Charles University
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# Nuclear hormone receptors in *Caenorhabditis elegans*: NHR-60 regulates embryonic development

*PhD thesis*

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## Content:

# 1. The aim of the study

The aim of the study was to analyze the function and properties of one predicted *Caenorhabditis elegans* nuclear hormone receptor the NHR-60. NHR- 60 is a member of a small subgroup of 18 receptors with an atypical P-box sequence CNGCKT and belongs to the group of supnrs (Robinson-Rechavi *et al.*, 2005).

**The goal of this study was**

a) to determine whether NHR-60 is a functional NHR

b) to characterize its expression

c) to uncover its developmental functions if existing

d) to integrate NHR-60 into known regulatory network

## 2. Abbreviations

| | |
|---|---|
| ACBP | Acyl-coenzyme A binding protein |
| AF-1 | activation function-1 |
| AF-2 | activation function-2 |
| CBP | CREB binding protein |
| *C. briggsae* | *Caenorhabditis briggsae* |
| *C. elegans* | *Caenorhabditis elegans* |
| cDNA | complementary DNA |
| CGC | Caenorhabditis Genetics Center |
| COUP-TF | chicken ovalbumin upstream promoter-transcription factor |
| CTE | carboxy terminal extension |
| DAPI | diamidinophenyl indole |
| DBD | DNA binding domain |
| DNA | deoxyribonucleic acid |
| DR | direct repeats |
| dsRNA | double stranded RNA |
| EcR | ecdysone receptor |
| *E. coli* | *Escherichia coli* |
| e.g. | exempli gratia |
| ER | everted repeats |
| Fig. | figure |
| *ftz* | *fushi tarazu* |
| gDNA | genomic DNA |
| GFP | green fluorescent protein |
| HDAC | histone deacetylase |
| HNF4 | hepatocyte nuclear factor 4 |
| HRE | hormone response element |
| IPTG | isopropyl-β-D-thiogalactopyranoside |
| IR | inverted repeats |
| kb | kilobase |
| LBD | ligand binding domain |
| Mb | Megabase |

| | |
|---|---|
| mRNA | messenger RNA |
| NCoR | nuclear receptor corepressor |
| NGF | nerve growth factor |
| NGM | nematode grow medium |
| NHR | nuclear hormone receptor |
| NR | nuclear receptor |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| p/CAF | p300/CBP associated factor |
| PPAR | peroxisome proliferator-activated receptor |
| qPCR | quantitative PCR |
| RAR | retinoic acid receptor |
| RXR | retinoid X receptor |
| RNA | ribonucleic acid |
| ROR | RAR-related orphan receptor |
| rRNA | ribosomal RNA |
| RT-PCR | reverse transcription polymerase chain reaction |
| SD | standard deviation |
| SL1 | splice leader 1 |
| SL2 | splice leader 2 |
| SMRT | silencing mediator of retinoid and thyroid receptors |
| SRC-1 | steroid receptor coactivator-1 |
| ssRNA | single stranded RNA |
| supnr | supplementary nuclear receptor |
| TR | thyroid receptor |
| tRNA | transfer RNA |
| TTBS | Tris Tween Buffered Saline |
| VDR | vitamin D receptor |

# 3. Introduction

## 3.1. *Caenorhabditis elegans* - a model organism

### 3.1.1. Main features

*Caenorhabditis elegans* is a small, free-living, soil nematode that lives across most of the world. *C. elegans* feeds on bacteria. Due to these properties and its simplicity *C. elegans* became a useful model organism for genetic and developmental studies (Brenner, 1974).

It has a constant number of somatic cells. Adult hermaphrodites have 959 somatic nuclei, adult males 1031. Full cell lineage is described. Worms have short life cycle, small size and could be easily cultivated under laboratory conditions. They can be grown on petri dishes with nematode grow medium (NGM) seeded with *Escherichia coli*.

### 3.1.2. Life cycle

*C. elegans* has two genders – males (XO) and hermaphrodites (XX). Males produce only sperms and can fertilize hermaphrodites. Hermaphrodites produce both sperms and oocytes and are capable of self-fertilization but cannot fertilize each other. Males are found at low frequency (approximately 1:500). They arise spontaneously by non-disjunction of sex chromosomes.

Hermaphrodites lay about 300 eggs, which in twelve hours hatch into larvae. *C. elegans* develops through four larval stages (punctuated by molt) into adults during three days (Fig. 1). Adult animals are approximately 1 mm long. In the case of unfavorable environmental factors, a specific larval stage (dauer larvae) develops instead of the normal L3 larvae. Dauer larvae do not feed and survive several months. When food becomes available, dauer larvae molt to be normal L4 larvae (Hope, 1999).

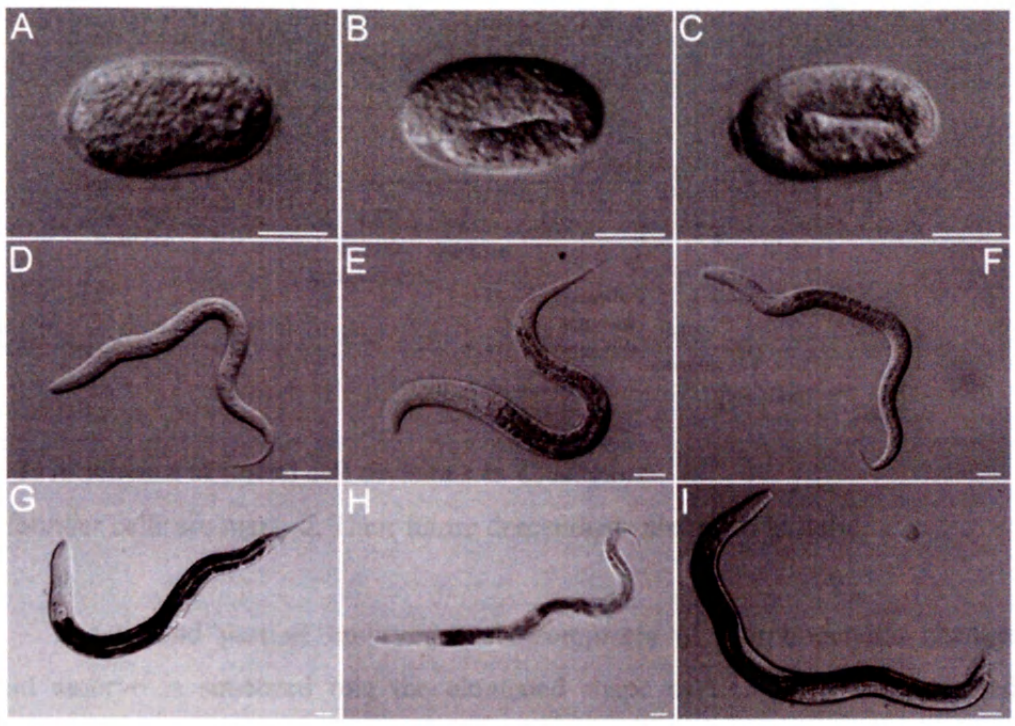


**Fig. 1: *C. elegans* developmental stages**

Individual *C. elegans* embryonic (comma (A), 1.5-fold (B) and 3-fold (C)) and larval (L1 larva (D), L2 larva (E), L3 larva (F) and L4 larva (G)) stages. Panel H shows an adult hermaphrodite worm and panel I an adult male.

Scale bar: 20 μm

### 3.1.3. Development

*C. elegans* embryogenesis has two distinct periods (Sulston *et al.*, 1983).

The first one comprises of mitotic cell divisions generating cells that form the first larval stage (558 cells in the hermaphrodite). Initial cell divisions generate six cells of unequal size called founder cells: AB, E, MS, C, D, and $P_4$ (Fig. 2). Each "stem" cell gives rise to a predetermined number of cells by a series of synchronous and symmetrical divisions. The state of determination is clonally inherited (Deppe *et al.*, 1977). E gives rise to intestinal cells, D to body-wall muscle cells and $P_4$ to germ line. Other founder cells produce several cell types.

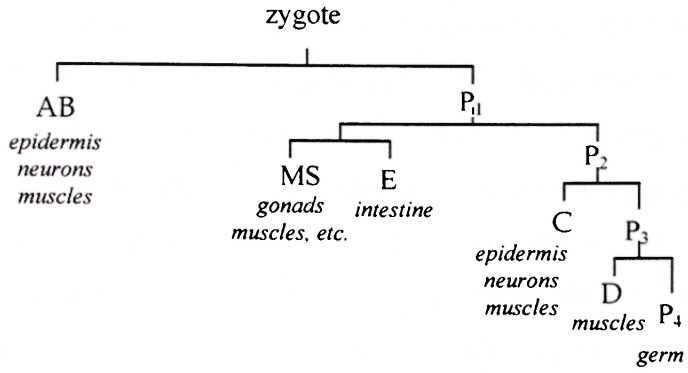


**Fig. 2: A schema of initial cell divisions in *C. elegans***
All founder cells are marked. Their future descendants are noted in italic.

The second part of embryogenesis comprises of morphogenetic changes. Ovoid embryo is squeezed into the elongated shape of L1. Elongation proceeds through several morphological forms: the comma, 1.5-fold, 2-fold, and 3-fold stage. In 3-fold stage the L1 cuticle is formed. Cells differentiate and embryo begins to roll in the chitin shell during elongation.

During post-embryonic development, germ line proliferates. Mature gonads are formed during L4 stage. The number of somatic cell nuclei increases to 959 in hermaphrodites and to 1031 in males. Metamorphosis is not involved in *C. elegans* post-embryonic development. All structures are formed while maintaining the same overall structures already generated during embryogenesis (Hope, 1999).

### 3.1.4. Genetics

#### 3.1.4.1. Basic introduction

Wild-type *C. elegans* hermaphrodites contain five pairs of autosomes and one pair of X chromosomes. Males contain five pair of autosomes and a single X chromosome. The condensed chromosomes are cytogenetically indistinguishable. The *C. elegans* chromosomes have diffuse kinetochores and lack a visible constriction (Albertson and Thomson, 1982). Recombination occurs in sperm of males and in both sperm and oocytes of hermaphrodites (Brenner, 1974).

The genome has a relatively small size - approximately 97 Mb (one-thirtieth the size of mammalian genomes). The *C. elegans* Genome Sequencing Consortium (at the Sanger Institute, Cambridge, UK [http://www.sanger.ac.uk/Projects/C_elegans/] and at the Genome Center, Washington University of St Louis, MI, USA [http://genome.wustl.edu/]) was established to determine the entire *C. elegans* DNA sequence. The essentially complete sequence was published in Science in December 1998. The sequence was annotated using ACeDB software [http://www.acedb.org]. The *C. elegans* research community uses several databases to share recent data such as *Caenorhabditis elegans* WWW Server [http://elegans.swmed.edu/], WormBase [http://www.wormbase.org/], WORMATLAS [http://www.wormatlas.org/] and many others.

### 3.1.4.2. Operons and *trans*-splicing

*C. elegans* and its relatives differ from other animals in having operons. Approximately 15% of *C. elegans* genes are organized in operons, 2-8 genes long (Blumenthal *et al.*, 2002). Polycistronic *C. elegans* operons are processed by internal cleavage. Polyadenylation follows to form 3′ends of mRNA. At the same time, approximately 100 bp downstream of the newly created 3′end, *trans*-splicing creates the 5′end of the downstream mRNA (Liu *et al.*, 2003). During the *trans*-splicing, a short RNA leader is attached to the 5′end of mRNA. Two types of splice leader were described in *C. elegans* – SL1, which is *trans*-spliced to 5′ends of monocistronic genes and first genes in operons and SL2, which is *trans*-spliced to genes downstream in the operons.

### 3.1.4.3. Protein-coding genes

Protein-coding genes are distributed over the whole genome. The genome encodes approximately 22,000 genes. Most of them are short genes covering around 3 kb. Genes contain usually short exon (median size 123 bases) and intron (median size 47 bases) sequences. Alternative splicing is observed. The number of splicing isoforms per gene is low. More than 90% of genes with alternatively spliced forms have only one or two isoforms (Spieth and Lawson, 2006).

### 3.1.4.4. Pseudogenes

Pseudogenes are non-functioning copies of genes in genomic DNA. Two types of pseudogenes could be found in *C. elegans* genome. Processed pseudogenes are created by reverse transcription of mRNA into cDNA and its subsequent insertion into gDNA. Processed pseudogenes are sporadic in *C. elegans* genome. Unprocessed pseudogenes are more often and arise by gene duplication and subsequent disablement. They usually have frameshifts, premature STOP codons, insertions, nucleotide substitutions etc. (Harrison *et al.*, 2001). The number of pseudogenes in *C. elegans* (561 annotated) is still not well estimated.

### 3.1.4.5. RNA genes

18S, 5.8S, and 26S rRNA genes form a large tandem repeat up to 150 copies on chromosome I. Each repeat contains just one copy each of 18S, 5.8S and 26S rRNA gene. 5S rRNA gene forms 100 copies tandem repeat on chromosome V.

608 nuclear tRNA and 22 mitochondrial tRNA genes are encoded in *C. elegans* genome. They usually have only one exon (Spieth and Lawson, 2006).

## 3.1.5. Anatomy

### 3.1.5.1. Basic anatomy

The anatomy of *C. elegans* is simple. It has a tubular body. An external cuticle covers the epidermal body wall. Beneath the epidermis are four longitudinal rows of body wall muscle cells located subventrally and subdorsally. The mouth leads into the bilobed muscular pharynx, which pumps food through the tubular intestine to the rectum and anus. The nervous system consists of a circumpharyngeal nerve ring, dorsal and ventral nerve cords and a variety of sensory receptors and ganglia. The reproductive system of adult hermaphrodites is formed by two gonadal arms. Each arm contains an ovary, oviduct, spermatheca and leads into the uterus. Germ line nuclei are produced in ovarial syncytium. The nuclei are first mitotic. They reach meiotic diakinesis in the oviduct prior to fertilization. Single nuclei become enclosed by membrane and form oocytes. The oocytes enlarge and mature passing through the

oviducts, which terminate at the spermatheca. Fertilized eggs at early stage of embryogenesis cumulate in the uterus before they are laid. Gonads terminate at the vulva. The reproductive system of adult males consists of a single testis connected with rectum via vas deferens. The vas deferens and the rectum unites posteriorly, forming a cloaca. Sexual structures are located in the male tail (Sulston and Horvitz, 1977).

#### 3.1.5.2. Epithelia

There are two major forms of extracellular matrix in *C. elegans*, the cuticle (exoskeleton) and basement membranes. Basement membranes provide mechanical stability to tissues and ensheathe organs (Kramer, 2005). The *C. elegans* epithelial cells have two different regions of plasma membrane – the basolateral and the apical. Basement membranes are adjacent to the basal surface and the cuticle is laid down adjacent to the apical surface (White, 1988).

Epithelial cells together with secreted cuticle establish the body form of the animal. Epithelial cells are connected together by belt desmosomes, which have an adherens junction-like structure (Priess and Hirsh, 1986). In *C. elegans*, epithelial cells include epithelium of the alimentary tract, epidermis (named also hypodermis), interfacial cells, and somatic gonad cells.

##### 3.1.5.2.1. Epidermis

*C. elegans* epidermis is an external epithelial layer of cells directly underlying (and secreting) the cuticle. Many of epidermal cells are multifunctional. They eliminate lot of cells undergoing programmed cell death (dead cells are phagocyted), serve for storage of several substances and act as blast cells (Sulston *et al.*, 1983). Epidermis also surrounds the animal.

There are three broad categories of epidermis in *C. elegans*: a) major epidermis consisting of dorsal and ventral hypodermis and seam cells, b) small epidermal syncytia covering head and tail and c) interfacial epidermal cells connecting internal epithelial organs to the epidermis (Michaux *et al.*, 2001).

