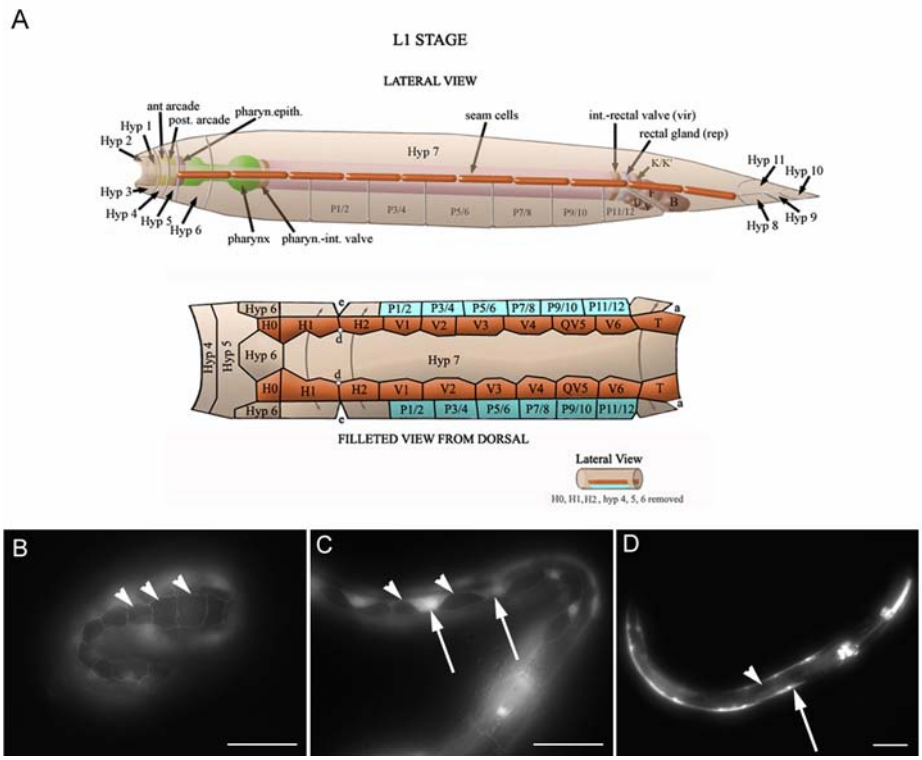

Figure 11. Double GFP expression of *nhr-40::gfp* #4586 on the *ajm-1*::*gfp* background

A. Scheme of hypodermal cells including seam cells and precursor P cells (hypFIG5 http://www.wormatlas.org/handbook/hypodermis/hypodermis.htm).

B. 1.5 fold stage embryo expressing GFP. Arrowheads show seam cells that do not express GFP.

C. Expression is observed in epidermal precursors P cells (arrows) and is not observed in seam cells (arrowheads) in L1 stage.

D. GFP expression of *nhr-40::gfp* is present in pharyngeal muscles and in body wall muscle cells (arrow). Seam cell is marked with arrowhead.

Scale – 20 μm.

5.6 Function of NHR-40

The function of *nhr-40* gene was studied using several different approaches. To block the natural function of the gene we used method RNA interference (RNAi). We also used constructs for overexpression or dominant negative experiments where either all cDNA of *nhr-40b* and *nhr-40d* are under the regulation of heat shock promoter (hsp). The constructs for overexpression are marked as #6076 and #6077. The construct for dominant negative experiment, in which a part of cDNA is under the regulation of heat shock promoter, is labeled as #6010. Finally, we studied mutated strain RB840 that has an intronic genetic deletion allele *nhr-40(ok667)*.

5.6.1 RNA mediated interference of *nhr-40*

We tested inhibition of *nhr-40* gene using several RNAi approaches in which dsRNA was delivered by soaking, feeding and microinjection. Soaking was tested with two constructs #4528 and #4534 and we did not find any significant changes in development or tissue. For feeding we prepared two constructs #4576 and #4577. We have not found any significant changes in development of *C. elegans* using feeding technique.

The best approach to block the natural function of the gene *nhr-40* appears to be microinjection to ovarial syncitium of young adult hermaphrodites. For microinjection we used two in vitro prepared dsRNAs, #4528 and #4534. #4528 dsRNA was prepared from construct containing cDNA from coding region containing exons 2 – 9. This region was amplified with primers #4500 and #4415. #4534 dsRNA was prepared from appropriate construct containing cDNA of the ligand binding domain (exon 5 – 9) amplified with primers #4417 and #4415 (Figure 12).

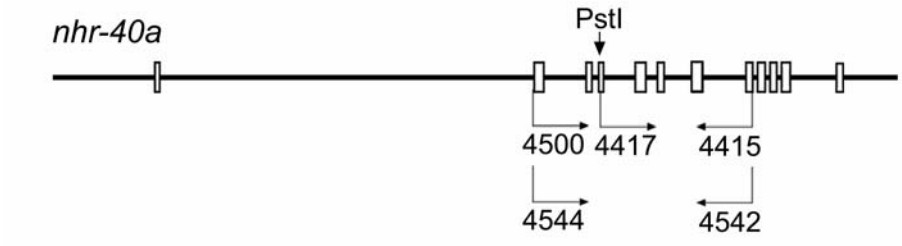

Figure 12. Scheme of *nhr-40a* isoform, which was used for preparation of dsRNA

Primers are indicated by position of vertical bars. Their orientation is marked by horizontal arrows (not in scale).

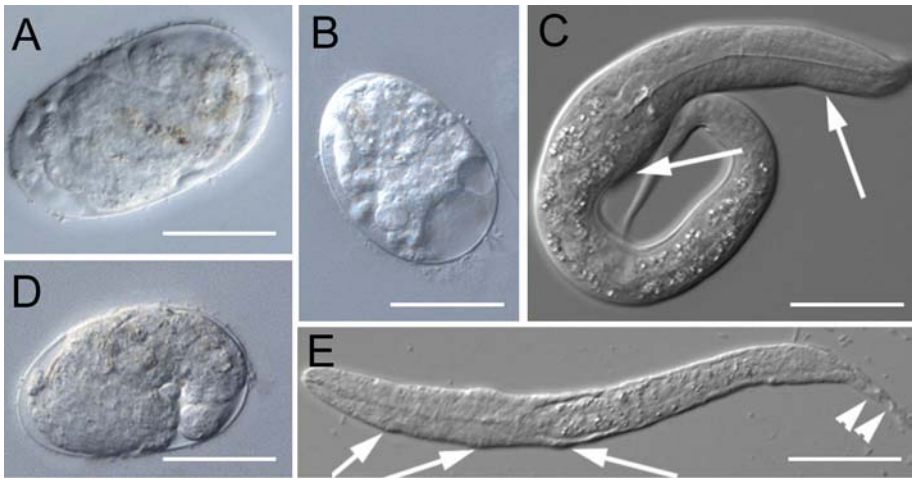

Figure 13. Phenotypes induced by *nhr-40* RNAi

A., D. Figures show arrested embryos at different stages of embryogenesis.

B. Arrested embryo with vacuoles typical for *nhr-40* RNAi.

C. L1 larva with bulges (arrows).

E. L1 larva with bulges (arrows) and vacuoles (arrowheads).

Scale – 20 μm.

Block of natural function of *nhr-40* by RNAi resulted in embryonic and larval arrest in L1 stage. dsRNA was incorporated by microinjection to the ovarial syncitium of young hermaphrodites and the progeny was observed and scored for developmental changes under the high power microscope. We have found affected embryos, which were arrested before and at the 2 fold stage. We found defects in elongation and morphogenesis. Affected L1 larvae showed similar phenotypical changes as the presence of bulges (bmd – body morphology defects), smaller body size (dpy phenotype) and slower movement than wild type L1 animals. RNAi with dsRNAs prepared from both constructs #4528 and #4534 resulted in similar phenotypes (Figure 13). We have found 10% of arrested embryos and approximately 20% of affected L1 larvae (Table 1).

Table 1. The developmental defects induced by inhibition of *nhr-40*

Treatment	RNAi	
	Exon 2-9 cDNA #4528	Exon 5-9 cDNA #4534
Embryonic arrest	10% (n = 5,252)	9% (n = 2,477)
Larval arrest/Lethality	19% (n = 672)	18% (n = 383)

n = number of assayed progeny

5.6.2 *nhr-40* RNAi on strains expressing epidermal markers

To better distinguish which tissue is affected we used *nhr-40* RNAi on transgenic lines expressing epidermal and muscle markers. A strain *ajm-1*::*gfp* (SU93) was used as epidermal marker. Inhibition of *nhr-40* by dsRNA #4534 in SU93 worms revealed that affected embryos contain epithelial seam cells that have wild type appearance (Figure 14).