#### 3.1.5.2.1.1. Epidermal precursors

The initial specification of epidermis requires the GATA family Zinc finger transcription factor ELT-1. *elt-1* null mutants completely fail to undergo morphogenesis and contain extra neurons and muscles instead of epidermis. ELT-1 is expressed from about the 28-cell stage in cells that will give rise to, among other cells, the major epidermal cells (Page *et al.*, 1997). The major epidermal precursors are born in embryo at approximately a 365-cell stage and are located on the dorsal surface of the early embryo (Chisholm, 2005).

ELT-1 turns on regulatory genes including ELT-3, ELT-5, ELT-6 and LIN-26. LIN-26 expression is continuous in major epidermal cells and is required to keep their proper fates. *lin-26* mutants cause epidermal cells to die and embryonic lethality (defects in ventral enclosure and elongation) (Labouesse *et al.*, 1994, Labouesse *et al.*, 1996). Major epidermis, when assembled, consists of six bilaterally symmetric longitudinal rows of cells (Sulston *et al.*, 1983). Cells of three different parts of major epidermis (dorsal, lateral and ventral) have different functions and express different regulatory genes. ELT-3 (induced by ELT-1) induces gene expression in non-seam cells (dorsal and ventral hypodermis). ELT-5 and ELT-6 redundantly specify seam cell identity. ELT-5 and ELT-6 repress ELT-3 expression in seam cells (Koh and Rothman, 2001).

#### 3.1.5.2.1.2. Intercalation of dorsal epidermal cells

After the terminal division of epidermal precursors in embryo, two rows of dorsal epidermis undergo exceeding rearrangement (dorsal intercalation). During the intercalation, dorsal cells become wedge shaped and their pointed tips are oriented toward the dorsal midline and interdigitate. Each dorsal cell is inserted between its two contralateral neighbors. Dorsal epidermal cells elongate and make contact with lateral epidermis (seam cells) also on opposite side of the embryo (Sulston *et al.*, 1983; Williams-Masson *et al.*, 1998). Finally, dorsal epidermis forms a single row of cells across the dorsal midline. These cells fuse and form the hyp6 and hyp7 syncytia (Podbilewicz, 2006).

#### 3.1.5.2.1.3. Ventral enclosure

Ventral enclosure is the process of migration of ventral epidermal cells towards the ventral midline to envelop the whole embryo with an epithelial monolayer (Chisholm, 2005). Ventral enclosure occurs shortly after dorsal intercalation begins. The first two pairs of ventral cells reaching the ventral midline are so called leading cells. These cells extend long protrusions towards the midline and initiate the migration (Williams-Masson *et al.*, 1997). The migratory activity of these cells is essential for enclosure, since ventral enclosure can be completely blocked by laser inactivation of these two pairs of cells (Williams-Masson *et al.*, 1997). After the leading cells make contact at the ventral midline, cells posterior to these cells (so called pocket cells) reach the midline. Ventral cells then form junctions with their contralateral neighbors to complete ventral enclosure (Williams-Masson *et al.*, 1997). Finally, the anterior epidermal cells cover the head.

#### 3.1.5.2.1.4. Elongation

During the second part of embryogenesis (after the cell proliferation phase), the embryo changes its shape dramatically. Due to elongation, the bean shape of embryo is changed into the normal worm-like shape. Elongation of the embryo reflects the elongation of epidermal cells along the anterior-posterior axis (Priess and Hirsh, 1986), so epidermis is thought to provide the driving power for the elongation. Elongation itself proceeds in two phases called early and late elongation.

Early elongation leads to the change of embryo from comma to two-fold stage. Actin filaments and microtubules were shown to be required for successful elongation (treatment with their inhibitors leads to the failure of elongation). After ventral enclosure, both filaments are reposed circumferentially till the elongation is complete. Contractions of these filaments result in contractions of epidermal cells and cell shape changes (Priess and Hirsh, 1986). This machinery is predominantly acting in lateral epidermis - in seam cells. Dorsal and ventral epidermal cells perhaps do not actively constrict during early elongation. They probably react on seam cell derived forces transmitted through adherens junctions (mutations in genes coding proteins of adherens junctions lead to ventral enclosure and elongation defects) (Chisholm, 2005).

Late elongation (beyond the two-fold stage) is driven by different mechanisms than early elongation. Mutants lacking muscle function fail to elongate after this stage. The failure of elongation could be the consequence of a failed interaction between muscle and hypodermal cells (Williams and Waterston, 1994).

After elongation, epidermal cells begin to secret the cuticle, which keeps epidermal cells in place. Defects in cuticle secretion lead to late-onset defects in elongation (Priess and Hirsh, 1986).

#### 3.1.5.2.1.5. Major epidermis

The major epidermis of embryo is comprised of the dorsal hyp7 syncytium, the ventral P cells, and the lateral seam cells (Koh and Rothman, 2001). Dorsal hyp7 syncytium containing 23 nuclei originated during morphogenesis. During the postembryonic development, other 110 epidermal cells fuse with hyp7 (Podbilewicz and White, 1994). Dorsal epidermis with 133 nuclei forms the biggest syncytium in *C. elegans*. At hatching, the ventral hypodermis contains 12 P blast cells. During postembryonic development, these cells divide and some daughter cells join the dorsal hyp7 syncytium. Other daughter cells give arise either ventral cord motoneurons (form ventral nerve cord) or vulval cells in the hermaphrodite (Sulston and White, 1988). Finally, the hyp7 epidermal syncytium replaces the P cells on the ventral part of the body.

#### 3.1.5.2.1.6. Seam cells

The seam cells form lateral epidermis. Seam cells of L1, dauer, and adult stages produce specific cuticular ridges called alae. These linear ridges run at the sides of the animal along the whole body. Gaps in the line of seam cells cause the gaps in alae. Alae likely serve to make the movement of the animal easier.

Two pairs of ten seam cells ($H_0 - H_2$, $V_1 - V_6$ and T) are present along each side of the newly hatched L1 larva. Almost all of them (except the $H_0$) are blast cells. Seam cells undergo stem cell division at the beginning of each subsequent larval stage (Fig. 3). Seam daughter cells become epidermal cells joining the hyp7

syncytium, neuroblasts, and seams in hermaphrodites. In males, different divisions of $V_5$, $V_6$ and T seam cells occur. Their daughter cells give rise to the sensory rays of a male tail. Seam cells coming from divisions of these three cells in males do not fuse and do not form alae (Sulston and Horvitz, 1977).

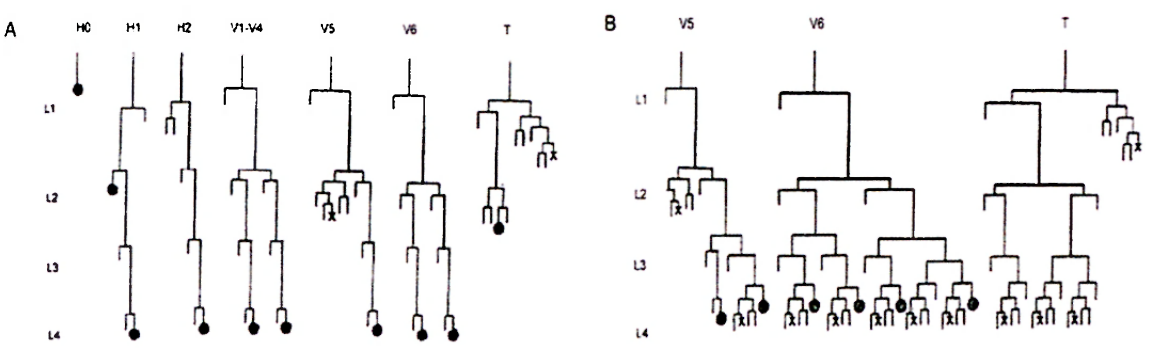


**Fig. 3: A schema of seam cell divisions**

A: A schema of seam cells divisions in *C. elegans* hermaphrodite. Seams are indicated as black dots.

B: A schema of male V5, V6, and T seam cells divisions.

Seam's daughter stem cells elongate and migrate on the top of their sisters (that fuse with hyp7 or become a neuroblast or a ray cell). When seam stem cells reach their seam stem neighbors the division starts again (Podbilewicz and White, 1994). At the end of the L4 stage, seam cells fuse. Fusion occurs between sister seam cells in the anterior and posterior parts of the cells (Podbilewicz and White, 1994).

### 3.1.5.2.1.7. Epidermal syncytia covering head and tail

The epidermis of the head surrounds the buccal cavity. This epidermis is formed by six concentric annular syncytia called hyp1 to hyp6. These small syncytia contain from two to six nuclei. The tip of the head has many sensory endings. The sensilla are bound to the cuticle of hyp2 and hyp3. The epidermis of the tail is formed by four mononucleate cells called hyp8 to hyp11 (except the binucleate hyp10) (White, 1988).

#### 3.1.5.2.1.8. Interfacial epidermal cells

The interfacial epidermal cells are cells connecting internal epithelial organs to the epidermis and specialized cells forming an opening in the epithelium. These cells have usually a toroidal structure (White, 1988).

### 3.1.5.2.2. Alimentary tract

The alimentary tract (gastrointestinal system) is formed by a single tube (including pharynx, intestine, rectum and anus). The alimentary tract includes also related tissues as valves, glands, muscles, tissues of buccal cavity etc.

#### 3.1.5.2.2.1. Pharynx

The gastrointestinal system starts in a buccal cavity. The cavity and the pharynx are separated by a cuticular constriction.

The pharynx (the *C. elegans* foregut) is a self-contained neuromuscular system. It is the organ, which grinds the food (bacteria) and pushes it by rhythmic pumping into the gut. The pharynx has triradiate symmetry and is composed of cell of six main types – epithelial, neuronal, muscular, gland, marginal, and valve cells (Albertson and Thompson, 1976). Several distinct regions could be recognized in the pharynx: an anterior procorpus, a bulb-shaped metacorpus, an isthmus, a terminal bulb with the grinder, and a pharyngeal-intestinal valve (Fig. 4).

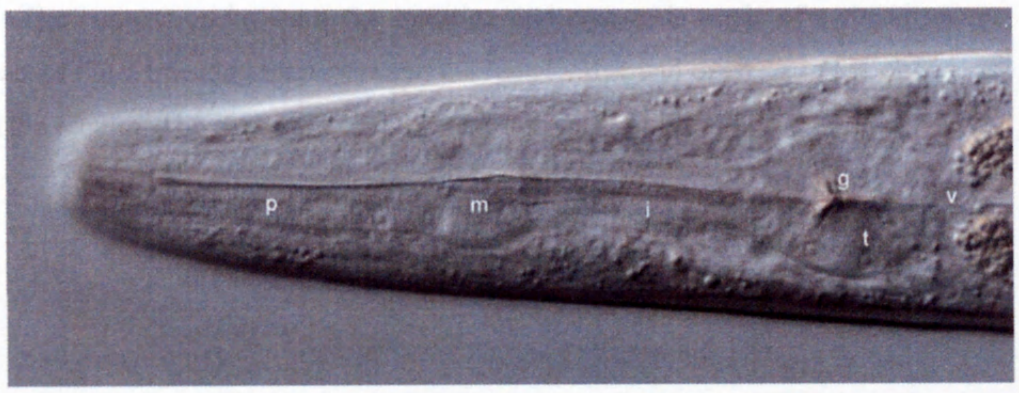


**Fig. 4: The *C. elegans* foregut**

A picture of *C. elegans* pharynx: procorpus (p), metacorpus (m), isthmus (i), terminal bulb (t) with the grinder (g) and pharyngeal-intestinal valve (v) are marked.

Two types of gland cells are present in the pharynx: g1 (two cells with three nuclei between them) and g2 (two cells with two nuclei between them). Gland cell bodies are located in the terminal bulb. They send processes that connect into the pharyngeal lumen. The glands appear to be innervated from pharyngeal motoneurons. Glands are expected to secrete digestive enzymes during feeding and to aid in degrading chitin and cuticle during molting (Mango, 2007).

Pharyngeal-intestinal valve makes connection between the pharynx and intestine.

#### 3.1.5.2.2.2. Intestine

*C. elegans* intestine is a tube formed by 20 large cells. These cells are organized in pairs bilaterally symmetric around the lumen. Each cell pair forms a ring. A whole intestine is made of nine rings, because the first anterior ring is formed by four cells. At hatching, intestinal cells are mononucleate. During postembryonic development, cells became larger, binucleate and polyploidic. Intestinal nuclei undergo endoreduplication prior to each molt (Hedgecock and White, 1985). The intestinal cells are multifunctional. They secrete digestive enzymes into the lumen, absorb nutrients and serve as storage organs (White, 1988).

#### 3.1.5.2.2.3. Rectum

The connection between the intestine and rectum is made of a rectal valve. The part of the alimentary tract between the rectal valve and opening to the outside is called the rectum. The opening itself is called the anus.

## 3.2. Nuclear hormone receptors

### 3.2.1. Introduction

NHRs form a large superfamily of transcription factors of Metazoan species. These proteins work in concert with set of coactivators and corepressors to activate or repress a specific target gene expression (Beato *et al.*, 1995; Robyr *et al.*, 2000; Horwitz *et al.*, 1996). Nuclear receptors play key roles in many important biological processes including development, defense against xenobiotics, regulation of metabolism and homeostasis, etc. (Mangelsdorf *et al.*, 1995; Glass, 1994).

Small lipophilic molecules that include steroid and thyroid hormones and metabolites regulate transcriptional activities of these receptors. The coordinated expression of multiple genes is dependent on the action of many transcription factors acting in combination on large numbers of gene promoters (Weinberg, 2007).

Most NHRs identified throughout the Metazoa are evolutionary conserved and belong to six major subfamilies NR1 to NR6 (Laudet, 1997; Gissendanner *et al.*, 2004; Robinson-Rechavi *et al.*, 2003). NHRs can bind the promoters of regulated genes as monomers, homodimers or heterodimers (Mangelsdorf *et al.*, 1995; Glass, 1994).

### 3.2.2. Structure of NHRs

All NHRs have conserved structure (Fig. 5), which consists of five domains.

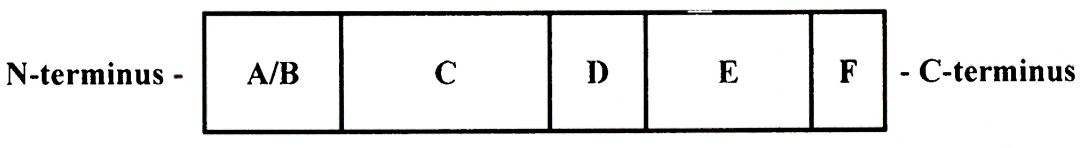


**Fig. 5: A schema of NHR structure**

#### 3.2.2.1. A/B domain

The first N-terminal domain (A/B) is not conserved among the members of the superfamily. The length and sequence of the A/B domain are variable. This domain contains a specific AF-1 activation function, which is autonomous and contributes to

interactions with transcriptional machinery (Robyr *et al.*, 2000). This region may also interact with cell-specific cofactors (Giguere, 1999).

### 3.2.2.2. DNA binding domain

The most conserved DNA binding domain (DBD) (domain C) is responsible for recognition of specific sequences in promoters of target genes. Sequences recognized by DBD are known as hormone response elements (HREs). NHRs are defined by the structure of their DBDs (Mangelsdorf and Evans, 1995; Mangelsdorf *et al.*, 1995).

The DBD is formed by two "zinc fingers", each consisting of 4 cysteins. Several subdomains could be distinguished in the DBD (Fig. 6). At the end of the first "zinc finger", there is a P box. The P box is an amino acid stretch recognizing and binding the core site. The core site itself is not sufficient to provide an exact NHR - target gene DNA binding. NHRs use also a secondary interaction to expanded DNA sequence (not only the core site). HRE is defined by spacing and orientation of core half-sites in the promoter of target genes and helps to differentiate the binding specificity among NHRs with the same P-box. The A-box is a structural element located in the carboxy-terminal extension (CTE) of DBD. The A box mediates recognition of residues upstream of HRE. The D box contributes to the dimerization properties of NHRs.