#4534 RNAi inhibition of *nhr-40* in JR667 strain (which is wild type integrated strain of the seam cell GFP marker), revealed wild type pattern of seam cells during embryonic arrest. The affected larvae have the normal number of seam cells despite severe defects in body shape and bulges (Figure 15).

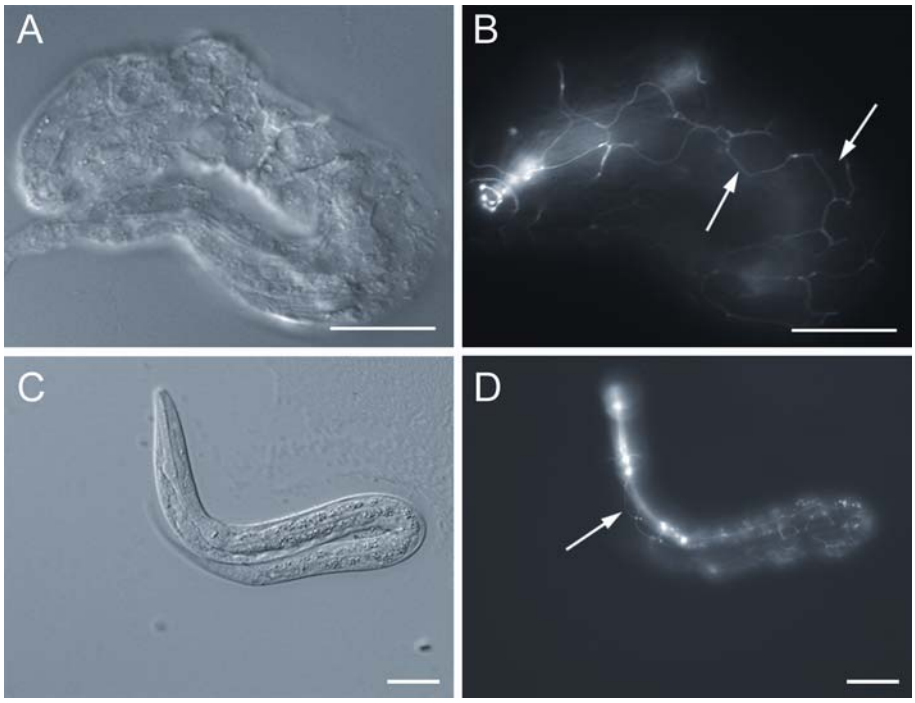

Figure 14. ***nhr-40*** **RNAi on the** ***ajm-1*****::*****gfp*** **background**

A. Affected embryo shortly before hatching in Nomarski optic.

B. GFP image of the same embryo. Seam cells are not affected (arrows).

C. Nomarski optics of affected L1 larva after *nhr-40* RNAi .

D. GFP image of the same L1 larva shows non-affected seam cells (arrow).

Scale bar – 20 μm.

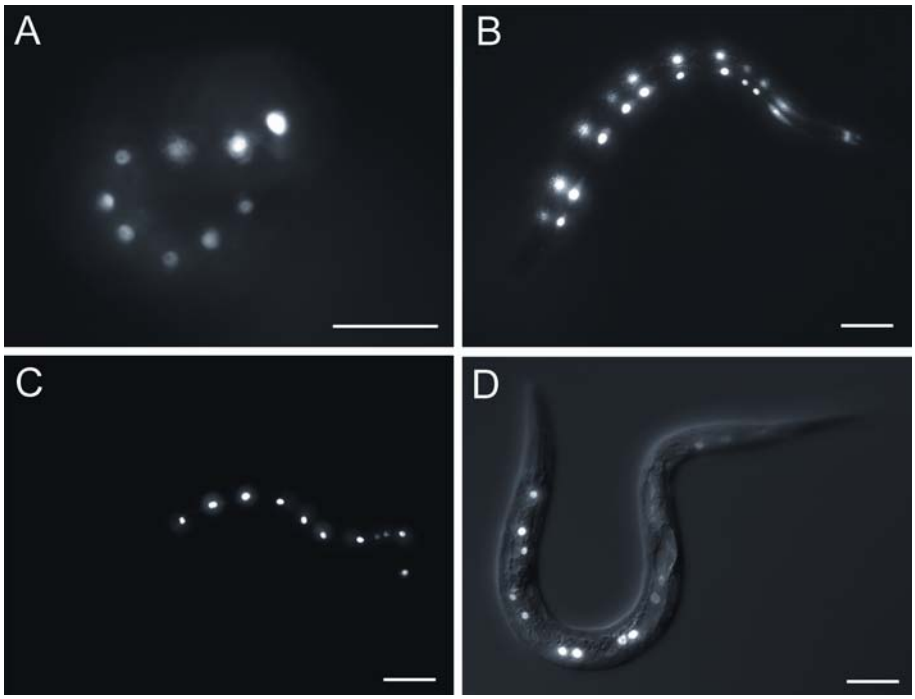

Figure 15. ***nhr-40*** **RNAi on JR667 strain**

A. Arrested embryo expressing GFP in seam cells.

B. Wild type L1 larva expressing GFP. GFP is expressed in two rows of seam cells.

C., D. GFP expression in seam cells of affected L1 larvae after *nhr-40* RNAi. The number of seam cells is not affected.

Scale bar – 20 μm.

5.6.3 *nhr-1* and *nhr-47* RNAi do not change expression of GFP of *nhr-40::gfp* worms

The closest homologues of *nhr-40* gene in *C.elegans* are genes *nhr-1* and *nhr-47*. We prepared dsRNAs of *nhr-1* and *nhr-47* from the templates that represented ligand the binding domains of these genes. In vitro prepared *nhr-1* dsRNA and *nhr-47* dsRNA were injected to ovarial syncitium of young adult hermaphrodites of *nhr-40::gfp* strains #4586 and #4523.

Screen of progeny after *nhr-1* RNAi revealed developmental defects in 3.4% embryos (42/1,254) and body morphology defect in 0.9% of L1 larvae with normal developed head and affected intestine (11/1,293). We focused on the change of GFP expression in *nhr-40::gfp* strains (in body wall muscle cells and neuronal cells). We did not find significant changes of GFP expression. This finding suggests that NHR-1 does not regulate *nhr-40* expression in *C.elegans* during embryogenesis and in L1 stage.

nhr-47 RNAi was done according the same conditions as *nhr-1* RNAi and we did not find any changes of GFP expression in *nhr-40::gfp* embryos and L1 larvae. This result also suggested that NHR-47 does not regulate expression of *nhr-40*.

We also tested the influence of *nhr-1* + *nhr-40* dsRNAs and *nhr-47* + *nhr-40* dsRNAs mixtures. We have found 7.2% (29/403) of arrested embryos after RNAi with the mixture of *nhr-1* + *nhr-40* dsRNAs and 10.6% (13/123) of affected embryos after RNAi with the mixture of *nhr-1* and *nhr-47* dsRNAs. No significant change in the number of affected progeny after these RNAis suggested that decrease of expression of products neither of *nhr-1* gene nor *nhr-47* gene do not influence *nhr-40* expression. NHR-1 and NHR-47 are not involved in regulation of expression together with NHR-40.

5.6.4 *hlh-1* RNAi does not change GFP expression of *nhr-40::gfp* worms

HLH-1 activity is required during embryonic development for the proper differentiation and function of body wall muscle cells. HLH-1 is expressed in nuclei and first appears in body wall muscle precursors at the ~80-cell stage of embryogenesis (Krause, 1995). *nhr-40* is expressed in precursors of body wall muscle cells during embryogenesis at the 1.5 fold and expression continues in these cells throughout larval development. Study of mutated *hlh-1(cc450)* strain revealed body morphology defects in L1 stage which are similar to those which we found

after *nhr-40* RNAi. We studied the influence of the change of *hlh-1* expression level on *nhr-40::gfp* expression. For this purpose we prepared *hlh-1* RNAi experiment using region of exons 1 to 3 of *hlh-1* gene to prepare dsRNA. This region is present in all three isoforms of *hlh-1* gene. dsRNA was injected to ovarial syncitium of *nhr-40::gfp* transgenic worms. Progeny was followed under the high power microscope. We found developmental changes during L1 stage with body morphology defects similar to those which can be detected after *nhr-40* RNAi but we did not detect any change of GFP expression in *nhr-40::gfp* transgenic animals. This result did not suggest the direct influence of HLH-1 on *nhr-40* expression.

5.6.5 *nhr-40* is important for development of body wall muscle cells

hlh-1::gfp transgenic line is a marker of body wall muscle cells (Krause et al., 1994). We injected *nhr-40* dsRNA #4534 to *hlh-1::gfp* transgenic worms. We have found disorganized and missing muscle cells in affected animals.