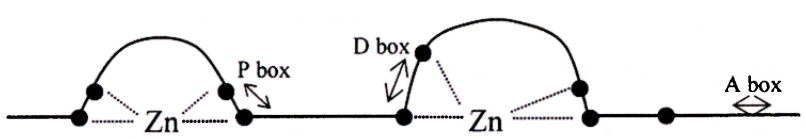


**Fig. 6: DNA binding domain of nuclear receptors**

A schematic representation of two "zinc fingers" with depicted subdomains. All conformationally important cysteins are marked with black dots.

#### 3.2.2.3. Hinge region

The D domain is a flexible hinge region. This region of nuclear receptor is also highly variable in the length and sequence. Hinge region allows DBD to rotate and enables some receptors to bind as dimers to both direct and inverted HREs (Giguere, 1999; Glass, 1994). D domain also interacts with corepressors (Horwitz *et al.*, 1996).

#### 3.2.2.4. Ligand binding domain

A ligand binding domain (LBD, E domain) is conserved among nuclear receptors. It is responsible for ligand binding, dimerization, interaction with heat-shock proteins, nuclear localization, and transactivation (Giguere, 1999; Yen, 2001). Ligand dependent transactivation is mediated by a highly conserved motif (activation function-2, AF–2) localized at the carboxy-terminal region of LBD. Ligand dependent transactivation involves the recruitment of coactivators (Robyr *et al.*, 2000; Giguere, 1999).

#### 3.2.2.5. F domain

The F domain is highly variable and its function is unknown in case of many NHRs.

### 3.2.3. Orphan nuclear receptors

Orphan nuclear receptors are NHRs with no known ligands. It is not clear, if orphan receptors act in a ligand independent manner or if their specific ligands are recruited from metabolites and the environment (Park *et al.*, 2003; Laudet, 1997). Based on phylogenetic studies, nuclear receptors could acquire ligand binding abilities independently during evolution. There is probably no relationship between the position of the receptor (gaining the ligand) in the tree and the chemical nature of its ligand. The ancestors of the nuclear receptor superfamily were probably also orphan receptors (Mendonca *et al.*, 1999).

### 3.2.4. Nomenclature

A majority of NHRs identified throughout the Metazoa are evolutionary conserved and belong to six major subfamilies (Laudet, 1997; Gissendanner *et al.*, 2004). Based on sequence alignments these subfamilies were defined:

- Subfamily NR1 - thyroid hormone receptors (TRs), retinoic acid receptors (RARs), peroxisome proliferator-activated receptors (PPARs), vitamin D receptors (VDRs), ecdysone receptors (EcRs) and numerous related orphan receptors (e.g. RORs)
- Subfamily NR2 - retinoid X receptors (RXRs), chicken ovalbumin upstream promoter-transcription factors (COUP-TFs), hepatocyte nuclear factor four (HNF4), etc.
- Subfamily NR3 - steroid receptors
- Subfamily NR4 - nerve growth factor (NGF) orphan receptors
- Subfamily NR5 - *ftz* (*fushi tarazu*) transcription factor 1 (FTZ-F1) orphan receptors
- Subfamily NR6 – the single GCNF1 orphan receptor

(Laudet, 1997; Robinson-Rechavi *et al.*, 2003).

### 3.2.5. Mechanism of binding

Nuclear receptors bind to target genes to activate or repress their expression. The binding specificity between nuclear receptors (NRs) and their target genes is based on two structural interactions (Van Gilst *et al.*, 2002). Primarily, P-box recognizes a specific six base pairs DNA fragment (a core site) in the promoter of the target gene. Majority of NRs has CxGCKGFFxR P-box motif recognizing an AGGTCA core site (Umesono and Evans, 1989; Giguere, 1999). The receptors for mineralocorticoids, glucocorticoids, progesterone and androgens recognizes AGAACA core site (Beato *et al.*, 1995). The core site itself is not sufficient to provide specific NR – target gene binding (Glass, 1994), a secondary interactions between NRs and target genes include recognition of more expanded DNA region in the promoter of target gene (HRE). Hormone response elements allow NRs with the same P-box specifically identified their target genes (Umesono and Evans, 1989).

NRs could be divided into three groups based on their secondary interactions. They bind as monomers or could form homodimers or heterodimers (Mangelsdorf *et al.*, 1995; Glass, 1994). NRs binding as monomers recognize a single core site. NRs binding as homodimers or heterodimers (usually in complex with RXR) recognize HREs composed of two core sites. The specificity of binding is ensured by the spacing and orientation of these two core sites (Glass, 1994; Giguere, 1999). Core sites in HREs for homodimers and heterodimers could be oriented as direct repeats (DR), inverted repeats (IR) or everted repeats (ER) (Mangelsdorf and Evans, 1995).

### 3.2.6. Coactivators and corepressors

Many proteins were described to influence the transcriptional activity of genes regulated by nuclear hormones receptors. NHRs could repress or enhance transcription by recruiting of coregulatory factors (corepressors or coactivators) to the transcription complex (Robyr *et al.*, 2000; Horwitz *et al.*, 1996).

Coactivators are expected to interact with the activation function domain of a NR and enhance the receptor activation function. They should interact with components of basal transcriptional machinery but should not be able to activate the transcription by their own (Robyr *et al.*, 2000). To date, the best known coactivator is steroid receptor coactivator-1 (SRC-1). Also CBP/p300 (CREB - binding protein) or P/CAF (p300/CBP – associated factor) are well-studied coactivators (Ding *et al.*, 1998; Kamei *et al.*, 1996). Interestingly, many of coactivators have histone acetyltransferase activity resulting in chromatin decondensation (Yang *et al.*, 1996; Robinson –Rechavi *et al.*, 2003)

Corepressors interact directly with the unliganded receptor. This binding leads to enhancement of transcription repression. Corepressors have an autonomous repression domain and should interact with components of basal transcriptional machinery (Robyr *et al.*, 2000). Most noted nuclear hormone corepressors are NCoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptors) (Horwitz *et al.*, 1996). These two corepressors are known to recruit a histone deacetylases (HDACs) through bridging molecules (Robyr *et al.*, 2000).

### 3.2.7. NHRs in *C. elegans*

NHRs have undergone an explosive expansion and diversification in *C. elegans* (Sluder *et al.*, 1999; Sluder and Maina, 2001). To date, the genome sequence of *C. elegans* revealed 284 predicted nuclear receptor genes. It is unusually large number contrary to human (48 NHRs) or *Drosophila* (18 NHRs) (Robinson-Rechavi *et al.*, 2001; Adams *et al.*, 2000; Maglich *et al.*, 2001; King-Jones and Thummel, 2005). Such diversity appears to be a characteristic of nematode and could be a mechanism by which nematodes adapt to survive in the environment which is the source of a great diversity of potential ligands (Sluder and Maina, 2001; Enmark and Gustafsson, 2001).

Only 15 of the *C. elegans* NHR genes have orthologs in insects and vertebrates. The remaining nuclear hormone receptors in *C. elegans* appear to originate from extensive duplications and diversifications of an ancestral gene encoding a protein related to vertebrate orphan receptor HNF4. These 269 nuclear receptors are called supplementary nuclear receptors (supnrs) and form a group of nematode specific NHRs in the subfamily NR2 (Robinson-Rechavi *et al.*, 2005).

Only several of 284 *C. elegans* NHRs have known function. They play important roles in the regulation of development and metabolism. *nhr-6*, *nhr-67*, and *nhr-85* are important for nematode reproduction (Gissendanner *et al.*, 2004), *nhr-23* and *nhr-25* function among other in epidermal differentiation (Kostrouchova *et al.*, 1998; Kostrouchova *et al.*, 2001; Asahina *et al.*, 2000; Silhankova *et al.*, 2005; Gissendanner and Sluder, 2000), *nhr-8* is required for xenobiotic resistance (Lindblom *et al.*, 2001) etc.

### 3.2.8. HNF4 and ACBP

Hepatocyte nuclear factor 4 (HNF4), a conserved member of a NR2 nuclear hormone receptor subfamily, is a transcription factor important for development and liver specific gene expression in vertebrates (Jiang *et al.*, 1995; Sladek, 1993; Li *et al.*, 2000). HNF4 is an orphan receptor and activates among other genes involved in cholesterol, fatty acid, and glucose metabolism and in liver differentiation (Sladek, 1993). HNF4 resides primarily in nuclei of expressing cells and binds DNA as

homodimer (Jiang *et al.*, 1995; Jiang *et al.*, 1997a). HNF4 subnuclear localization correlates with the HNF4 ability to transactivate its target genes. A proper intranuclear localization of HNF4 is dependent on tyrosine and serine/threonine phosphorylation (Ktistaki *et al.*, 1995; Jiang *et al.*, 1997b).

Acyl-coenzyme A binding proteins (ACBP) bind long chain acyl-CoA esters. Long chain acyl-CoA esters are known to be intermediates in lipid synthesis and function in the gene regulation (Knudsen *et al.*, 2000). Acyl-coenzyme A binding proteins were isolated from many eukaryotic species including yeasts. The proteins have highly conserved amino acid sequences with 83-103 residues. ACBP in yeasts was shown to be a housekeeping gene (Knudsen *et al.*, 1999; Kragelund *et al.*, 1999). ACBP was expected to be cytosolic but detailed analysis of ACBP distribution in rat hepatic and hepatoma cells localized ACBP also into the nucleus (Elholm *et al.*, 2000).

The function of ACBP in the nucleus remains unknown except its interaction with hepatocyte nuclear factor 4α. ACBP and HNF4α were shown to interact physically and functionally in the nucleus of rat hepatoma cells (Petrescu *et al.*, 2003).

*C. elegans* genome contains four ACBP orthologs called ACBP-1, ACBP-2, ACBP-3, and ACBP-4. ACBP-1 is the most conserved ACBP gene and is highly homologous to mammalian ACBP.

# 4. Materials and methods

## 4.1. Materials

### 4.1.1. *C. elegans* strains

**N2** – wild type animals (var. Bristol)

**JR667** – strain expressing seam cell GFP marker

**SU93** - strain expressing *ajm-1*::GFP marker , pRF4

(*ajm-1* encodes a member of the apical junction molecule)

**PD7963** – strain expressing *hlh-1*::GFP marker, pRF4

(*hlh-1* encodes a basic helix-loop-helix (bHLH) transcription factor expressed in body wall muscle cells and their precursors)

**#4991** (*chr3::gfp* (*nhr-23::gfp*)) – strain expressing *chr-3*::GFP fusion protein

**N2, JR667** and **SU93** strains were kindly afforded by the CGC (Caenorhabditis Genetics Center, University of Minnesota, Minneapolis, MN, USA), **PD7963 and #4991** strains were obtained as a kind gift from Dr. Michael Krause (NIH, Bethesda MD, USA).

Wild type ***C. briggsae*** (related nematode species) worms were kindly afforded by the CGC as well.

### 4.1.2. *E. coli* strains

**OP50** – used as a natural food for *C. elegans* and *C. briggsae*

**HT115** - carry an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 RNA polymerase gene contained within a stable insertion of a modified lambda prophage λ DE3. The strain is deficient for RNase III, an enzyme that normally degrades a majority of dsRNAs in bacterial cells (Timmons *et al.*, 2000)

**DH5α** - competent cells for transformation

**TOP10** – competent cells for transformation

### 4.1.3. Vectors

**pRF4** – vector containing the *rol-6(su1006)* mutant collagen gene. Its expression causes exhibition of a helically twisted cuticle and body (Mello *et al.*, 1991)

**L4440 (pPD129.36)** – vector containing two convergent T7 polymerase promoters in opposite orientation separated by a multicloning site

**pCR®4-TOPO® Cloning Vector** (Invitrogen) – vector for the direct insertion of *Taq* polymerase-amplified PCR (polymerase chain reaction) products (using covalently bound topoisomerase I) containing T3 and T7 polymerase promoters

**pPD95.67** – promoterless gfp vector with a nucleolar localization signal

**pPD95.75** – promoterless gfp vector without a nucleolar localization signal

**pPD49.83** – ectopic expression vector with the hsp16-41 promoter sequence

Vectors **L4440**, **pPD95.67**, **pPD95.75** and **pPD49.83** were a kind gift from Dr. Andrew Fire (Stanford University School of Medicine, Stanford CA, USA).

### 4.1.4. Solutions and media

| | |
|---|---|
| **DNA lysis buffer:** | 0.1 M Tris pH 8.3, 50mM EDTA, 200 mM NaCl |
| **Worm lysis buffer:** | 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 0.45% Tween 20, 0.45% NP-40, 0.01% gelatin, 200 µg/ml Proteinase K |
| **RNA lysis buffer:** | 10 mM Tris-HCl pH 7.5, 10 mM EDTA, 5% 2-mercaptoethanol, 0.5% SDS |
| **Lysis buffer:** | 50 mM Tris pH 8, 300 mM NaCl, 10 mM $MgCl_2$, 0.4% NP-40 |
| **Dilution buffer:** | 50 mM Tris pH 8, 0.4% NP-40, 2.5 µM $CaCl_2$, 1 µl of DNase I |

**Wash buffer:** 50 mM Tris pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 0.4% NP-40

**1x TTBS:** 100 mM Tris pH 7.5, 0.9% NaCl, 0.1% Tween 20

**10x TBE:** 60.5 g of Trisbase, 30.85 g of $H_3BO_3$, 3.72 g of EDTA 2Na $2H_2O$, $H_2O$ to 1l, autoclave

**10x PBS:** 80 g of NaCl, 2 g of KCl, 14.4 g of $Na_2HPO_4$, 2.4 g of $KH_2PO_4$, $H_2O$ to 1l, pH 7.4, autoclave

**LB medium:** 10 g of Trypton, 5 g of Yeast Extract, 10 g of NaCl, 1 ml of 1 M NaOH, $H_2O$ to 1l, autoclave

**LB agar:** 10 g of Trypton, 5 g of Yeast Extract, 10 g of NaCl, 1 ml of 1 M NaOH, 15 g of Bacto Agar, $H_2O$ to 1l, autoclave

**NGM:** 17 g of Bacto Agar, 2.5 g of Bacto Pepton, 3 g of NaCl, 1 ml of Cholesterol (5 mg/ml in Ethanol), $H_2O$ to 1l, autoclave, 25 ml of 1 M $KH_2PO_4$, 1 ml of 1 M $CaCl_2$, 1 ml of 1 M $MgSO_4$ add after autoclaving

**M9 medium:** 6 g of $Na_2HPO_4$, 3 g of $KH_2PO_4$, 5 g of NaCl, 0.5 g of $MgSO_4$, $H_2O$ to 1l, autoclave

## 4.2. Methods

### 4.2.1. Molecular biology methods

#### 4.2.1.1. gDNA isolation

Animals (mixed stages) grown on 2% agarose plates (common NGM plates covered with 2% agarose layer with OP50 on it) were washed with water and frozen at –80°C. DNA lysis buffer was added to thaw worms to 5 ml. 0.5 ml of 10% SDS and 250 µl of Proteinase K (20 mg/ml) (Q-BIO gene, MP Biomedicals, Irvine, CA, USA) were added. Worms were vortexed and heated for 30 min at 65°C. Phenol-chloroform extractions were performed two times (5 ml of phenol was added, a whole mixture was rocked for 30 min and then spun for 5 min at 5,000 RPM. The upper phase was transferred to a fresh tube and the same procedure with chloroform followed.). Ethanol precipitation followed: 3 M NaOAc (0.1 volume of transferred solution) and cold 96% ethanol (2 volumes) were added. gDNA came out of solution after gentle mixing. gDNA wound on a glass pipette was transferred to a fresh tube, air-dried and resuspended in 3 ml of water. RNase A (ICN, MP Biomedicals, Irvine, CA, USA) was added (to the final concentration 10 µg/ml) and incubated for 30 min at 37°C. Next phenol-chloroform extraction and ethanol precipitation followed. The gDNA pellet was air-dried and resuspended in water.

#### 4.2.1.2. Total RNA isolation

Animals (mixed or individual stages), grown on 2% agarose plates, were washed with water and frozen at –80°C. The frozen pellet was resuspended in 0.5 ml of RNA lysis buffer with 12.5 µl of Proteinase K (20 mg/ml). After 60 s vortexing, the mixture was incubated for 60 min at 55°C. Phenol-chloroform extraction followed. In experiments that yielded too viscous upper layer, extra water and phenol were added. For 500 µl of sample, 1 µl of glycogen (20 mg/ml), 50 µl of 10% NaOAc and 1.25 ml of cold ethanol were added. The mixture was incubated for 30 min on dry ice and spun for 10 min at 4°C. The air-dried pellet was dissolved in water and treated with 1 unit of DNase I (Promega, Madison, WI, USA) per 1 µg of total RNA for 30

min at 37°C. Phenol-chloroform extraction and ethanol precipitation followed and RNA was resuspended in water.

#### 4.2.1.3. RT-PCR

cDNA was prepared by reverse transcription (Superscript II kit, Invitrogen, Carlsbad, CA, USA) of previously isolated DNA-free RNA. 1 µg of total RNA was mixed with 100 ng of random hexamers and heated for 5 min at 65°C. Then 4 µl of first strand buffer, 1 µl of RNase Inhibitor (Bioline USA Inc, Randolph, MA, USA), 1 µl of 100 mM DTT, 1 µl of dNTP's and 1 µl of Superscript II were added. The mixture was incubated for 50 min at 42°C, 15 min at 70°C and cooled down. First strand cDNA served as a template for subsequent PCRs with specific primers.

#### 4.2.1.4. PCR

PCR reactions were performed according to a standard protocol with specific primers and templates (N2 gDNA, N2 cDNA or *C. briggsae* cDNA and gDNA) using BIO-X-ACT Short DNA polymerase (Bioline) for short fragments up to 1,500 bp, BIO TAQ DNA polymerase (Bioline) and Taq DNA polymerase (Invitrogen). For all PCR reactions PTC-100™ and PTC-200 ™ Peltier thermal cyclers (MJ Research Inc., Waltham, MA, USA) were used.

#### 4.2.1.5. Single worm PCR (to confirm presence of the construct in the animal)

A single worm was transferred to a 5 µl drop of worm lysis buffer in a PCR tube and frozen for 10 min at –80°C. Then the tube was heated 60 min at 60°C, 15 min at 95°C and finally kept at 4°C. 2.5 µl of prepared single worm DNA mixture served as a template for standard PCR reaction to amplify specific DNA fragment contained in the construct.

#### 4.2.1.6. Real-time PCR (Q-PCR)

Quantitative PCR was performed as described by Sun (Sun *et al.*, 2004) with modifications. Real-time PCR was performed in PTC0200 DNA EngineR Thermal

Cycler equipped with ALS 0296 96-well Sample Block (MJ Research) using DyNAmoTM HS SYBR Green qPCR Kit (Finnzymes, Espoo, Finland). The amplicons of *nhr-60* (primers #6165 and #4566), *ama-1*(primers #5169 and # 5170) and *act-1* (primers #5293 and #5294) selected regions were amplified using RT-PCR from 5 µg of total *C. elegans* RNA (from individual developmental stages). The amplified fragments for determination of calibration curves were purified from the agarose gel. The amount of DNA was determined spectrophotometrically. Each sample was analyzed in dublet and at least two times. The results obtained for *nhr-60* were normalized against RNA polymerase II large subunit (*ama-1*) or against actin (*act-1*) using Opticon MonitorTM Version 3.0 software.

#### 4.2.1.7. Cloning

**Using T4 DNA ligase:** PCR amplified DNA fragments and appropriate vectors were digested with specific restriction enzymes. After purification, the DNA fragments were ligated into the vectors by T4 DNA ligase (Invitrogen, Fermentas) for 1 hour at room temperature or overnight at 16°C.

**TOPO TA Cloning:** PCR amplified fragments (synthesized with Taq DNA polymerase) were cloned into the pCR®4-TOPO vector using TOPO TA Cloning®Kit for Sequencing (Invitrogen).

#### 4.2.1.8. Constructs

Primers used for PCR amplification of DNA fragments of our interest, the sizes of amplified fragments, used DNA templates and vectors are mentioned in this section. The primer sequences and applied restriction sites are shown in the list of primers (Appendix).

**4.2.1.8.1. Constructs for *nhr-60*::GFP expression** (transcriptional fusion)

*nhr-60*::GFP A - primers #4535 (sense) and #4536 (antisense), N2 gDNA template 2,160 bp DNA fragment (1,950 bp of *nhr-60* upstream region) cloned into pPD95.67

*nhr-60*::GFP B - primers #6046 (sense) and #4536 (antisense), N2 gDNA template
790 bp DNA fragment (580 bp of *nhr-60* upstream region) cloned into pPD95.67

*nhr-60*::GFP C - primers #6063 (sense) and #4536 (antisense), N2 gDNA template
550 bp DNA fragment (340 bp of *nhr-60* upstream region) cloned into pPD95.67

**4.2.1.8.2. Constructs for ACBP-1::GFP expression** (translational fusion)

ACBP-1::GFP S - primers #7016 (sense) and #7014 (antisense), N2 gDNA template
1,050 bp DNA fragment (530 bp of *acbp-1* upstream region) cloned into pPD95.75

ACBP-1::GFP L - primers #7013 (sense) and #7014 (antisense), N2 gDNA template
1,700 bp DNA fragment (1,180 bp of *acbp-1* upstream region) cloned into pPD95.75

**4.2.1.8.3. Constructs for in vitro transcriptions**

*nhr-60* in L4440 - primers #4520 (sense) and #4521 (antisense), yk470e6 EST clone template
690 bp DNA fragment cloned into L4440

*nhr-116* in L4440 - primers #6051 (sense) and #6052 (antisense), cDNA mix template
520 bp DNA fragment cloned into L4440

*nhr-60 unrelated* in L4440 - primers #6045 (sense) and #6060 (antisense), gDNA template
760 bp DNA fragment cloned into L4440

*acbp-1* in pCR®4-TOPO – primers #5169 (sense) and #5170 (antisense), cDNA mix template
260 bp DNA fragment cloned into pCR®4-TOPO vector

*acbp-2* in pCR®4-TOPO - primers #7000 (sense) and #7001 (antisense), cDNA mix template
560 bp DNA fragment cloned into pCR®4-TOPO vector

*acbp-3* in pCR®4-TOPO - primers #7002 (sense) and #7003 (antisense), cDNA mix template

170 bp DNA fragment cloned into pCR®4-TOPO vector

*acbp-4* in pCR®4-TOPO - primers #7004 (sense) and #7005 (antisense), cDNA mix template

270 bp DNA fragment cloned into pCR®4-TOPO vector

#### 4.2.1.8.4. Constructs with a heat-shock regulated promoter

*nhr-60/all cDNA* - primers #6014 (sense) and #6015 (antisense), yk470e6 EST clone template

1,410 bp DNA fragment cloned into pPD49.83

*nhr-60/AF2-* - primers #6014 (sense) and #6016 (antisense), yk470e6 EST clone template

1,290 bp DNA fragment cloned into pPD49.83

### 4.2.1.9. Transformation

Ligation or TOPO TA Cloning was followed by transformation into suitable competent cells. The ligation (TOPO TA Cloning) mixture was incubated with competent cells for 10 min on wet ice. Heat-shock was done at 41°C for 1 min. LB medium was added and the culture was incubated shaking at 37°C for 45 min. The culture was spread on LB agar plates with Ampicillin (ICN, MP Biomedicals, Irvine, CA, USA) (100 μg/ml) or Kanamycin (50 μg/ml) (Serva, Heidelberg, Germany) and grown overnight at 37°C. The construct was released from bacteria using JETQUICK plasmid Miniprep Spin Kit (GENOMED, Lőhne, Germany) or UNIQ-10 Spin Column Plasmid DNA Minipreps Kit (Bio Basic Inc., Ontario, Canada). The cloning of DNA fragments into the vectors was confirmed using a control digestion with appropriate restriction enzymes with nucleic acid electrophoresis and sequencing.

Sequencing of constructs and PCR products was provided on ABI Prism 3100-*Avant* sequencer (Applied Biosystems, Foster City, CA, USA) kindly performed by Helena Myšková (Institute of Inherited Metabolic Disorders, Prague).

#### 4.2.1.10. In vitro transcription

Constructs for in vitro transcriptions were prepared by PCR amplification of *C. elegans* cDNA with appropriate primers and subsequent ligation into L4440 vector or TOPO TA cloning into pCR®4-TOPO vector. Prior to in vitro transcription, the construct was linearised at the ends of inserted DNA using unique restriction sites. 500 ng of each linearised construct was mixed with 20 µl of rNTP´s, 10 µl of 5x transcription reaction buffer, 1 µl of 100 mM DTT, 1 µl of RNase Inhibitor, 4 µl of T7 polymerase (Promega) (2.5 µl for the first hour, next 1.5 µl for the second hour) and water up to final volume 50 µl. Both mixtures (for sense and antisense ssRNAs) were incubated at 37°C for 2 hours. Equal amounts of both ssRNAs (confirmed by electrophoresis) were mixed and incubated for 10 min at 70°C and for 30 min at room temperature to anneal. dsRNA was purified by phenol-chloroform extraction and ethanol precipitation.

### 4.2.2. Maintenance of *C. elegans*

#### 4.2.2.1. Worm cultivation

*C. elegans* are maintained in the laboratory on nematode growth medium (NGM) agar, which has been aseptically poured in Petri dishes (Brenner, 1974). NGM agar plates are seeded with OP50 *E. coli* lawn. *C. elegans* are visualized using a dissecting microscope Olympus SD30 (Olympus, Tokyo, Japan). Worms could be transferred on a new plate using a chunk of agar from an old plate. Worms could be also picked individually using a platinum wire with a hook at the end, which could be easily sterilized in a flame.

#### 4.2.2.2. Worm culture synchronization

*C. elegans* plates with gravid hermaphrodites were washed with water. Worms were collected in 12 ml centrifuge tubes and washed with water several times. 2 ml of 5 M NaOH and 1 ml of bleach (SAVO) (Bochemie, Bohumín, Czech Republic) were added to 7 ml of water with worms. The mixture was shaken well for 6 min until worms disappeared and only embryos were visible. Then the solution was divided into two tubes, water was added. Centrifugation (1,000 rpm for 5 min) followed.

Supernatants were discarded and new water added. Centrifugation (1,000 rpm for 5 min) followed. Wash (with water) was repeated six times. The pellet of released embryos was diluted in 10 ml of M9 and incubated overnight at room temperature, shaking. Next day, the worm culture was synchronized in L1 stage.

#### 4.2.2.3. Preparation of transgenic *C. elegans* strains

The plasmid DNA of construct at the concentration 50 ng/µl was co-injected with pRF4 plasmid (50 ng/µl) into the ovarial syncytium of young adult N2 hermaphrodites (Olympus Inverted System Microscope IX70 equipped with Narishige Micromanipulator (Olympus, Tokyo, Japan), kindly performed by Hana Prouzová (Prague, Institute of Inherited Metabolic Disorders)). Progeny of microinjected animals was screened to pick worms rolling due to expression of pRF4. The presence of transgenic construct in rolling animals was confirmed using single worm PCR.

Two *C. elegans* transgenic strains were prepared for each of all *nhr-60*::GFP and ACBP-1::GFP fusion constructs. Three *C. elegans* transgenic strains expressing *nhr-60/all cDNA* and *nhr-60/AF2-* were prepared.

#### 4.2.2.4. Determination of GFP expression

Transgenic worms carrying specific GFP-transcriptional or GFP-translational constructs were observed using Olympus SZX12 Stereomicroscope System or Olympus BX60 System Microscope both equipped with a light fluorescence attachment. BX60 Microscope is equipped also with Olympus DP30BW camera allowing taking pictures of observed objects. SZX12 microscope allows observing worms directly on NGM plates. To observe worms using BX60 microscope worms must be put into drop of water on a 2% agarose pad on the slide.

#### 4.2.2.5. RNA interference

##### 4.2.2.5.1. Soaking

5-10 wild type worms of individual developmental stages were placed into the drop of in vitro prepared dsRNA (c = 1-2 μg/μl) in an eppendorf tube overnight at room temperature. Worms were placed on ordinary NGM plates with OP50 and followed for phenotypical or behavioral changes throughout their development.

##### 4.2.2.5.2. Feeding

Preparation of a culture for feeding: An appropriate construct was transformed into HT115 *E. coli* strain. The culture was grown overnight at 37°C in LB medium with Ampicillin (100 μg/ml) to OD (595) = 0.4. Production of specific dsRNA from the construct in the bacterial culture was induced by IPTG (ICN, MP Biomedicals, Irvine, CA, USA) (0.4 mM) and subsequent cultivation at 37°C for 4 hours.

Preparation of feeding plates: common NGM plates with Ampicillin (100 μg/ml) and IPTG (0.4 mM) were inoculated with culture for feeding.

L1 synchronized wild type worms were placed on feeding plates and followed for phenotypical or behavioral changes during development.

##### 4.2.2.5.3. Microinjections

dsRNA (c = 1-2 μg/μl) was microinjected into ovarial syncytium of young adult hermaphrodites (N2 worms or specific GFP expressing strains). Worms were placed on NGM plates and their progeny was followed to detect phenotypical and behavioral changes.

#### 4.2.2.6. Heat-shock experiments

Worm cultures (carrying construct with heat-shock regulated promoter) on Petri dishes were incubated at 31°C for 2 hours or at 34°C for 30 min. The incubation

at 6°C (for 30 min) followed. Finally, worms and their progeny were grown under standard conditions and followed for phenotypical or behavioral changes.

#### 4.2.2.7. NHR-60 antibody preparation

15 aa long polypeptide (PKDLVMRVIEDIMDS) was designed from the C-terminus of NHR-60. The synthesized polypeptide (Invitrogen) was used to immunize two rabbits. Polyclonal antibodies #4529 and #4530 from both rabbits from bleeds at 4, 8 and 10 weeks were made. Their specificity was confirmed by ELISA using synthetic peptide and by Western blots. Antibody #4529 (bleed at 8 weeks) was used in all experiments.

#### 4.2.2.8. Immunocytochemistry

Embryos, larvae or adult animals were washed several times with water and placed on poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA) coated slides. 10 µl of sediment containing animals were placed on the slide together with 10 µl of 5% paraformaldehyde diluted with 1x PBS, covered with cover glass and incubated in a wet chamber for 10 min at room temperature and then frozen for 10 min on an aluminum block that had been chilled on dry ice. After that, cover glasses were cracked out promptly (so-called freeze crack). Fixation was preformed in two ways:

Methanol fixation and methanol/TTBS rehydratation: Slides after freeze crack were placed into cold methanol (-20°C) for 10 min and re-hydrated for 10 min each in series of re-hydration buffers methanol/TTBS in ratio 9:1, 7:3, 1:1 and 1:4.

Methanol/acetone fixation: Slides after freeze crack were placed in cold methanol (-20°C) and then in cold acetone (-20°C) each for 10 min.

After fixation, the primary antibody #4529 was used in dilution 1:300 (with 1x TTBS). Slides were kept in a wet chamber at 4°C over night. Next day, the slides were washed 3 times in 1x TTBS and a secondary goat anti rabbit IgG antibody conjugated to Alexa Fluor 568 (Molecular Probes, Eugene, OR, USA) in dilution 1:400 was used. Slides were incubated at room temperature for 2 hours, washed 3x in

1x TTBS, and 5 µl of DAPI (diamidinophenyl indole, 1 µg/ml) was added and mounted with 10 µl of mounting medium (Shandon, Pittsburgh, Pa, USA). Fluorescent labeling of detected proteins was observed using Olympus BX60 System Microscope equipped with the light fluorescence attachment or Nicon Eclypse E800 microscope equipped with a C1 confocal head and 543 nm laser line (Nicon, Spectra-Physics Lasers).