Body wall muscle cells were not only miss-localized, but we found decrease of GFP expression in several muscle cells and change of the morphology of these cells (Figure 16).

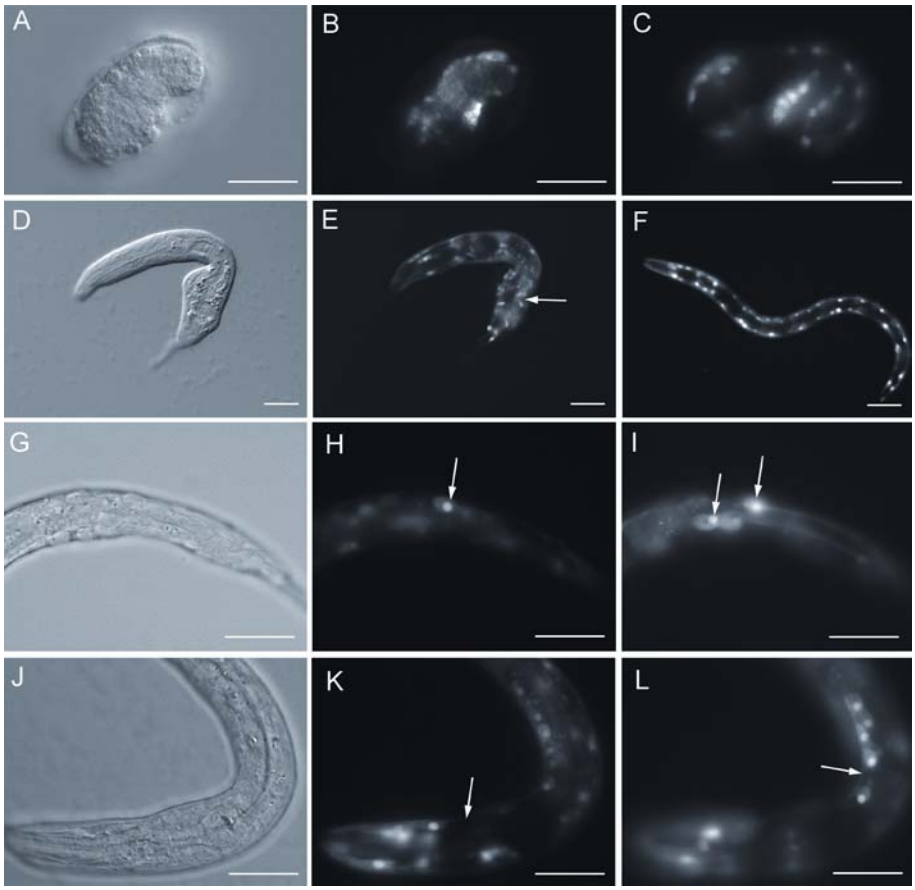

Figure 16. Developmental defects of muscle cells in *hlh-1::gfp* worms treated with *nhr-40* RNAi

A. Affected embryo before 1.5 stage in Nomarski optics.

B. GFP image of the same embryo showing disorganized and missing body wall muscle cells.

C. Wild type embryo expressing *hlh-1::gfp* showing normal organization of body wall muscle cells detected by GFP.

F. *hlh-1::gfp* L1 larva with normal pattern of GFP expressing body wall muscle cells.

D., G., J. panels show L1 larvae with developmental defects, bulges and strictures in Nomarski optics.

E., H., I., K., L. panels show L1 larvae with defective muscle cells and with mispositioned muscle cells that have defective ovoid shape, marked with arrows in panels H and J and missing body wall muscle cells in K and L.

Scale – 20 μm.

Body wall muscle cells (BWM) in *C. elegans* are longitudinal and organized in four quadrants. L1 larva after hatching has 21 body wall muscle cells in DR and DL quadrants, 20 cells in VR quadrant and 19 cells in VL quadrant. The number of body wall muscle cells (19 – 21 cells per quadrant) in worms subjected to *nhr-40* RNAi was determined using an *hlh-1::gfp* strain that marks these cells (Krause et al., 1994). We scored 2 quadrants in 15 L1 larvae *hlh-1::gfp* strain and 20 L1 larvae of *hlh-1::gfp* strain after #4534 RNAi. The average number of cells per quadrant expressing the transgene was 18.0 (SD 2.2) in control animals and 15.1 (SD 3.0) in *nhr-40* RNAi – treated animals. This result is statistically significant assuming the null hypothesis at 99% probability ($p<0.0001$).

5.6.6 *cyp-4* RNAi does not change GFP expression in *nhr-40::gfp* worms

cyp-4 encodes a highly conserved cyclophilin. The *cyp-4* transcript is expressed in early larval stages exclusively in body wall muscle cells. *cyp-4* RNAi resulted in progeny with morphological defects with effect on body wall muscle cell development (Page and Winter, 1998). We tested influence of CYP-4 on *nhr-40* expression using *cyp-4* RNAi on *nhr-40::gfp* line. We did not find any significant changes in progeny after RNAi.

5.7 Overexpression of *nhr-40* and overexpression of *nhr-40* AF-2$^-$

5.7.1 Overexpression of *nhr-40*

We have prepared two *nhr-40* constructs #6076 and #6077 containing cDNA corresponding to mRNA of *nhr-40* gene (*nhr-40b* and *nhr-40d* respectively) (Figure 17).

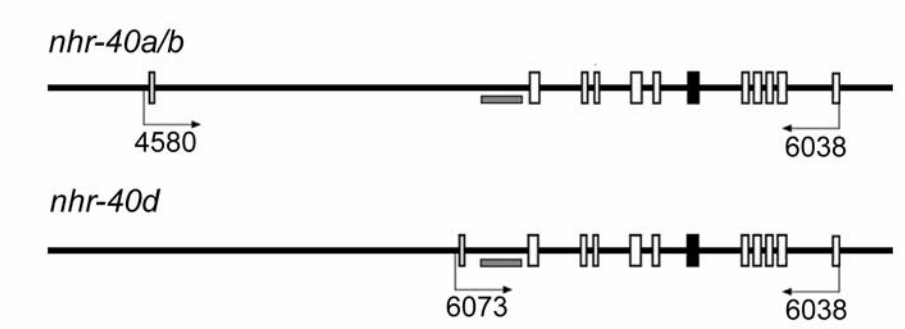

Figure 17. Scheme of *nhr-40a/b* and *nhr-40d* gene isoforms with marked primers #6076 and #6077 used for preparation of constructs for overexpression experiments

Primers are indicated by position of vertical bars. Their orientation is marked by horizontal arrows (not in scale). Gray bar shows deletion that is described below.

To make constructs for overexpression all cDNAs of *nhr-40a/b* and *d* isoforms were cloned under the regulation of heat shock promoter (hsp). Transgenic lines of *C. elegans* prepared by microinjection using hsp construct and pRF4 vector as the positive marker of microinjection were tested. Population of transgenic gravid hermaphrodites carrying *nhr-40* overexpressing construct and population of N2 gravid hermaphrodites as control were transferred to 100 µl of M9 solution and heated at 31°C for 2 h in PCR cycler. After heat shock the population was cooled down 16°C for 30 min and then transferred on NGM plates with OP50. Adult hermaphrodites were transferred each 12 hours and progeny was followed. Induction of transgene in adult hermaphrodites resulted in phenotypes very similar to *nhr-40* RNAi. We found embryonic and larval arrest of the progeny of heated hermaphrodites (Figure 18). We found 18% and 12% of affected embryos after heat

shock of strains #6076 and #6077 respectively and 21% of affected L1 larvae (Table 2).

Table 2. The developmental defects induced by overexpression of *nhr-40*

Treatment	Overexpresion	
	nhr-40b (#6076)	*nhr-40d* (#6077)
Embryonic arrest	18% (n = 402)	12% (n = 321)
Larval Arrest / Lethality	21% (n = 271)	22% (n = 208)

n = number of assayed progeny

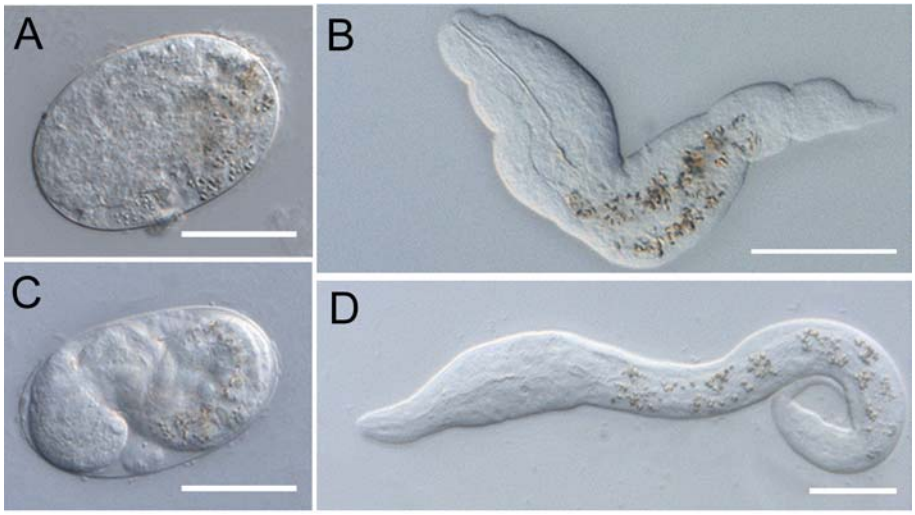

Figure 18. Phenotypes caused by *nhr-40* overexpression

Panels A., C. show affected embryos of transgenic line carrying either #6076 or #6077 construct after heat shock.