#### 4.2.2.9. Western blot analysis

Worms from mixed and individual stages were washed in water and spin at 1,000 rpm and frozen at -80°C in Eppendorf tubes. After thawing, the equal amount of Tris-Glycine SDS Sample Buffer (2x) (Invitrogen) was added. The samples were boiled for 5 min and chilled. The protein concentration from supernatant was estimated using BCA kit (Pierce, Rockford, IL, USA). 1 µl of 2-mercaptoethanol was added to the sample before gel loading. For standard Western blot analysis 60 µg of protein samples were separated by 4%/12% SDS PAGE. Proteins were blotted onto nitrocellulose membrane. Membrane was incubated overnight at 4°C in T-PBS-M (phosphate buffered saline (1x PBS) pH 7.4 with 0.1% (v/v) Tween 20 and 5% low-fat milk powder (w/v)). Incubation in T-PBS-M with primary antibody followed for 1 hour at room temperature, rocking. The membrane was washed in T-PBS (phosphate buffered saline (1x PBS) pH 7.4 with 0.1% (v/v) Tween 20) for 10 min at room temperature six times, rocking. Incubation with secondary antibody coupled to horseradish peroxidase (Sigma, St. Louis, MO, USA) diluted in T-PBS followed. Membrane was washed in T-PBS for 10 min at room temperature four times, rocking. SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) was used for detection of peroxidase activity.

#### 4.2.2.10. Co-immuno precipitation

N2 and ACBP-1::GFP worms were grown and collected. Samples were frozen in 20 µl of $dH_2O$ at -80°C. 100 µl of Lysis buffer with added antiprotease (1 mini tablet per 10 ml, Roche, Mannheim, Germany) was added to melted samples and incubated for 30 min at 4°C (on wet ice). 5 ultrasound treatments (5x 10 s with bar inside the tube) were followed by centrifugation (16.000 rpm, 20 min, 4°C).

Subsequently 100 µl of Dilution buffer was added. Next, 40 µl of Protein G Plus/Protein A Agarose Suspension (Calbiochem (Merck), Darmstadt, Germany) was added and the lysate was incubated and rocked for 45 min at 4°C. After centrifugation (1,000 rpm, 1 min, 4°C), the supernatant was incubated with 2 µl of the primary anti NHR-60 IgG polyclonal antibody #4529 for 1 hour at 4°C, rocking. Then 20 µl of Protein G Plus/Protein A Agarose Suspension was added and the sample was incubated for 1 hour at 4°C, rocking. Three washes with 500 µl of Wash buffer followed. 1 µl of 2-mercaptoethanol and 5 µl of Tris-Glycine SDS Sample Buffer (2x) were added to the final agarose pellet. After 3 min of boiling, samples were prepared for WB analysis. ACBP-1::GFP was detected using the primary goat IgG anti-GFP antibody and the secondary anti-goat IgG antibody conjugated with horseradish peroxidase.

# 5. Results

## 5.1. Characterization of the *nhr-60* gene

Based on the comparison of NHR-60 amino acid sequence with other *C. elegans* NHRs, NHR-60 belongs to Class 1 of nuclear receptors. *nhr-60* is a member of subgroup of 18 *C. elegans* supnrs that have the P-box sequence CNGCKT (Fig. 7).

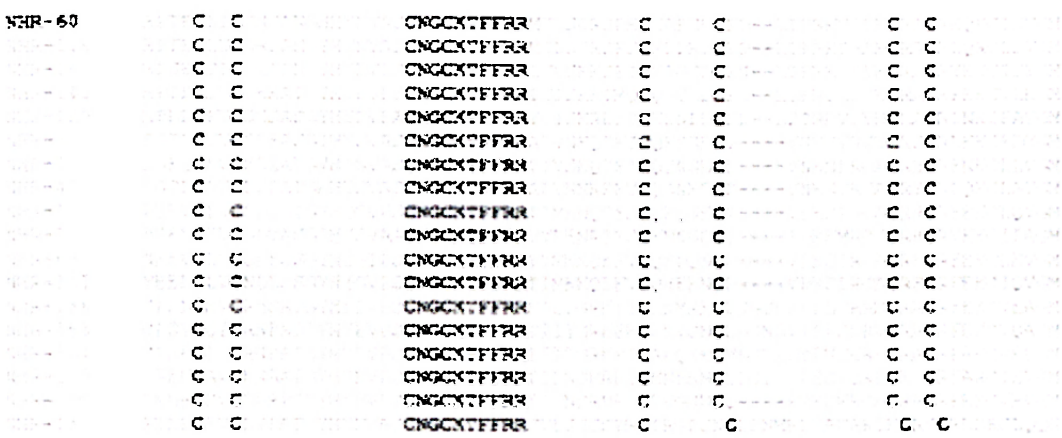


**Fig. 7: An alignment of DBD amino acid sequences of 18 *C. elegans* supnrs sharing the same P-box CNGCKT**

The P-box and the conserved cysteins are marked in bold.

The *nhr-60* gene is localized on chromosome V (cosmid F57A10.5) and consists of seven exons spanning approximately 2.7 kb (Fig. 8). The gene prediction is shown on WormBase web (www.wormbase.org, release WS150, Nov. 30, 2005).

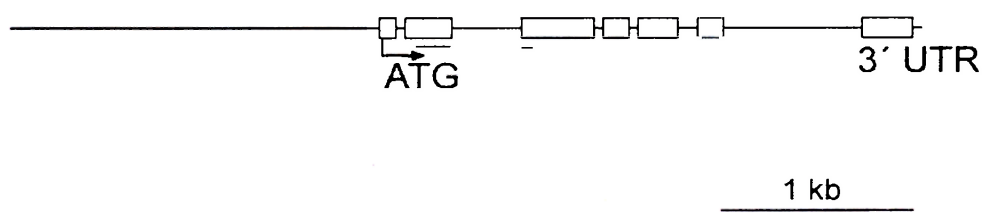


**Fig. 8: Schematic representation of *nhr-60* genomic organization**

*nhr-60* exons are marked as open rectangles. The DBD domain is underlined.

The 5' end of *nhr-60* cDNA was sequenced to determine if *nhr-60* is trans-spliced to SL1 or SL2 splice leader. RT-PCR from total RNA (mix of all developmental stages) with *nhr-60* specific antisense primer #4536 and sense primer SL1 or SL2 respectively was performed. Amplified DNA fragment from PCR with SL1 and #4536 primers was purified from the agarose gel and sequenced. PCR with SL2 and #4536 did not lead to amplification of any DNA fragment. The sequencing revealed that *nhr-60* is trans-spliced to SL1 several nucleotides (gtccat) upstream of the predicted start codon.

EST clone yk470e6 (obtained from Dr. Y. Kohara, National Institute of Genetics, Japan) or reversely transcribed total RNA from mixed stages served as templates for *nhr-60* full length amplification. Cloning and sequencing of PCR products (sense primer #6014, antisense primer #6015) confirmed the gene structure prediction with a single prominent splicing isoform.

### 5.2. *nhr-60* gene homolog in *Caenorhabditis briggsae*

After the search in *C. briggsae* database, a single *nhr-60* homolog (CBG22907) was identified. The predicted CBG22907 protein product (CBP05478) has 70% similarity and 62% identity to CeNHR-60 at the amino acid level. The predicted CBG22907 sequence does not contain start codon. Based on comparison of *nhr-60* and CBG22907 nucleotide sequences we suggested different CBG22907 first exon and designed primers (#5156 and #5157) for CBG22907 full length cDNA amplification. Total cDNA from *C. briggsae* was prepared and CBG22907 cDNA was amplified. Sequencing confirmed a miss prediction of CBG22907 sequence in the database. Based on our results, both *C. elegans* and *C. briggsae nhr-60* genes contain the same number of exons with conserved sizes (Fig. 9). We suggest naming CBG22907 as *Cbnhr-60*.

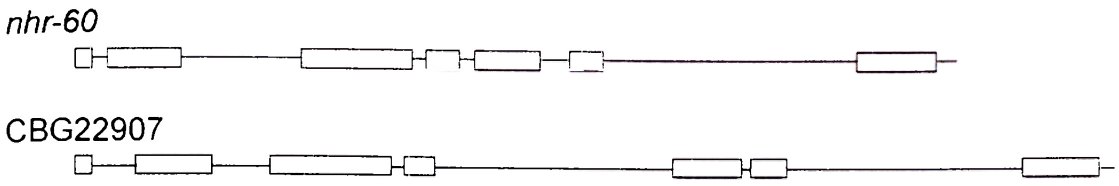


**Fig. 9: A comparison of genomic organization of *C. elegans nhr-60* and *C. briggsae* CBG22907**

Exons are marked as open rectangles. Note the conserved number and length of exons of *nhr-60* and CBG22907.

### 5.3. NHR-60 homologs in *Caenorhabditis elegans*

We performed P-BLAST searches in *C. elegans* database with NHR-60 full length amino acid sequence as well as with DBD and LBD only. The most similar to NHR-60 are *C. elegans* NHR-116 and NHR-129.

### 5.4. *nhr-60* expression levels

To determine *nhr-60* expression levels in *C. elegans* we employed two approaches – semiquantitative and quantitative PCRs. For both types of PCR reactions, we prepared two set of templates (cDNAs from total RNA from embryos, individual larval stages and adult animals).

For semiquantitative PCR, *nhr-60* primers #4520 and #4521 were used. PCR was designed to terminate during exponential phase (35 cycles). *ama-1* DNA fragments (primers #4684 and #4685) were co-amplified in the same reactions as internal controls. *ama-1* encodes the large subunit of RNA polymerase II, which is transcribed to the same level throughout all *C. elegans* development. The primers for *ama-1* amplification were added during the $5^{th}$ cycle to reach the exponential phase at the same time as *nhr-60*. The results revealed that *nhr-60* is expressed in all developmental stages and the expression decreases in L4 and adult stage (Fig. 10).

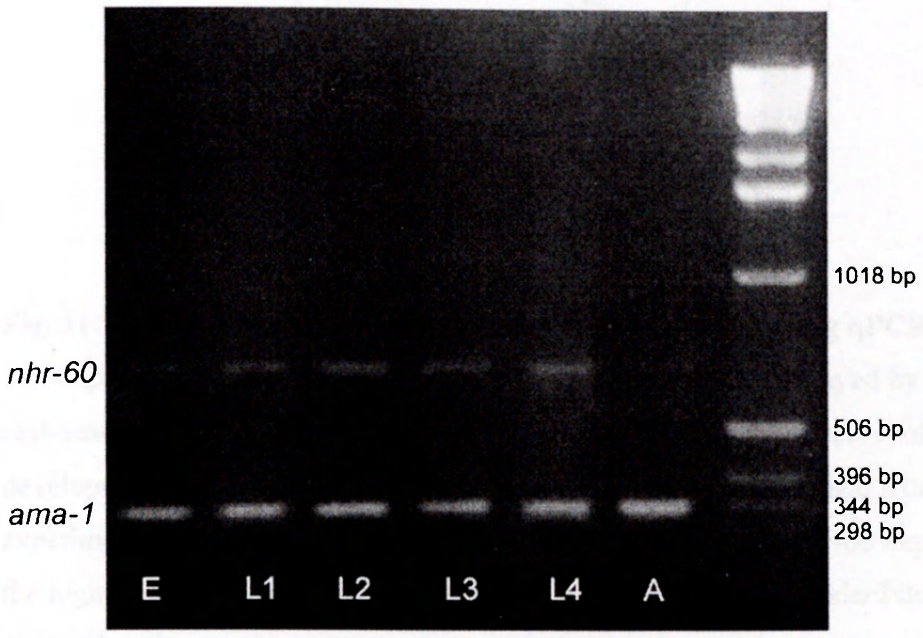


**Fig. 10: *nhr-60* expression profile using semiquantitative PCR with *nhr-60* and *ama-1* primers**

Quantitative real-time PCR (qPCR) was done with *nhr-60* primers #6165 and #4566. *nhr-60* expression levels during individual developmental stages were calculated from calibration curves. The obtained results were normalized to *ama-1* (primers #4684 and #4685) or actin, *act-1* (primers #5293 and #5294) respectively. Using qPCR, we confirmed that *nhr-60* is expressed in all *C. elegans* developmental stages. We found that nhr-60 expression is increased in L3 stage and then decreases in L4 and adult animals (Fig. 11).

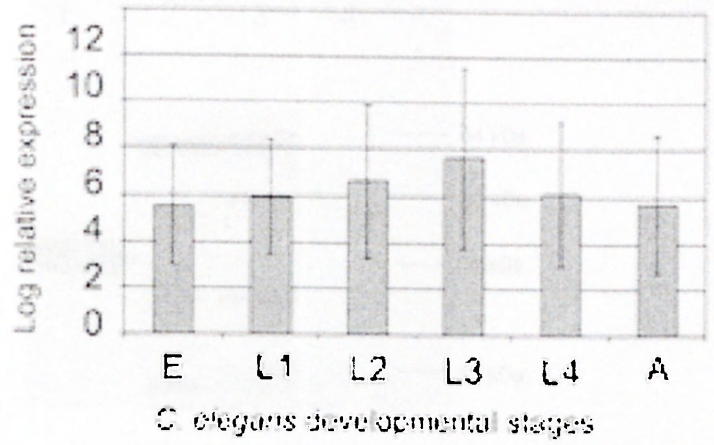


**Fig. 11: *nhr-60* expression during *C. elegans* development using qPCR**

The expression of *nhr-60* during *C. elegans* development was assayed by quantitative real-time PCR from cDNAs prepared from total RNA extracts of individual developmental stages. Relative levels of expression were determined from calibrated experiments normalized to expressions of *ama-1* and/or *act-1*. *nhr-60* expression was the highest in L3 stage and decreased in L4 and adult stages. Standard deviation bars are indicated.

### 5.5. NHR-60 distribution

NHR-60 specific polyclonal antibody (#4529) raised in rabbits was used for Western blot analysis of *C. elegans* protein extracts and for immunocytochemistry.

Western blot analysis of *C. elegans* protein extracts from mixed populations and from individual developmental stages of wild type N2 worms with #4529 antibody were performed. The analysis of protein extracts from mixed stages revealed the presence of two NHR-60 specific bands. The prominent band is migrating at 65 kDa and the minor band (detected only in some preparations) is migrating at 50 kDa (the size correlating with NHR-60 prediction). The minor band has up to approximately 10% of the intensity of the prominent band (Fig. 12). The 50 kDa NHR-60 protein was detected by Western blots in case when high quantities of protein lysates were used for the analysis (80 µg of protein extract per a lane). An additional band (migrating at 36 kDa) was detected in all Western blots with protein extracts containing not only worm lysate but also material from *E. coli* OP50. *C. elegans* are fed on OP50 and their guts are filled with the bacteria.

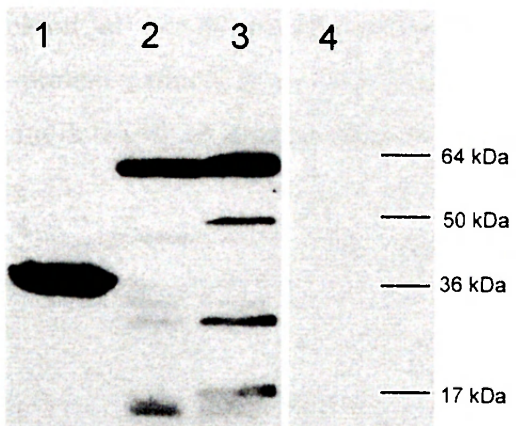


**Fig. 12: Analysis of wild type NHR-60 by Western blot**

Protein lysates from *E. coli* (lane 1) or *C. elegans* N2 worms (lanes 2 to 4) were analyzed by Western blot using rabbit polyclonal antibody #4529 against NHR-60 (lanes 1 to 3) or preimmune serum (lane 4). 40 µg of protein extract was loaded in lanes 1, 2, and 4 and 80 µg in the lane 3. Antibody detects a *C. elegans* protein migrating at 64 kDa (lanes 2 and 3). The antibody in bigger protein loading (lane 3) detected a protein migrating at expected size 50 kDa. A protein migrating at 36 kDa (lane 1) that is detected in some experiments by the #4529 antibody is a non-specific interaction with some protein from bacterial lysate (all worms are fed on bacteria).

Using western blot analysis for individual developmental stages we detected prominent 64 kDa band in embryos, all larval stages and adult animals as well (Fig. 13). The minor band was detected in stages when big quantities of lysates were used.

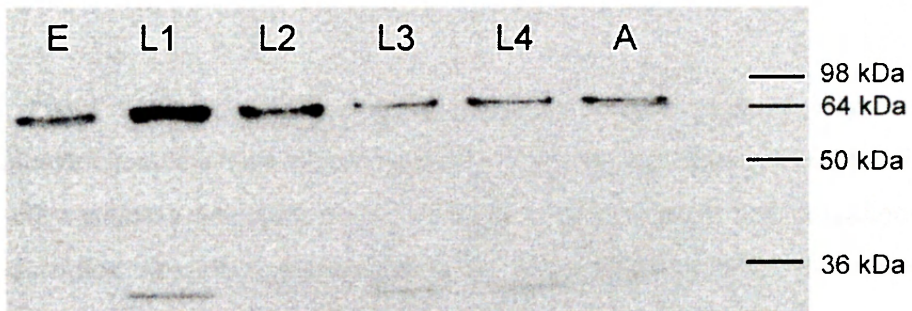


**Fig. 13: Detection of NHR-60 in *C. elegans* developmental stages using Western blot analysis**

Protein lysates from developmental stages of *C. elegans* were used for Western blot detection of NHR-60 using #4529 antibody against this protein. NHR-60 is expressed in all stages. The detection of 36 kDa protein is the consequence of contamination with bacteria.

Inhibition of *nhr-60* expression by RNAi and subsequent Western blot analysis of protein extracts from control and RNAi-treated animals revealed the increase of the intensity of the prominent band (64 kDa), the minor band was not detected (Fig. 14).

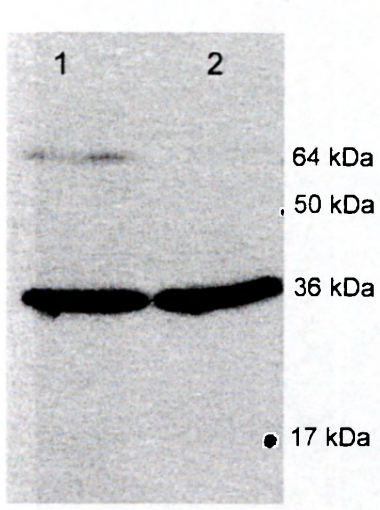


**Fig. 14: Western blot analysis of NHR-60 in wild type embryos and in embryos following *nhr-60* RNAi**

Protein lysates from wild type embryos (lane 1) and embryos subjected to *nhr-60* RNAi (lane 2) were analyzed by Western blot using #4529 antibody against NHR-60. *nhr-60* RNAi resulted in decreased detection of protein migrating at 64 kDa by the antibody. The 36 kDa protein detected in both lanes comes from contamination with bacteria.

Fixed wild type N2 embryos, larvae and adult animals were used for immunocytochemistry with #4529 antibody. NHR-60 was detected in all cell nuclei. NHR-60 was detectable from the 1-cell stage of development until adulthood. Such early detection strongly suggests a maternal contribution of NHR-60. Interestingly, immunostaining revealed prominent staining at the periphery of nuclei in all cells. Although ubiquitous throughout the development, *nhr-60* expression seems to be specifically increased in larval seam cells as well as in the germ line (Fig. 15).

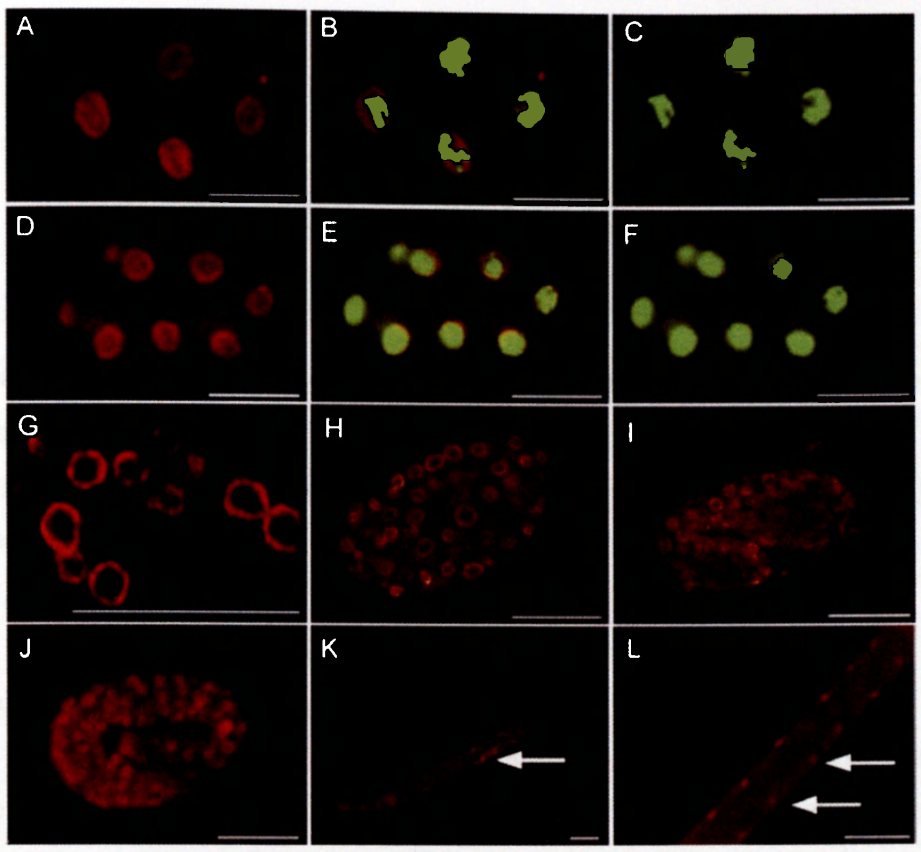


**Fig. 15: Detection of NHR-60 in embryos and larvae**

Rabbit polyclonal antibody against NHR-60 was used to detect the distribution of NHR-60 in embryos and larvae. NHR-60 is detected at 4 cell stage (A) and continues to be expressed in 8 cell embryo (D) in nuclei as indicated by DAPI staining (C and F) and the merged images (B and E). Note the prominent localization of NHR-60 at the nuclear periphery that becomes evident in confocal microscopy (G, H, and I) in 50-cell (G), comma (H), and 1.5-fold (I) stage embryos. Panel J shows a 3-fold embryo with ubiquitous expression, panel K an L1 larva with prominent staining in germ line nuclei (arrow), and panel L an L3 larva with prominent staining in seam cell nuclei (arrows). Scale bar: 20 µm

We used immunocytochemistry not only for staining of wild-type *C. elegans* but for worms with decreased (by RNAi) or increased (by overexpression) NHR-60 level as well. The NHR-60 antibody signal was either reduced to near background level after *nhr-60* RNAi or increased in transgenic animals overexpressing full length NHR-60 from the heat-shock driven promoter (Fig. 16).

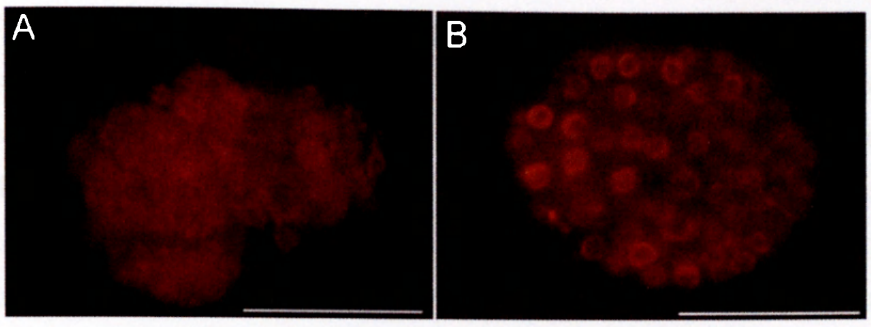


**Fig. 16: Detection of NHR-60 in embryos following NHR-60 inhibition or overexpression**

The NHR-60 antibody staining pattern is decreased after *nhr-60* RNAi (A) and increased after *nhr-60* overexpression (B). Scale bar: 20μm

### 5.6. *nhr-60*::GFP reporter genes expression

In order to determine *nhr-60* expression pattern, we prepared various transgenic lines expressing GFP from *nhr-60*::GFP transcriptional fusion constructs. *nhr-60*::GFP A, B, and C are extra-chromosomal arrays containing the putative promoter regions and limited coding sequence of the gene. The constructs (A, B, and C) included either 1,950 bp, 580 bp or 340 bp upstream of the predicted start of translation and genomic DNA up to the part of the second exon (Fig. 17).

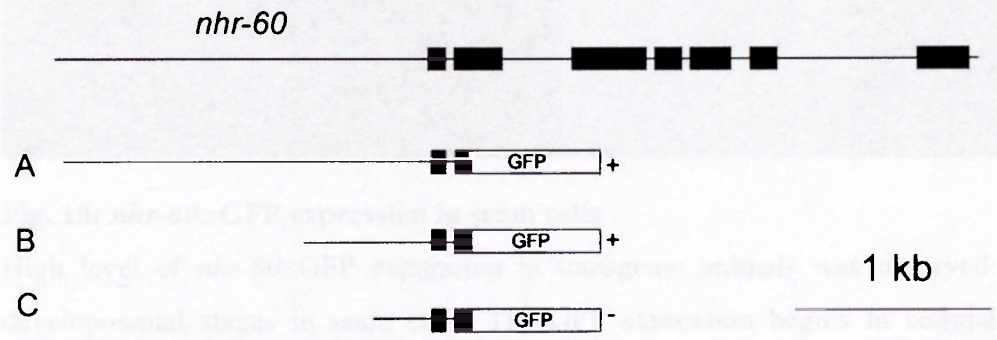


**Fig. 17: A schematic representation of *nhr-60* genomic organization and *nhr-60*::GFP transcriptional fusion constructs**

Three *nhr-60* putative promoter regions (1,950 bp in A, 580 bp in B, and 340 bp in C) and *nhr-60* gDNA up to the part of the 2nd exon were used for preparation of *nhr-60*::GFP fusion constructs. Constructs *nhr-60*::GFP A and B revealed GFP expression contrary to construct *nhr-60*::GFP C.

Constructs *nhr-60*::GFP A and B are expressed in many different cell types and reveal the same expression pattern. Construct *nhr-60*::GFP C did not show any expression although the presence of transgene was confirmed by single worm PCR. Such lack of GFP expression suggests that the region within 340 bp upstream of the translational start is not sufficient to promote *nhr-60* expression. The strongest expression of *nhr-60*::GFP was observed in seam cells, consistent with the antibody localization of NHR-60 protein.

The expression in seam cells started in embryonic precursors of seam cells at about 260 min after fertilization and continued in all larval stages. The level of GFP expression seemed constant during the whole larval development. The GFP signal in seam cells disappeared in adult animals after seam cells undergo homotypic cell fusion (Fig. 18).

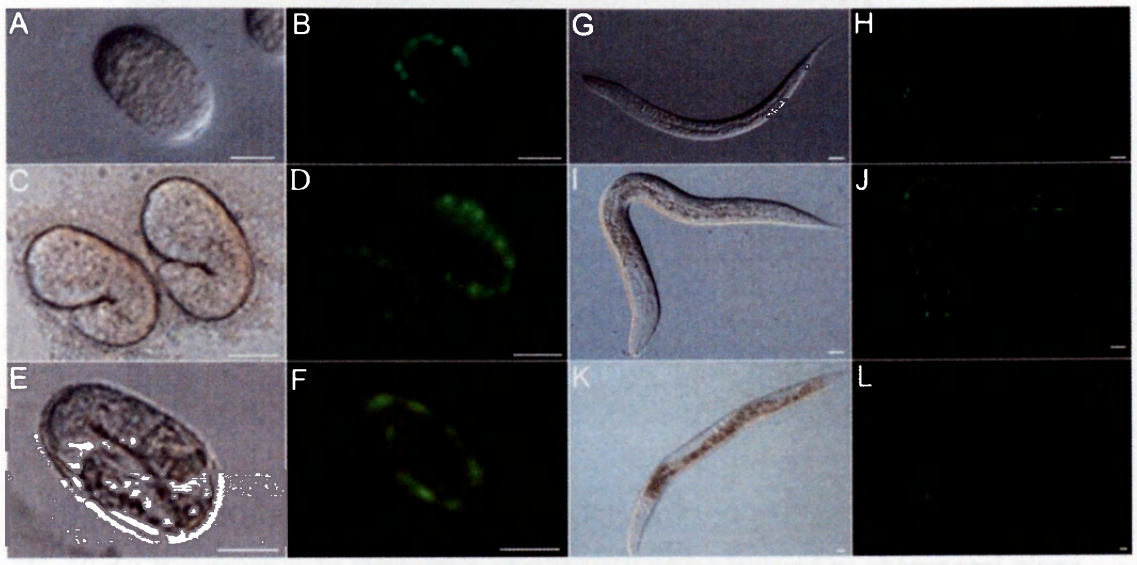


**Fig. 18: *nhr-60*::GFP expression in seam cells**

High level of *nhr-60*::GFP expression in transgenic animals was observed in all developmental stages in seam cells. The GFP expression begins in comma stage embryos (B) continues throughout the whole larval development (D, F, H, J, and L) until seams fuse. Seams are arranged as two rows of ten cells on both sides of embryo or newly hatched L1 larvae.

A, B: Nomarski and GFP images of comma stage embryo; C, D: Nomarski and GFP images of 1.5-fold embryo; E, F: Nomarski and GFP images of 3-fold embryo; G, H: Nomarski and GFP images of L1 larva; I, J: Nomarski and GFP images of L3 larva; K, L: Nomarski and GFP images of L4 larva. Scale bar: 20 μm

We observed strong GFP signal in all pharyngeal gland cells, VC4 and VC5 neurons, and in the hermaphrodite uterine vulval UV1 cells (4 nuclei). In males, both *nhr-60*::GFP expressing transgenes were expressed in ray cells in L4 and in the mature tail in addition (Fig. 19).

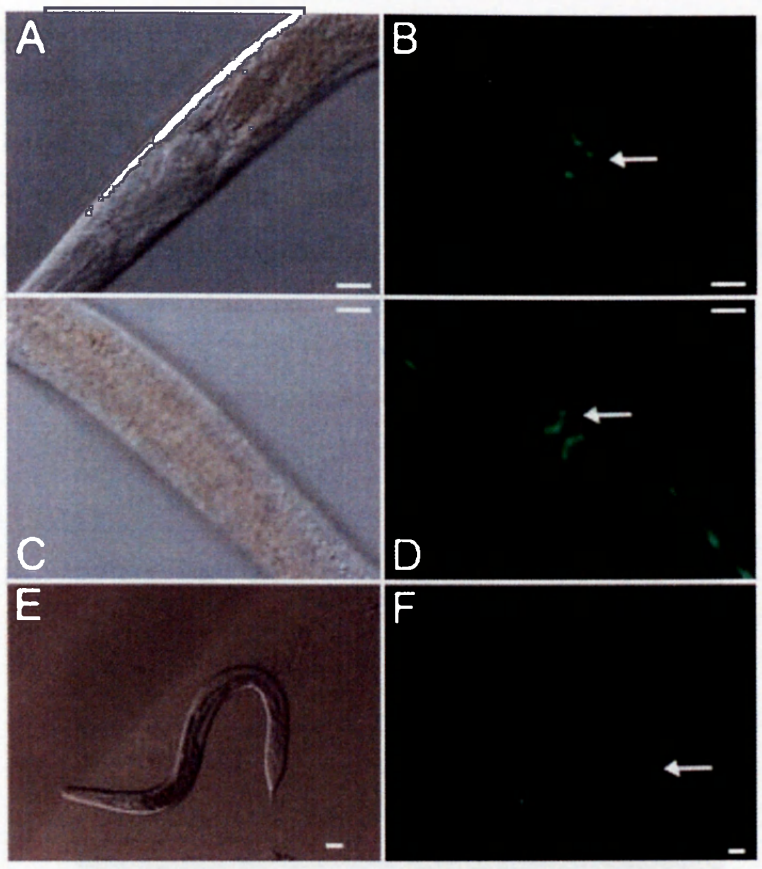


**Fig. 19: *nhr-60*::GFP expression in other cell types**

*nhr-60*::GFP expression in transgenic animals was also observed in pharyngeal gland cells (B), uterine vulval cells (D) and in ray cells of a male tail (F).