Panels B., D. show affected L1 larvae of the same transgenic lines.

Scale bar – 20 μm.

5.7.2 Overexpression of *nhr-40* AF-2^{-} (dominant negative experiment)

We prepared *nhr-40* mutated construct #6010 containing DNA corresponding to *nhr-40a* cDNA with deletion at 3´end of ligand binding domain (Figure 19).

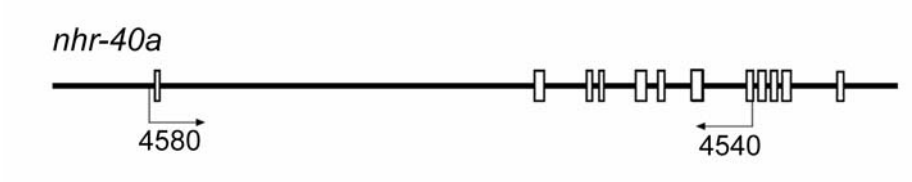

Figure 19. Scheme of *nhr-40a* gene isoform with primers used for preparation of #6010 construct used for dominant negative experiment

For dominant negative experiment we prepared a part of *nhr-40a* isoform cDNA representing region from 5´UTR to exon 9 under the regulation of heat shock promoter. Transgenic gravid hermaphrodites of #6010 *C. elegans* strain prepared by microinjection using hsp construct and pRF4 vector were tested by heating at 31°C for 2 h. N2 population of gravid hermaphrodites was used as the control. All experiments were done in PCR cycler and animals were kept in M9 solution. After heat shock worms were cooled down at 16°C for 30 min and transferred on NGM plate with OP50. Adult mothers were transferred each 12 h and progeny was followed and scored. We have found very similar phenotypical changes as with *nhr-40* RNAi: embryonic arrest and body morphology defects, short size of body and bulges (Figure 20).

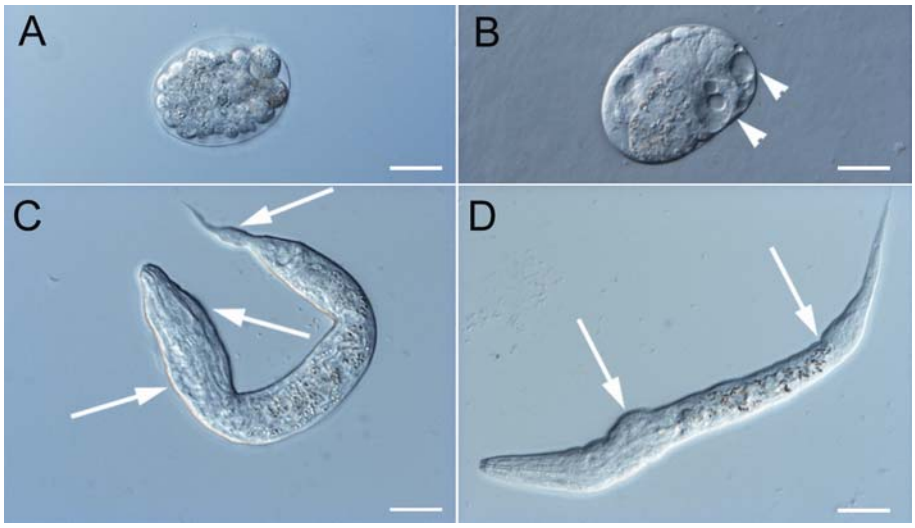

Figure 20. Phenotypes caused by overexpression of *nhr-40a* AF-2$^-$

Panels A., B. show affected embryos with vacuoles (arrowheds) of transgenic line carrying #6010 construct after heat shock.

Panels C., D. show affected L1 larvae of the same transgenic line after heat shock showing bulges (arrows), strictures and body morphology defects.

Scale bar – 20 μm.

Transgenic line #6010 has approximately 30% of transgenic animal per 1 hermaphrodite. We found 260 rollers from 861 scored animals. After heat shock experiment we found 32 affected L1 from 510 studied under the high power scope. With respect to the fact that the line #6010 has only 30% of transgenic animal we calculated that 20.9% of L1 were affected (32/153).

5.8 RB840 strain carrying *nhr-40(ok667)* deletion allele

We sought a genetic deletion allele of *nhr-40* to better understand the developmental function of the gene. To date, only a single *nhr-40* deletion allele has been generated and was provided us by the *C. elegans* Gene Knockout Consortium at the Oklahoma Medical Research Foundation. To confirm the deletion described by *C. elegans* Gene Knockout Consortium we prepared gDNA from RB840 strain and from this template three overlapping PCRs to find a predicted region of deletion. The first PCR was prepared with primers #6041 and #6043, the second with primers #4595 and #4502 and the third with primers #6044 and #6042. Sequencing of the second PCR product revealed 840 bp long deletion within the first intron of all *nhr-*

40 isoforms. Deletion is positioned 62 bp upstream of the start of exon 2. In addition, this allele had an insertion of 6 bp (aaaaga) in 3´ end of the deletion (Figure 21).

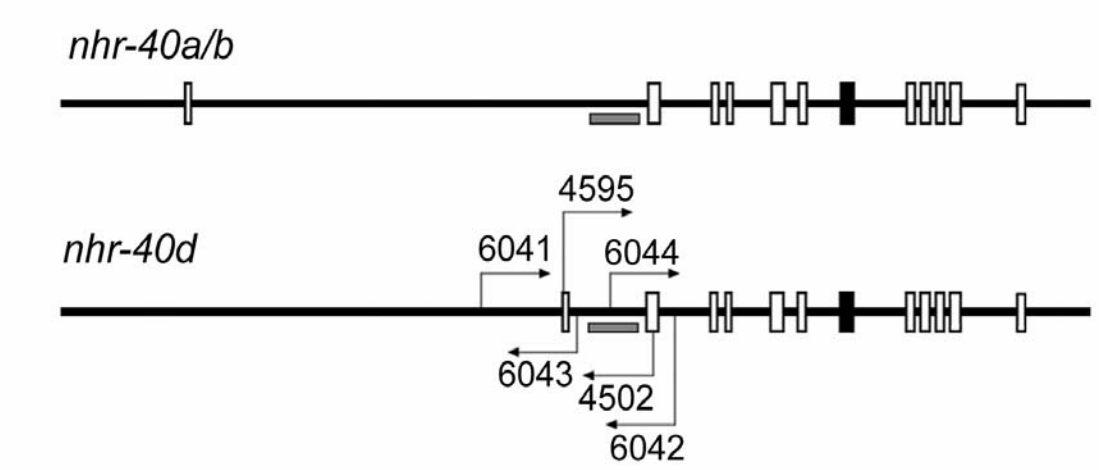

Figure 21. Scheme of mutated *nhr-40* gene with *(ok667)* deletion allele
Primers are indicated by position of vertical bars. Their orientation is marked by horizontal arrows (not in scale). The region of deletion is shown with gray bar.

Mutated homozygotes were out-crossed three times with wild type N2 males. After each out-cross 2 single worm PCRs were done to confirm in the first step heterozygotes and then in the second step homozygotes carrying the mutation. This PCR was done with primers #4595 and #4502. Expected size of the wild type allele is 1,403 bp and size of the mutated is 569 bp. Mutated animals were then screened for phenotypical changes under the high power microscope and scored. We have found phenotypes paralleled to those observed after *nhr-40* RNAi or *nhr-40* overexpression (Figure 22). Table 3 shows results of segregation of RB840 progeny: 10% of non-developed embryos and 20% of affected L1 larvae with body morphology defects.

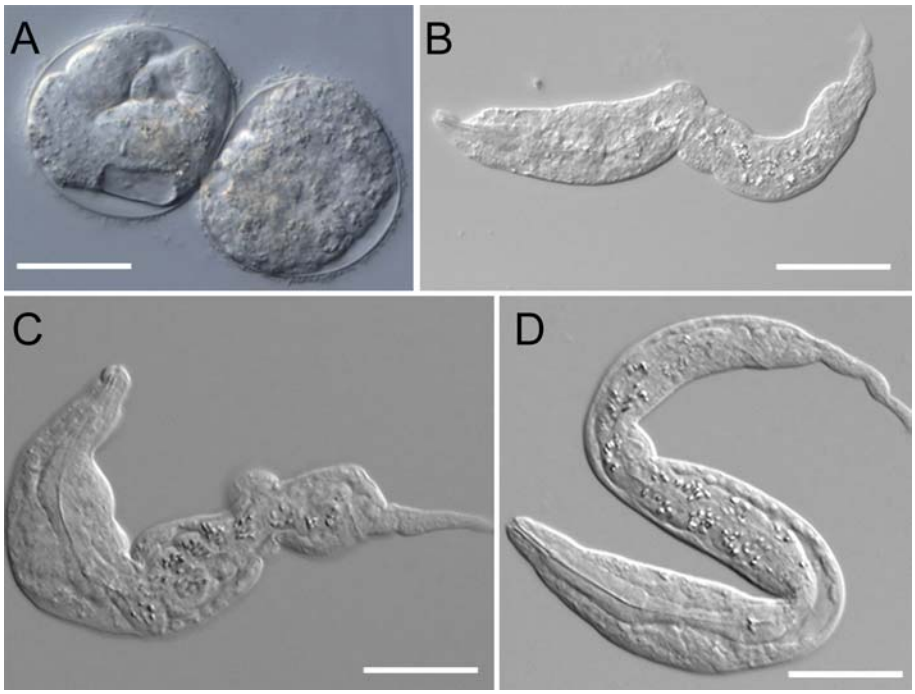

Figure 22. Phenotypic changes found in RB840 line

A. Embryonic arrest occurred at the 1.5 – 2 fold stage of embryogenesis with defects in morphogenesis and appearance of vacuoles.

B. – D. L1 larvae with severe defects of body shape, bulges and strictures and with dpy phenotype.

Scale – 20 μm.

The location of the *nhr-40(ok667)* deletion in RB840 strain that is 62 bp upstream the exon 2 suggested it could affect the splicing to the second exon. We used RT-PCR to examine the expression of *nhr-40* transcripts *a*, *b* and *d* that represent products from the two promoters of the gene. We found that while majority of message corresponded to wild type cDNAs, there were aberrant transcripts detectable for both the *nhr-40a/b* and *nhr-40d* messages. For this purpose we prepared PCR reactions with primers #4580 and #4581 for *nhr-40a/b* message of and primers #6036 and #4581 for *nhr-40d* message. Direct sequencing of amplified fragments revealed that both exons 1 were frequently and inappropriately spliced to exon 3. Coding regions of these aberrant exon 1/3 splicing resulted in out-of-frame splicing transcripts with premature stop codon and if translated it would produce a severely truncated protein product (Figure 23).

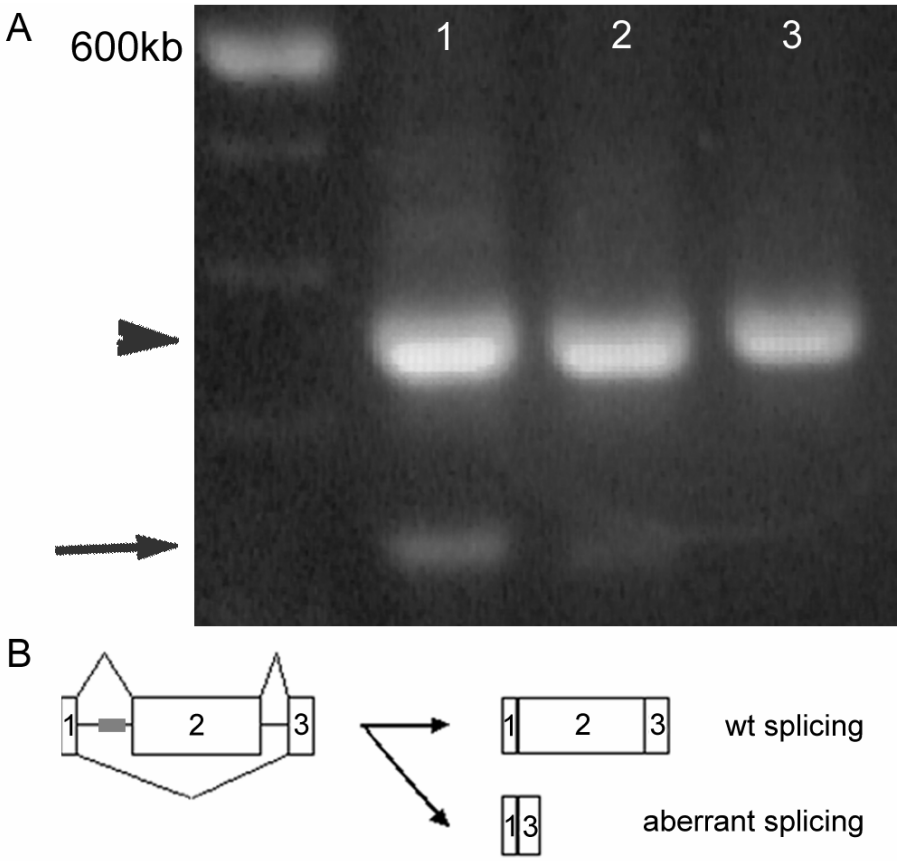

Figure 23. Detection of abberant splicing of *nhr-40* isoforms

A. Electrophoresis gel with detection of PCR products after PCR with primers #4580 and #4581 (line 1, template cDNA from RB840), primers #6036 and #4581 (line 2, template cDNA from RB840), primers #4580 and #4581 (line 3, template wild type N2 strain as the control, 344bp, arrowhead). A band 119 bp revealed 1/3 aberrant splicing (arrow) in line 1 and 2.

B. Scheme of wild type and aberrant splicing.

C. Figure shows that the minority of the message aberrant exon 1/3 splicing resulted in out-of-frame splicing transcripts with premature stop codon in RB840 strain.

We studied *nhr-40* promoter 2 in *nhr-40(ok667)* animals. gDNA prepared from RB840 served as the template for PCR with primers #4501 and #4502. The part of intron 1, normally promoting *nhr-40d*, from RB840 strain was cloned into the promoterless GFP vector pPD95.73 and named #6093. We prepared four independent transgenic lines. A comparison of reporter gene expression between strains harboring the wild type versus mutant promoter 2 was made. We found that the expression from mutant promoter 2 was very weak, reaching maximally only 10 to 20% of values obtained with the wild type promoter 2. This result suggests that *nhr-40* transcription is reduced in the deletion allele strain RB840 due to splicing abnormalities.

5.8.1 Rescue by gDNA

We attempted to rescue the embryonic and larval arrest phenotypes of the *nhr-40(ok667)* deletion allele using wild type genomic DNA. The wild type *nhr-40* locus (~15 kb) was amplified by several overlapping PCR fragments (Figure 24).

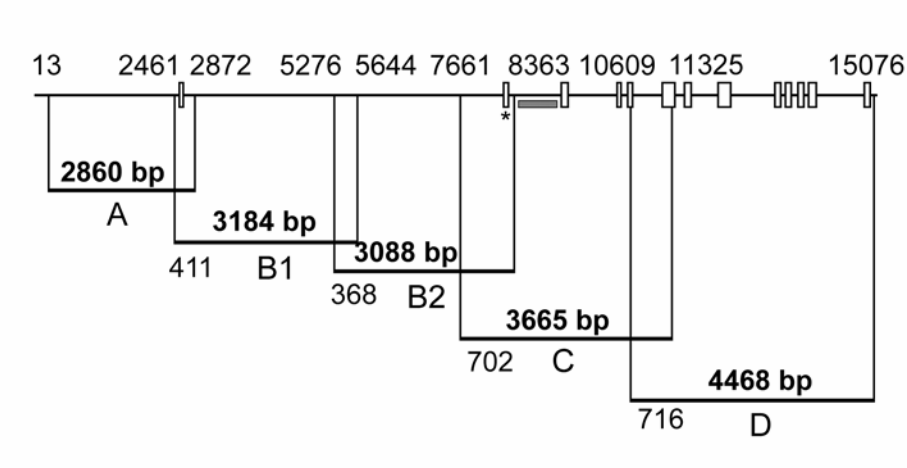

Figure 24. Schematic representation of *nhr-40* genomic regions used for rescue experiments

The origin of overlapping genomic PCR fragments used for rescue experiments of the *nhr-40* mutant strain RB840. The region of overlap among individual PCR products varied between 368 bp and 716 bp. The PCR products were marked A, B1, B2, C and D. The deletion in the strain RB840 is marked as the gray box under the sequence in the 1st intron. The asterisk marks the 1st exon of *nhr-40d* isoforms.