A, B: Nomarski and GFP images of *C. elegans* head expressing *nhr-60*::GFP in pharyngeal gland cells (arrow).

C, D: Nomarski and GFP images of the hermaphrodite uterine vulval cells (arrow).

E, F: Nomarski and GFP images showing expression of *nhr-60*::GFP in male L4 larva, expression in ray cells is marked with an arrow.

Scale bar: 20 µm

### 5.7. *nhr-60* expression in seam cells is downstream of NHR-23

Since the expression of *nhr-60*::GFP in seam cells overlaps with the expression of *nhr-23* (*chr-3*) (Kostrouchova *et al.*, 1998), we tested the possibility that the regulation of these two genes is linked. We followed the expression of *nhr-60*::GFP transgene in worms undergoing *nhr-23* RNAi. *nhr-23* RNAi on worms expressing *nhr-60*::GFP caused not only *nhr-23* related affected phenotype but also a decrease of *nhr-60*::GFP expression in seam cells in embryos and larval stages. The effect of *nhr-23* RNAi on *nhr-60*::GFP expression was seam cell specific because the expression in pharyngeal gland cells was unaltered (Fig. 20). In contrast, *nhr-60* RNAi had no effect on the expression of *nhr-23*::GFP although *nhr-60* RNAi related affected phenotype was observed.

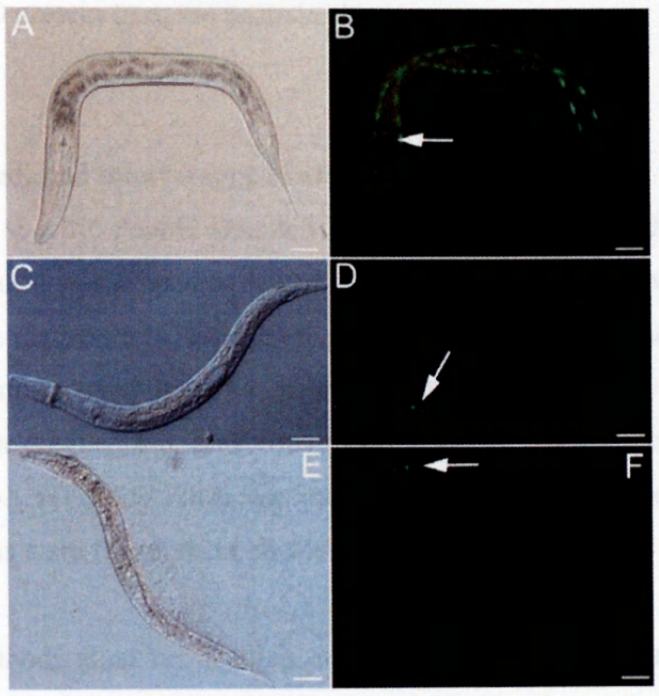


**Fig. 20: The effect of NHR-23 inhibition on *nhr-60*::GFP expression**

Nomarski (A) and fluorescent (B) images of *nhr-60*::GFP expressing L3 larva. Expression in pharyngeal gland cells is marked with an arrow. The expression of *nhr-60*::GFP in seam cells is lost in worms following *nhr-23* RNAi while the *nhr-60*::GFP expression in the pharyngeal gland cells is unaffected (panels D and F, arrows). Panels C and E show nomarski images of the same larvae.

Scale bar: 20 µm

### 5.8. NHR-60 function

The function of *nhr-60* in *C. elegans* was studied by several approaches. We blocked a natural function of the gene by RNA interference. In vitro prepared gene specific dsRNA introduced into animals is processed into 21-24 bp long RNA duplexes (small interfering RNAs – siRNAs) by the RNaseIII enzyme Dicer. After unwinding, siRNA single strands are incorporated into the multi-subunit RNA-induced silencing complex (RISC) and lead the complex to cleave naturally produced RNA with sequence identical to the siRNAs (Fire *et al.*, 1991; Timmons and Fire, 1995, Voinnet, 2005). dsRNA was introduced into worms either by soaking, feeding or by microinjecting. Natural function of *nhr-60* was blocked and studied also by expression of a dominant-negative NHR-60 isoform. Different approach to study *nhr-60* function was to enhance natural function of the gene by overexpression of full length NHR-60 protein from the heat-shock promoter.

#### 5.8.1. RNA mediated interference of *nhr-60*

*nhr-60* specific double stranded RNA (in vitro transcribed from "*nhr-60* in L4440" construct) was prepared. A part of *nhr-60* cDNA (covering exons 3 to 6 in length 690 bp), that served as a template, encodes the putative ligand binding domain. The in vitro synthesized dsRNA was delivered into worms either by microinjecting or by soaking. For feeding, the same construct (*nhr-60* in L4440) was transformed into bacteria *E. coli* HT 115. After the induction, bacteria produced *nhr-60* specific dsRNA. Worms were fed on these bacteria.

All methods (soaking, feeding, microinjecting) gave similar results but with variable degrees of penetrance. The most severe effects were seen in progeny of hermaphrodites after *nhr-60* dsRNA microinjections. Following microinjections, 21% of the progeny (n = 5,858, whereas "n" means number of assayed progeny) failed to hatch (Fig. 21). The majority of the affected embryos were arrested at the two-fold stage of embryogenesis. They appeared to have incomplete ventral enclosure resulting in a protrusion of cells ventrally. Arrested embryos had severe defects in morphogenesis including problems with elongation as well. Small portion of arrested embryos had an atypical spherical shape instead ovoid.

Defects were also observed in a small fraction of L1 progeny of hermaphrodites injected with *nhr-60* dsRNA. Affected larvae developed from affected embryos, which were after all able to hatch, presumably reflecting the less effective knockdown of NHR-60 activity. These larvae had morphological abnormalities along with vacuoles indicative of general cell death (Fig. 21).

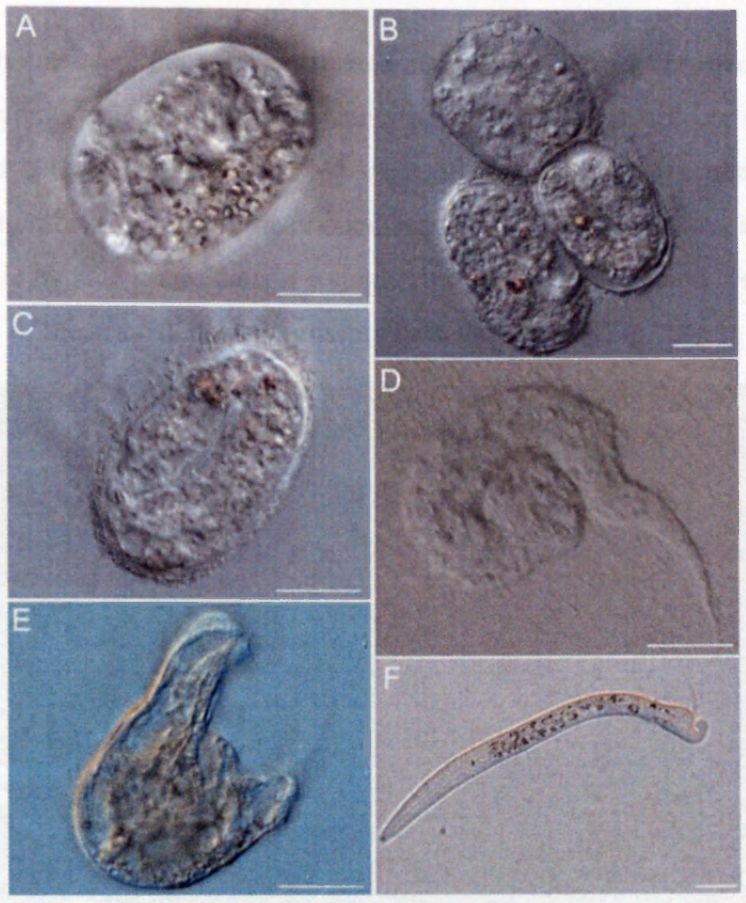


**Fig. 21: *nhr-60* RNAi developmental defects induced on N2 worms**

A-C: Arrested embryos with affected ventral enclosure;

D, E: Worms failing to hatch properly show morphological defects and vacuoles;

F: L2 larva with body morphology defect. Scale bar: 20 µm

The effect of *nhr-60* RNAi on the number of progeny was studied by soaking. L3 larvae (N2) were soaked in solution of *nhr-60* dsRNA and in water as a control. The inhibition of *nhr-60* function by RNAi (soaking) led also to decreased number of progeny. The average number of progeny after *nhr-60* RNAi was 182 (SD 30, progeny of 10 hermaphrodites were scored, SD means standard deviation) in contrast to 222 in controls (SD 44.3, progeny of 12 worms were scored).

### 5.8.2. *nhr-60* RNAi on GFP reporter genes expressing strains

To identify the most affected tissues after *nhr-60* RNAi, we employed several GFP reporter genes. These GFP reporter strains allowed us to assay defects in seam cells (strains JR667 and *nhr-60*::GFP), epidermal and seam cells (strains SU93 and *nhr-23*::GFP), and body wall muscle cells (strain PD7963).

Most tissues were not overtly affected and, in most cases, the arrest occurred after all embryonic cell divisions were completed. However, a striking observation was that seam cells in arrested embryos were often displaced. Frequently, *nhr-60* RNAi arrested embryos had miss-positioned seam cells in the head and tail regions, arranged as side by side rather than in a single row as in wild type embryos. In many embryos some of the seam cells were missing or disorganized (Fig. 22).

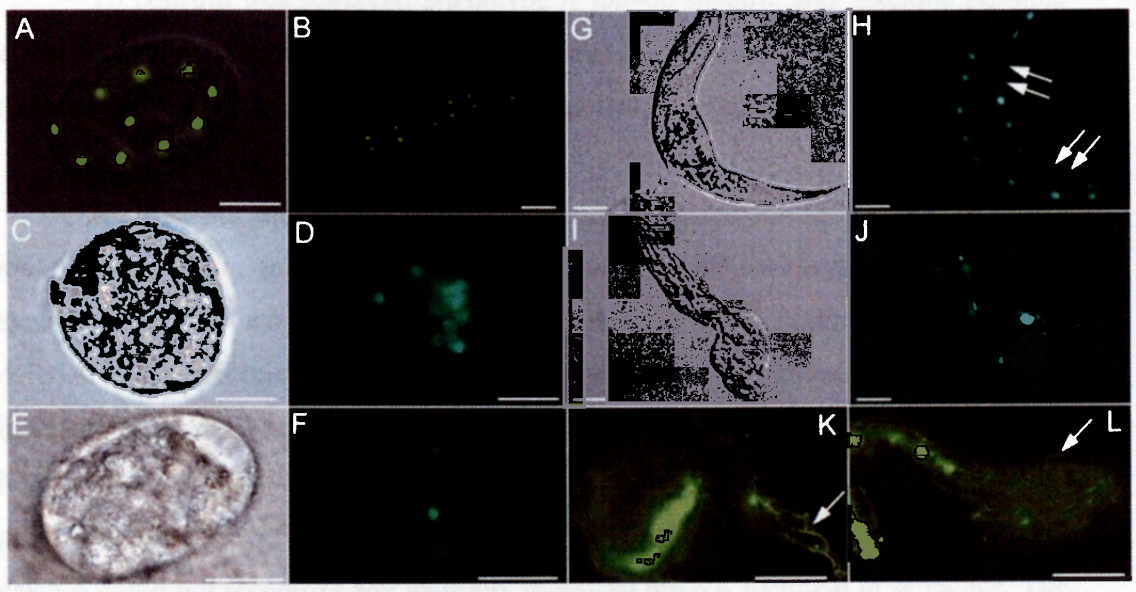


**Fig. 22: Developmental defects induced by *nhr-60* RNAi**

NHR-60 inhibition was done on JR667 (C to J) and SU93 worms (K and L). JR667 worms express GFP transgene in seam cell nuclei. SU93 worms express GFP transgene in adherent junctions of epithelial cell boundaries. Panels A and B show unaltered expression of transgene in JR667 embryo (A, a lateral view) and larva (B, a dorso-ventral view). Panels C, E show nomarski images of typical malformed embryos and D, F fluorescent images of the same embryos. Decreased number and miss-positioning of seam cells is visible. Larvae with typical body shape defects are shown in panels G to L. The body shape defects are accompanied by miss-position or completely missing seam cells (fluorescent images H, J, K, and L, arrows). Panels G and I show nomarski images of affected JR667 larvae shown in panels H and J. Scale bar: 20 μm

The average number of observable seam cells in JR667 embryos after *nhr-60* RNAi decreased to 9.8 seam cells per an animal (n = 20, SD 5.4). In control animals (embryos of non-treated JR667 hermaphrodites), 19.1 seam cells per an animal (n = 20, SD 1.0) were observed on the average. This indicates a statistically important difference.

Seam cells show high-level expression of *nhr-60*::GFP. They also show the most obvious phenotypic defect following *nhr-60* RNAi. This suggests that NHR-60 activity is particularly important for development of this cell type.

### 5.8.3. Control of RNAi specificity

To confirm that observed phenotype is *nhr-60* specific and exclude possible effect either of chemicals used for in vitro RNA synthesis or the procedure (microinjecting) itself we performed several control experiments.

At first, we did *nhr-60* coding sequence-unrelated RNAi. dsRNA from the *nhr-60* non coding (promoter) region (construct *nhr-60 unrelated* in L4440) was synthesized in the same way as *nhr-60* dsRNA used in all experiments. Unrelated dsRNA was microinjected into young wild type hermaphrodites. The progeny of injected worms was followed and scored in the same way as progeny of hermaphrodites after *nhr-60* RNAi. Because there is not mRNA transcribed from the promoter region in cell, siRNAs originating by Dicer cleavage of injected unrelated dsRNA could not recognize any naturally produced mRNA identical in sequence. The only observable effect after unrelated RNAi is either the effect caused by used chemicals or by the procedure of microinjecting. We scored 1,510 progeny following unrelated RNAi, only 0.8% on the average showed phenotypical defects (arrested embryonic development).

Second, together with each *nhr-60* dsRNA microinjection experiment we injected also water into several hermaphrodites as a control of the procedure itself. Usually we did not observe any affected progeny after microinjecting of water and the maximum of progeny with defects was never higher than 2%.

Taken together we can conclude that effects observed after *nhr-60* RNAi are caused by gene specific RNAi and not non-specifically due to used chemicals or technique.

### 5.8.4. RNA mediated interference of *nhr-116*

*nhr-116* belongs to the subgroup of supnrs with the same P-box (CNGCKT) as *nhr-60*. Based on the protein and nucleotide sequence as well, *nhr-116* is the closest homolog of *nhr-60* in *C. elegans*. To exclude the possible redundancy in *nhr-60* and *nhr-116* function we blocked also the function of *nhr-116* by RNAi.

*nhr-116* dsRNA was prepared in vitro (construct *nhr-116* in L4440) and microinjected into wild type hermaphrodites. After the RNAi, 1.1% of progeny (n = 1,390) had sporadic defects. We did not observe any statistically important effect on progeny following *nhr-116* RNAi.

We performed also double RNAi experiment, when *nhr-60* and *nhr-116* dsRNAs were microinjected into wild type hermaphrodites together in ratio 1:1. We observed the same affected phenotype as after *nhr-60* RNAi (arrested embryos with the defect of ventral enclosure and larvae with morphological abnormalities) but the penetrance was lower contrary to *nhr-60* RNAi alone (5.3%, n = 2,471). These results indicate that *nhr-60* and *nhr-116* are not redundant. The decrease in *nhr-60* RNAi effect could be explained as the consequence of lower concentration of *nhr-60* dsRNA used for microinjections (reduced to half).