Mix of PCR reactions was injected into the RB840 hermaphrodites together with pRF4 marker plasmid that causes a roller phenotype. This method is often used

to generate transgenic animals and to rescue mutant animals (Maryon et al., 1996; Maryon et al., 1998; Mercer et al., 2003; Watanabe et al., 2005). The fragments injected into the gonad of hermaphrodites recombine by homologous recombinations to reconstruct an intact *nhr-40* gene. Transgenic animals were recognized as rollers and their progeny was scored for developmental arrest. We found that there was a small but significant decrease in embryonic arrest and a nearly 3-fold decrease in larval arrest and lethality when wild type genomic DNA was introduced into the RB840 strain (Table 3). The significance of this result was supported by the analysis of the proportion of affected L1 larvae in progeny of RB840 hermaphrodites injected with amplified genomic DNA. The percentage of affected L1 larvae decreased from 26.2% (SD=15.6) per parent control animal to 8.6% (SD=7.8) per treated RB840 parent, indicating a significant rescue at 99% probability using the Student´s t-test ($p<0.001$).

These results strongly suggest that the developmental defects observed in mutant strain reflect loss of NHR-40 activity.

Table 3. Segregation of progeny RB840 *C.elegans* strain after rescue experiment

Treatment	None (%)	Rescue by gDNA
Embryonic arrest	10% (n = 2,521)	7% (n = 2,859)
Larval Arrest /Lethality	20% (n = 2,521)	7% (n = 2,859)

n = number of assayed progeny

5.8.2 Rescue of *nhr-40(ok667)* defects by *myo3::nhr-40d*

nhr-40 expression showed that both promoters were active in muscle cells of embryos, larvae and adults. We used tissue-specific muscle promoter driving the expression of *nhr-40* cDNA to see if they could rescue the phenotypes of *nhr-40(ok667)* homozygous animals. The *myo-3* is expressed in non-pharyngeal muscles (Fire and Waterston, 1989; Okkema et al., 1993). *myo-3* encodes MHC A, the minor isoform of MHC (myosin heavy chain) that is essential for thick filament formation and for viability, movement and embryonic elongation. *myo-3* is expressed in body muscle cells, in the somatic sheath cell covering the hermaphrodite gonad, in enteric muscles and vulval muscles of the hermaphrodite.

Progeny of transgenic adults were scored for embryonic and larval arrest. This resulted in good rescue of the larval arrest (~30% reduction) but did not affect the embryonic arrest phenotype (Table 4). The significance of this result was supported by statistical analysis of the proportion of affected larvae in the progeny of rescued RB840 mutant animals (per parent animal). Whereas RB840 mutants carrying a non-rescuing control plasmid gave rise to 21.9% (n=3,296 SD 8.51) affected progeny, the percentage of affected larvae from RB840 mutants with the rescuing construct was only 12.6% (n=1,742 SD 5.0). These results are significantly different at 99 % probability ($p<0.0036$). The lack of embryonic rescue may reflect a maternal need for NHR-40 activity that is not present in our rescued lines due to the inability of extrachromosomal transgenes to be expressed in the germline (Kelly and Fire, 1998; Kelly et al., 1997).

Table 4. Rescue of *nhr-40(ok667)* defects by tissue-specific constructs

Treatment	None (%)	*myo3::nhr-40d*
Embryonic arrest	10% (n = 2,521)	10% (n = 2,113)
Larval Arrest / Lethality	20% (n = 2,521)	14% (n = 2,113)

n = number of assayed progeny

5.8.3 Rescue of *nhr-40(ok667)* defects by *nhr-23::nhr-40d*

We attempted to rescue RB840 by expressing *nhr-40* in epidermal cells under the control of *nhr-23* promoter. Analysis of 686 embryos and larvae among the progeny of seven parental transgenic animals obtained after injection RB840 with *nhr-23::nhr-40* demonstrated that his construct did not rescue the mutant phenotypes. This result, combined with our *myo-3* promoter rescue data, suggests that body wall muscle defects are largely responsible for the *nhr-40* mutant phenotypes we observed.

5.9 Protein analysis

The antibody against NHR-40 was prepared by immunization of 2 rabbits denominated B4527 and B4528. The antibody was proposed to recognize 3´end of the protein and was tested using custom antibody ELISA test (Invitrogen).

Repeated Western blot analysis have not revealed any positive staining of total protein extract of *C. elegans*.

Antibody against NHR-40 was also tested by staining of fixed embryos and larvae on poly – L – lysine slides. We did not find positive staining of NHR-40 using B4527 and B4528 antibodies.

5.10 Phalloidin staining

Phalloidin predominantly stains actin which is present in body wall muscle cells. For staining we used synchronized population of L1 larvae of RB840 mutated strain and as control wild type N2 L1 larvae. After comparison of staining pattern and the shape of body wall muscle cells of wild type L1 and of mutated animals we found changes in the structure of body wall muscle cells and different shape of actin filaments suggesting changes in the distance of dense body in muscles (Figure 25).

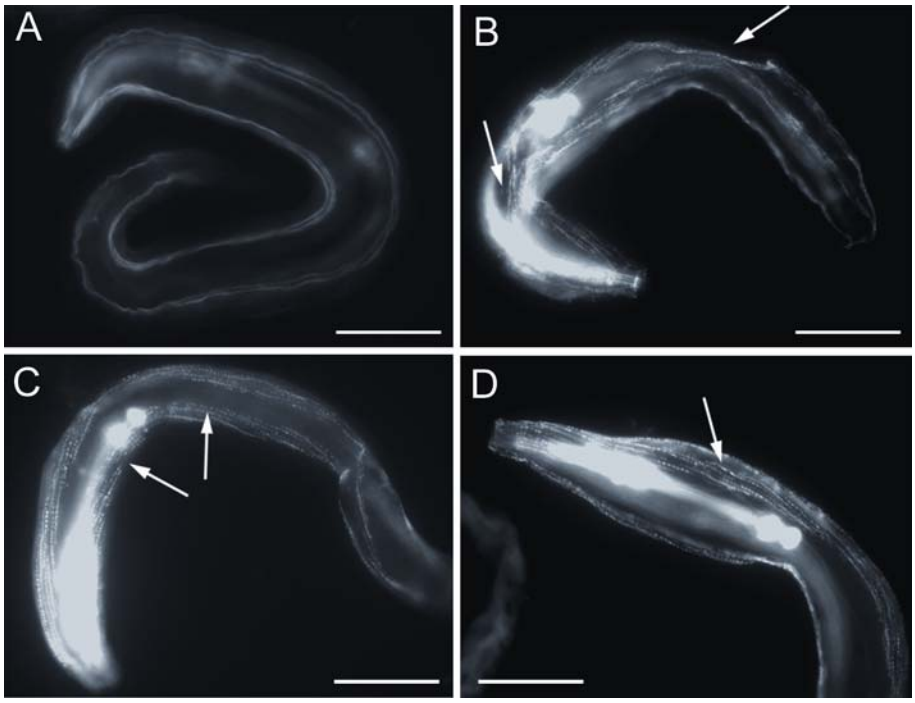

Figure 25. Staining of actin filaments using phalloidin

A. Phalloidin staining of wild type L1 larva. Staining of body wall muscle cells is regular and continuous.

B. – D. Phalloidin staining of affected L1 larvae of RB840 strain. Staining of body wall muscle cells revealed different shape of actin filaments (B) and their discontinuity (C, D).

5.11 Movement assay

Movement of RB840 L1 larvae was also affected. The larvae moved slowly, reversing direction several times and very often moving in circles (Figure 26).

The starving L1 animals were placed on a marked line on chemotaxis agar on a 9 cm Petri dish. A second line was made at a distance of 2 cm and was filled with OP50 bacteria. 1 ml of isoamylalcohol diluted 1:50 in ethanol was placed in the opposite side of the dish. Isoamylalcohol was used to attract worms toward the line of OP50. The movement of the worms was measured in 1, 2, 5 and 24 h interval.

Moving assay showed that between 23 – 25% of 200 non-selected RB840 L1 animals (n = 200) did not reach the OP50 after 24 hours. Similar test was done with 523 selected RB840 animals showing developmental body morphology defects and 100 wild type N2 L1 larvae. Comparison of these two groups of L1 revealed that only 9.6% of affected larvae were able to reach bacteria.

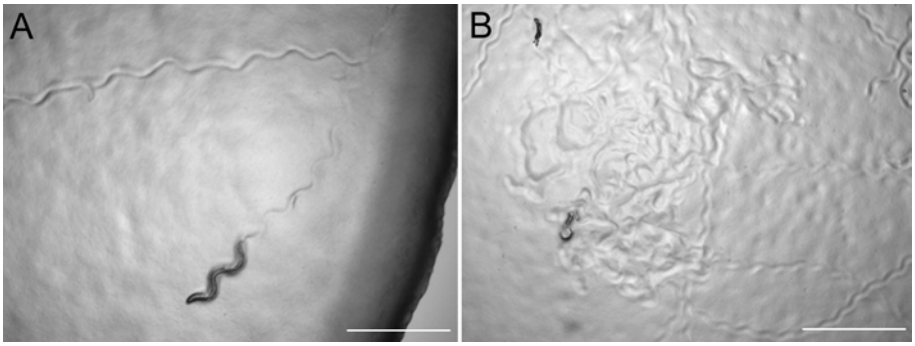

Figure 26. The movement of larvae on the NGM plate after 48h from hatching (cultivation at 22°C)

A. The normal movement of control N2 L3 larva.

B. Examples of mutated L1 larvae with slow development, body morphology defects and different trajectories of the movement.

Scale – 500 μm.

5.12 Motility assay

Single wild type N2 or RB840 L1 larvae were placed in 15 μl drops of M9 buffer and left to acclimate for 1 min. Each full sinus movement was counted.

In addition, the motility assay revealed that affected RB840 L1 larvae showed decreased movement than N2 wild type worms. 15 L1 were scored in each group. N2 values for two experiments were 111.4 ± 14 and 130.6 ± 15.9 compared to RB840 values 41.2 ± 9.9 and 28.9 ± 11.2.

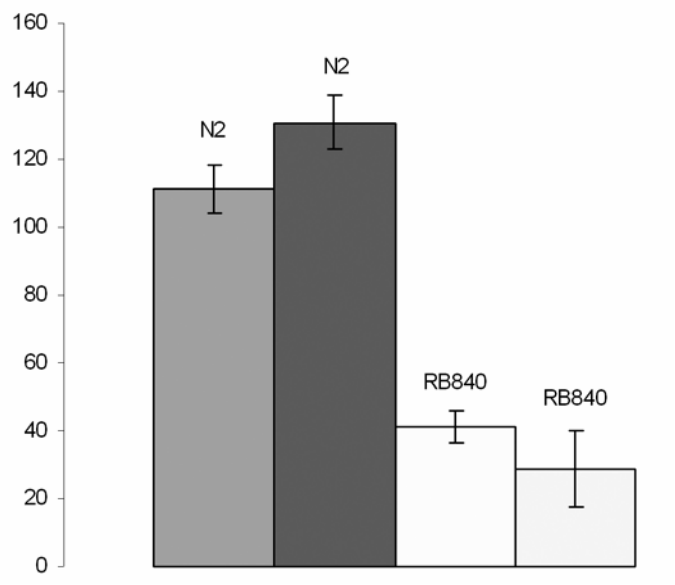

Figure 27. Graph showing a significant difference in motility between wild type N2 L1 and mutated RB840 L1 larvae

6 DISCUSSION

Our characterization of NHR-40 is the first analysis of the subclass of 18 supnrs that have the P box sequence CNGCKT. This subclass represents the closest DNA binding domain to the vertebrate receptors of subfamilies NR1 and NR2. The gene sequence and structure, including a very large first intron, is evolutionarily conserved in nematodes. To today 284 *C.elegans* NHR – encoding genes is known or predicted. We examined nine (*nhr-6*, *nhr-19*, *nhr-21*, *nhr-23*, *nhr-31*, *nhr-40*, *nhr-46*, *nhr-64*) that have similar gDNA organization with the size of first intron being larger then 3 kb. This finding is suggesting that multiple promoters may be a common feature of genes with this structure. The 9 nuclear hormone receptor encoding genes with this organization are not closely related to each other evolutionarily, suggesting that the presence of a large first intron is not simply a consequence of recent gene duplication events. Rather, this gene organization most likely provides some functional advantage, perhaps in allowing flexibility in the temporal and/or spatial pattern of expression.

In the case of *nhr-40*, the two promoters show very similar results in the study of expression by reporter gene constructs (expression in body wall muscle cells, neuronal cells in the head and the tail, ventral and dorsal nerve cord and in the cell of egg laying organ system) and both are expressed throughout development at a constant level. This suggests that under laboratory conditions the potential for flexibility in expression is subtle, if present at all.

Our functional studies demonstrate that NHR-40 regulates aspects of embryonic and larval development. Reduction of function via RNAi or an intronic deletion causes very similar phenotypes, namely, defects in morphogenesis, developmental arrest and lethality. Our characterization of promoter 2 and its alternate first exon provide a molecular mechanism by which a large deletion in the region could interfere with normal *nhr-40* expression. Phenotypes similar to our reduction-of-function studies were also observed following overexpression of *nhr-40* or its part (*nhr-40* AF-2$^-$) from a heat shock promoter in transgenic animals.

One possible mechanism is that the transgene, arranged in a concatenated extra chromosomal array, serves as a template of double stranded RNA formation resulting in an RNAi effect (Fire et al., 1991). Similar phenotypes from RNAi and overexpression have been reported for *pad-2* (Menzel et al., 2004) and *erm-1* (Gobel

et al., 2004). A second possibility is that overexpression results in increased expression from downstream target genes as was shown for *odr-3* (Roayaie et al., 1998) and such overexpression may interfere with normal development. Finally, high levels of NHR-40 may act as a dominant negative protein, by titrating other essential factors or altering DNA binding properties through heterooligomerization (Kurokawa et al., 1994; Nelson et al., 1996).

One surprising finding of our reduction – of – function phenotypes is its relatively low penetrance. Regardless of the perturbation of gene function, loss of NHR-40 activity results in about 10% embryonic and about 20% larval arrest and lethality. We believe that these phenotypes represent bone fide knockdowns of NHR-40 activity because 1) they are not observed in control experiments, 2) the phenotypes can be partially rescued by genomic *nhr-40* DNA, and 3) although not fully penetrant, the phenotypes are very consistent and reproducible. There are several possible explanations for the incomplete penetrance of the *nhr-40* reduction – of – function phenotypes. The most obvious, given it is a member of a gene family consisting of 284 genes, is redundancy of function with one, or more, NHR encoding genes. In a simple test of this model, we did double RNAi with *nhr-40* and its two closest supnrs based on sequence, *nhr-1* and *nhr-47*. Double RNAi between *nhr-40* and either *nhr-1* or *nhr-47* did not alter the phenotype or penetrance of *nhr-40* RNAi alone. It is possible that redundancy in function is provided by other NHR genes or by a gene(s) outside the NHR superfamily. Another possibility is that NHR-40 functions in dose – sensitive processes. Our *nhr-40* RNAi and mutant allele may only be sufficient to partially reduce NHR-40 activity. RNAi is known to not completely eliminate mRNA levels, particularly for genes expressed late in embryogenesis or in larvae (Kostrouchova et al., 2001). Moreover, our analysis of transcripts in the *nhr-40(ok667)* mutant shows that although splicing is affected, some wild type message is produced. The partially penetrant phenotypes we observe may only reflect partial *nhr-40* function; loss of *nhr-40* function may cause a more penetrant and more severe phenotype.

The *C. elegans* supnr family form a group of 269 orphan nuclear receptors that, based on computational analysis, arose from expansion of an ancestral gene encoding a protein related to vertebrate HNF4 (Robinson-Rechavi et al., 2005). In many organisms, orphan NHRs are involved in the metabolic response to xenobiotics (Lindblom et al., 2001; Mohan and Heyman, 2003; Moore et al., 2000; Xie and

Evans, 2001) and any given NHR can respond to a variety of dissimilar xenobiotics (Maglich et al., 2002). It has been suggested that the expansion of supnrs in *C. elegans* reflected a need to cope metabolically with the numerous and varied xenobiotics it encountered in its soil environment (Lindblom et al., 2001). In this model, most supnrs would not be expected to have functions in the laboratory setting where potential ligands of the NHRs would be absent. Our demonstration that *nhr-40* has a developmental role suggests that xenobiotics may not have been the only pressure driving expansion of this gene family. Another supnr, NHR-49, was shown to be a major regulator of fat metabolism, especially fatty acid beta oxidation (Van Gilst et al., 2005a; Van Gilst et al., 2005b). Regardless of the original pressure driving expansion of the supnrs in nematodes, it would appear that many of these genes have adopted essential developmental roles independent of interactions with the environment.

7 CONCLUSIONS

In this study, we have shown that the hypothetical nuclear hormone receptor *nhr-40* is a functional gene with important developmental regulatory functions.

We identified and characterized transcripts of *nhr-40,* characterized the expression pattern in developmental stages and tissues and identified a developmental regulatory function of *nhr-40*.

We found that *nhr-40* gene is expressed in 3 isoforms that we named *nhr-40a*, *b* and *d*.

We found that this receptor is expressed from embryos during all larval stages and in adulthood.

We found that expression of *nhr-40* is regulated from two promoters.

Promoter 1 regulates the expression of *nhr-40* in neurons of the head, in ventral and dorsal neuronal cords, in pharynx, in body wall muscle cells and vulva muscle cells in adult stage.

Promoter 2, which is localized in the first intron of the gene, regulates expression of *nhr-40* similarly as promoter 1 but differs in the number of neuronal cells in the head and tail, uterine-vulva cells and utse in adult stage.

We show that *nhr-40* regulates development of embryos and young L1 larvae.

We show that *nhr-40* loss of function induces defect of development of body wall muscle cells and that the defect has a neuromuscular character.

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9 APPENDIX

9.1 List of figures

9.2 Nomenclature of nuclear receptors
http://www.ens-lyon.fr/LBMC/laudet/nurebase/nurebase.html

Subfamilies and Group	Genes	Trivial Names	Accession numbers	Tetraploidy	Isoforms
1A	NR1A1	TR*a*, c-erbA-1, THRA	M24748	yes	yes
	NR1A2	TR*b*, c-erbA-2, THRB	X04707		yes
1B	NR1B1	RAR*a*	X06538		yes
	NR1B2	RAR*b*, HAP	Y00291		yes
	NR1B3	RAR*g*, RARD	M57707		yes
	NR1B4	RAR	AF378827		
1C	NR1C1	PPAR*a*	L02932		
	NR1C2	PPAR*b*, NUC1, PPAR*d*, FAAR	L07592		
	NR1C3	PPAR*g*	L40904		yes
1D	NR1D1	REVERB*a*, EAR1, EAR1A	M24898		
	NR1D2	REVERB*b*, EAR1*b*, BD73, RVR, HZF2	L31785		
	NR1D3	E75	X51548		yes
1E	NR1E1	E78, DR-78	U01087		yes
1F	NR1F1	ROR*a,* RZR*a*	U04897		yes
	NR1F2	ROR*b*, RZR*b*	Y08639		
	NR1F3	ROR*g*, TOR	U16997		
	NR1F4	HR3, DHR3, MHR3, GHR3	M90806		
		CNR3, CHR3	U13075		
1G	NR1G1	CNR14	U13074		
1H	NR1H1	ECR	M74078		yes
	NR1H2	UR, OR-1, NER1, RIP15, LXR*b*	U07132		
	NR1H3	RLD1, LXR, LXR*a*	U22662		
	NR1H4	FXR, RIP14, HRR1	U09416		
	NR1H5	FXRB	AY094586		
1I	NR1I1	VDR	J03258		
	NR1I2	ONR1, PXR, SXR, BXR	X75163		

	NR1I3	MB67, CAR1, CAR*a*	Z30425		
	NR1I4	CAR2, CAR*b*	AF009327		
1J	NR1J1	DHR96	U36792		
1K	NR1K1	NHR1	U19360		
2A	NR2A1	HNF4	X76930		yes
	NR2A2	HNF4G	Z49826		
	NR2A3	HNF4B	Z49827		
	NR2A4	DHNF4, HNF4D	U70874		
2B	NR2B1	RXRA	X52773		
	NR2B2	RXRB, H-2RIIBP, RCoR-1	M84820	yes	yes
	NR2B3	RXRG	X66225		yes
	NR2B4	USP, Ultraspiracle, 2C1, CF1, RXR1, RXR2	X52591		
2C	NR2C1	TR2, TR2-11	M29960		yes
	NR2C2	TR4, TAK1	L27586		
	NR2C3	TR2-4	AF378828		
2D	NR2D1	DHR78	U36791		
2E	NR2E1	TLL, TLX, XTLL	S72373		
	NR2E2	TLL, Tailless	M34639		
	NR2E3	PNR	AF121129		
	NR2E4	dissatisfaction	O96680		
	NR2E5	fax-1	Q9U4I0		
2F	NR2F1	COUP-TFI, COUPTFA, EAR3, SVP44	X12795		
	NR2F2	COUP-TFII, COUPTFB, ARP1, SVP40	M64497		
	NR2F3	SVP, COUP-TF	M28863		yes
	NR2F4	COUP-TFIII, COUPTFG	X63092		
	NR2F5	SVP46	X70300		
	NR2F6	EAR2	X12794		
	NR2F7	AmNR7	AF323687		
2G	NR2G1	HNF, RXR	AJ517420		
2H	NR2H1	AmNR4, AmNR8	AF323683		
3A	NR3A1	ERa	X03635		yes

	NR3A2	ERb	U57439		yes
3B	NR3B1	ERR1, ERRa	X51416		
	NR3B2	ERR2, ERRb	X51417		
	NR3B3	ERR3, ERRg	AF094318		
	NR3B4	Drosophila ERR	AE003556		
3C	NR3C1	GR	X03225		yes
	NR3C2	MR	M16801		
	NR3C3	PR	M15716		yes
	NR3C4	AR	M20132		yes
4A	NR4A1	NGFIB, TR3, N10, NUR77, NAK1	L13740		
	NR4A2	NURR1, NOT, RNR1, HZF-3, TINOR	X75918		
	NR4A3	NOR1, MINOR	D38530		
	NR4A4	DHR38, NGFIB	U36762		
		CNR8, C48D5	U13076		
5A	NR5A1	SF1, ELP, FTZ-F1, AD4BP	D88155		yes
	NR5A2	LRH1, xFF1rA, xFF1rB, FFLR, PHR, FTF	U93553		yes
	NR5A3	FTZ-F1	M63711	yes	yes
	NR5A4	FF1b	Q9IAI9		
5B	NR5B1	DHR39, FTZF1B	L06423		
6A	NR6A1	GCNF1, RTR	U14666		
	NR6A2	HR4, THR4, GRF	AL035245		
0A	NR0A1	KNI, Knirps	X13331		
	NR0A2	KNRL, Knirps related	X14153		
	NR0A3	EGON, Embryonic gonad, EAGLE	X16631		
	NR0A4	ODR7	U16708		
	NR0A5	Trithorax	M31617		
0B	NR0B1	DAX1, AHCH	S74720		
	NR0B2	SHP	L76571		

10 LIST OF AUTHOR´S PUBLICATIONS AND PRESENTATIONS

10.1 Publications

Libý, P., Kostrouchová, M., Pohludka, M., Yilma, P., Hrabal, P., Sikora, J., **Brožová, E.**, Kostrouchová, M., Rall, J.E. and Kostrouch, Z. (2006) Elevated and deregulated expression of HDAC3 in human astrocytic glial tumours. Folia Biol 52, 21-33.

Brožová, E., Šimečková, K., Kostrouch, Z., Rall, J.E. and Kostrouchová, M. (2006) NHR-40, a *Caenorhabditis elegans* supplementary nuclear receptor, regulates embryonic and early larval development. Mechanisms of Development, in press.

10.2 Presentations

Brožová, E., Šimečková, K., Kostrouch, Z. and Kostrouchová, M. (2004) Nuclear hormone receptor NHR-40 is important for Caenorhabditis elegans late embryonic and larval development. Poster presentation. *European worm meeting*, Interlaken, Switzerland, 22 – 25.5.2004.

Brožová, E., Šimečková, K., Kostrouch, Z. and Kostrouchová, M. (2005) The expression and function of *Caenorhabditis elegans* nuclear hormone receptor 40. Oral presentation. *6th Student Scientific Conference*, *1st Faculty of Medicine, Charles University in Prague,* Czech Republic, 23.5.2005.

Brožová, E., Šimečková, K., Kostrouch, Z. and Kostrouchová, M. (2005) NHR-40, a *Caenorhabditis elegans* nuclear hormone receptor is important for late embryonic and larval development. Poster presentation. *15th International C.elegans Meeting, University of California*, Los Angeles*,* USA, 25 – 29.2005.

Brožová, E., and Kostrouchová, M. (2005) NHR-40, a supplementary nuclear receptor is important for embryonic and larval development of *C.elegans*. Oral presentation. *Hradec Králové Medical Postgraduate Conference 2nd Meeting,* Hradec Králové, Czech Republic, 8 – 9.12.2005.

Brožová, E., Šimečková, K., Kostrouch, Z. and Kostrouchová, M. (2005) NHR-40, a supplementary nuclear receptor is important for embryonic and larval development of *C.elegans*. Oral presentation. *3rd Meeting of the Doctoral Schools of Charles University in Prague and Louis Pasteur University in Strasbourg,* Strasbourg, France, 11 – 14.2005.

Brožová, E., Šimečková, K., Kostrouch, Z. and Kostrouchová, M. (2006) NHR-40, a *Caenorhabditis elegans* supplementary nuclear receptor regulates the development of embryos and L1 larvae. Poster presentation. *European worm meeting,* Hersonissos, Crete, Greece, 29 – 3.2006.

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