### 5.8.5. Expression of NHR-60 dominant –negative isoform

NHRs lacking transcriptional activation domains can function as dominant negative factors (Liu *et al.*, 2002). In order to identify the putative activation domain 2 (AF-2), we performed Multiple sequence analysis of NHR-60 and several vertebrate nuclear receptors. The analysis indicates that *nhr-60* contains AF-2 at the end of the coding sequence, similarly as most vertebrate nuclear receptors.

We prepared *nhr-60/AF2-* construct with a heat-inducible promoter driving the expression of *nhr-60* cDNA lacking the coding sequence homologous to the part of the putative AF-2. Heat-shock induction of *nhr-60/AF2-* expression in transgenic worms was done either at 31°C for 2 hours or at 34°C for 30 min. Heat-shock induced expression of incomplete NHR-60 lacking putative AF-2 domain resulted in 21% of affected embryos (2 h at 31°C, n = 252) eventually 36% of affected progeny (30 min at 34°C, n = 313) supporting the idea, that the terminal part of NHR-60 may indeed function as AF-2.

As a control, we similarly induced expression of a full length *nhr-60* cDNA from the same heat-shock promoter. Incubation of transgenic worms carrying *nhr-60/all cDNA* construct at either 31°C or 34°C had no effect on progeny comparing to effect of heating on wild type N2 worms. N2 worms were exposed to the same conditions to determine this effect. Progeny of worms kept 2 hours at 31°C showed 2% of affected embryos. When worms were incubated at 34°C, the fraction of affected progeny increased to 6%.

NHR-60/AF2- caused both early and late embryonic arrest. The early embryonic arrest occurred at the several hundred-cell stage with embryos containing many vacuoles indicative of general cell death. The late embryonic arrest observed occurred at 2- and 3-fold stage with embryos failing to complete elongation (Fig. 23).

Because the NHR-60/AF2- protein lacks the C-terminal peptide used for antibody production, we were able to assay endogenous NHR-60 abundance and localization following heat-shock induction of the putative dominant negative isoform. We observed an obvious decrease in the intensity of NHR-60 antibody labeling in animals expressing NHR-60/AF2- in vivo or by Western blot analysis. These results demonstrate that NHR-60/AF2- is likely functioning as a dominant negative factor, adversely affecting development and displacing endogenous NHR-60 from sites of nuclear localization.

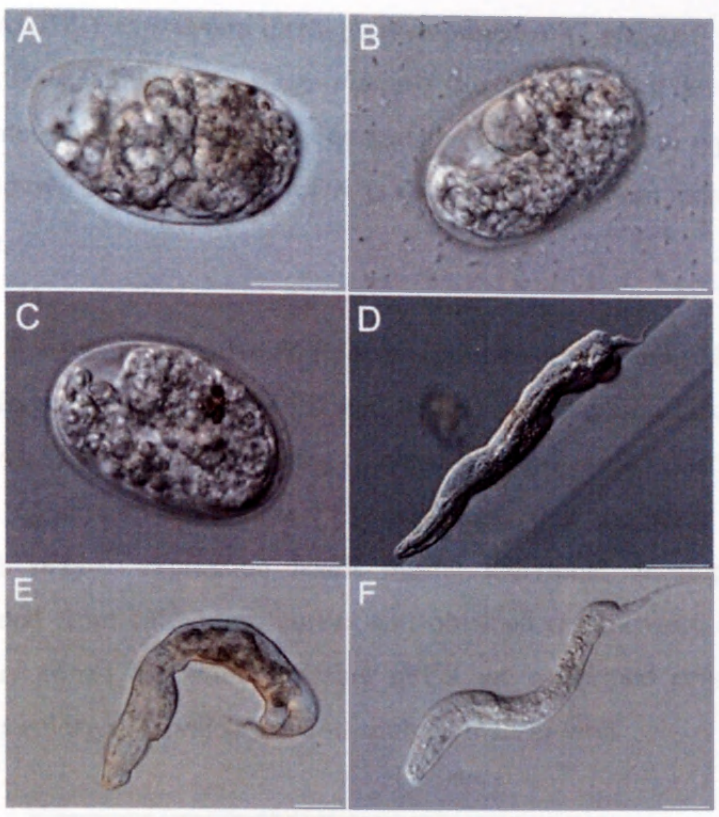


**Fig. 23: Developmental defects induced by expression of NHR-60 dominant–negative isoform**

Defects similar to NHR-60 inhibition were observed in embryos (panels A to C) and larvae (panels D to F) overexpressing NHR-60 isoform lacking the putative activation function AF2- domain. Scale bar: 20 μm

### 5.9. *C. elegans* Acyl-coenzyme A binding protein (ACBP-1)

The family of nuclear hormone receptors in *C. elegans* has undergone an extensive expansion and diversification (Sluder *et al.*, 1999; Sluder and Maina, 2001). NHR-60, a supplementary NHR, is expected to be evolved from an ancestral homolog of vertebrate orphan receptor HNF4-α (Robinson-Rechavi *et al.*, 2005). It was shown, that HNF4-α physically and functionally interacts with Acyl-coenzyme A binding protein (ACBP), a small protein transporting fatty acids in the form of acyl-coenzyme A (Petrescu *et al.*, 2003). Four genes with high homology to mammalian ACBP-1 are encoded in *C. elegans* genome (*acbp-1* to *acbp-4*). The closest homolog is *C. elegans* ACBP-1. We tried to find if these two *C. elegans* proteins (NHR-60 and ACBP-1) function together.

### 5.9.1. ACBP-1 expression during development of *C. elegans*

To determine if *C. elegans acbp-1* is expressed in all developmental stages we performed semiquantitative and quantitative PCRs. For both types of PCR reactions we used two set of templates (cDNAs from total RNA from individual developmental stages).

For semiquantitative PCR, *acbp-1* primers #5169 and #5170 were used. *ama-1* DNA fragments (primers #4684 and #4685) were co-amplified as internal controls (Fig. 24). The results showed that *C. elegans acbp-1* is expressed in all developmental stages. Quantitative real-time PCR was done with the same primers (#5169 and #5170). *acbp-1* expression levels during individual developmental stages were calculated from calibration curves and obtained results were normalized to *ama-1* (primers #4684 and #4685). Using qPCR we confirmed *acbp-1* expression in *C. elegans* embryos, larval stages and adult animals as well.

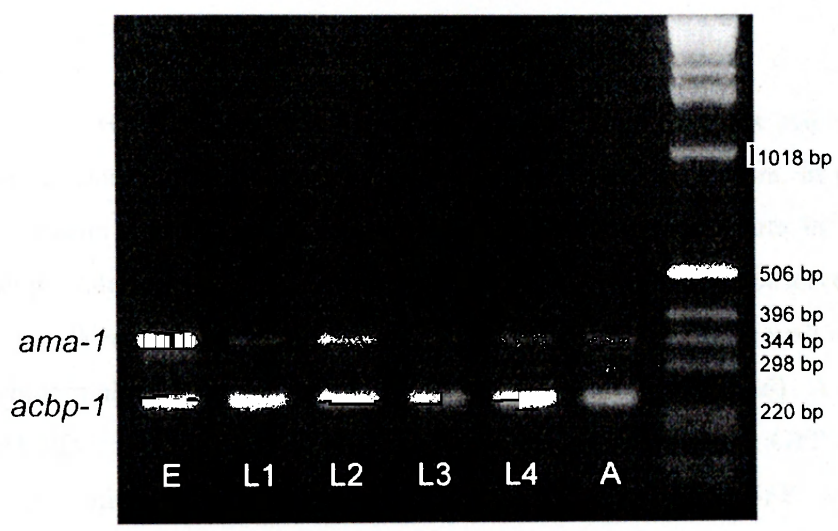


**Fig. 24: *acbp-1* expression during *C. elegans* development**

### 5.9.2. ACBP-1::GFP reporter genes expression

In order to find the *acbp-1* expression pattern we prepared several transgenic lines expressing GFP from two ACBP-1::GFP translational fusion constructs. ACBP-1::GFP S and L are extra-chromosomal arrays containing the putative promoter regions and complete coding sequence of *acbp-1*. The construct ACBP-1::GFP S

included 530 bp upstream of the predicted start of translation and genomic DNA up to the end of the coding sequence of the gene. The construct ACBP-1::GFP L differs from S in the length of the putative promoter region (1,180 bp) (Fig. 25).

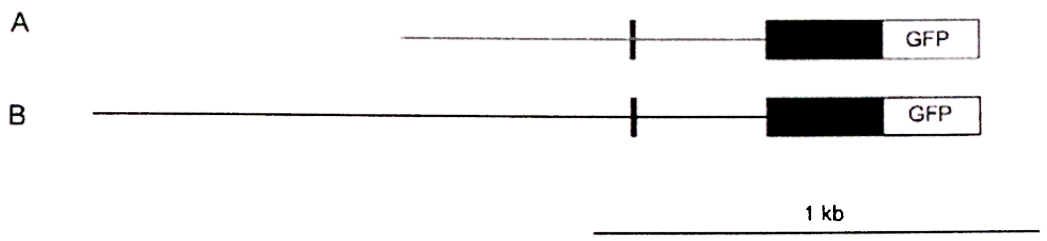


**Fig. 25: A schema of ACBP-1::GFP translational fusion constructs**
*acbp-1* exons are marked as black rectangles.
A: ACBP-1::GFP S construct (putative promoter length 530 bp)
B: ACBP-1::GFP L construct (putative promoter length 1,180 bp)

ACBP-1::GFP was found to be expressed in various cell types in embryos, larvae and adults. ACBP-1::GFP is localized in the cytoplasm, in peripheral regions of nuclei and inside of nuclei as well. The expression starts in embryos (comma stage) and continues until adulthood. Strong expression was observed in the intestinal cells. Weak but detectable expression was found also in body wall muscle cells, some epidermal cells, seam cells and in several neurons (Fig. 26). A small fraction of ACBP-1::GFP expressing worms did not expressed ACBP-1::GFP in the intestine. In these animals the intracellular distribution of ACBP-1::GFP was well observed otherwise obscured by strong fluorescence in the intestinal cells.

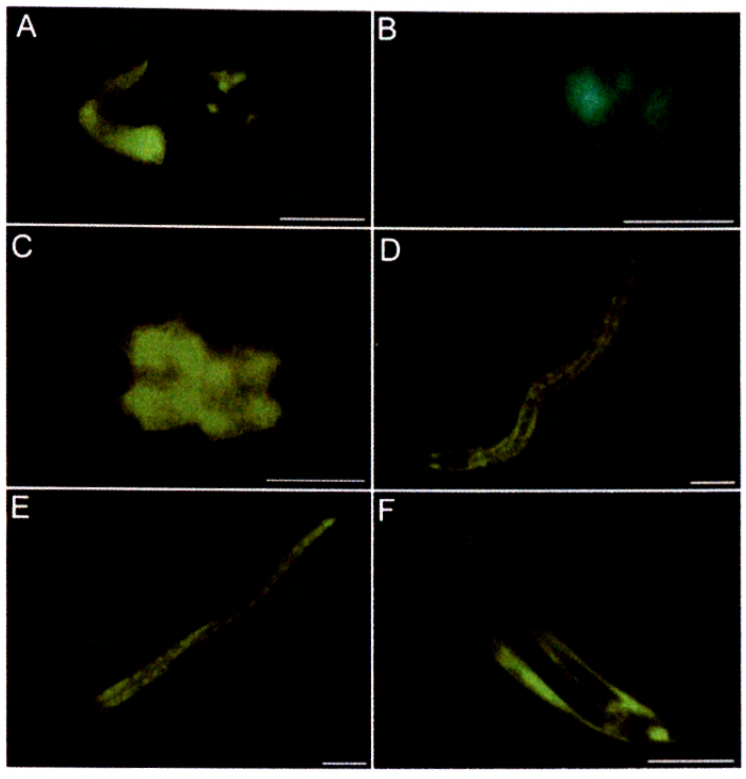


**Fig. 26: ACBP-1::GFP expression**

A-C: Embryos showing expression of ACBP-1::GFP translational fusion constructs. The expression was detected first in precursors of intestinal cells in comma stage embryo (C). Due to mosaicism, some embryos show expression only in intestinal cells (B) and some in precursors of epidermal and muscle cells in addition (A).

D-F: Larvae express ACBP-1::GFP mainly in intestinal cells (E). If not, also weak expression in epidermal (D) and body wall muscle (F) cell was detected.

Scale bar: 20 µm

### 5.10. *acbp-1* RNAi triggers affected phenotypes similar to *nhr-60* inhibition

*acbp-1* expression was inhibited by RNAi using microinjections of *acbp-1* dsRNA (in vitro transcription from *acbp-1* in pCR® 4-TOPO) into gonads of young adult hermaphrodites. The inhibition caused severe developmental defects in progeny of microinjected animals.

Embryos were arrested in early and late embryonic stages. The early arrested embryos showed defects of ventral enclosure. Embryos had ventrally everted cells.

Some of arrested embryos were irregularly lobulated and developed in anomalous shape. The late embryonic defects had characteristics of defective elongation in the 2-fold stage. Some affected embryos developed into the 3-fold stage. These embryos were severely dysmorphogenic with defective elongation. Larvae hatched from some affected embryos had body morphology defects with defective elongation and body shape (Fig. 27).

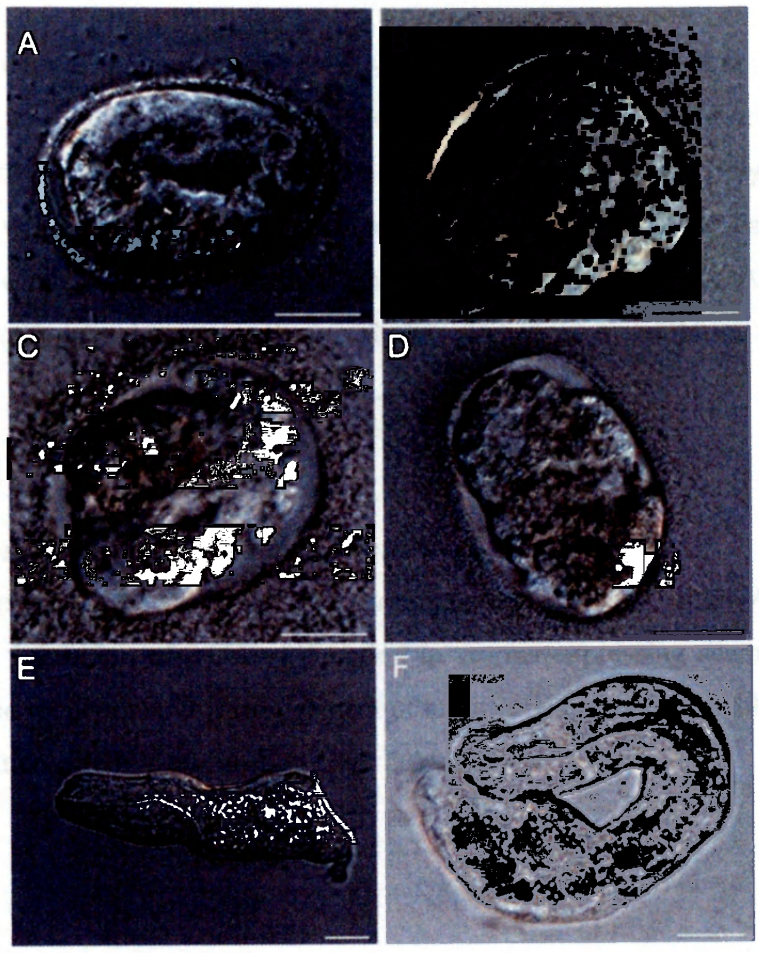


**Fig. 27: *acbp-1* RNAi developmental defects induced on N2 worms**

A-D: Arrested embryos showing body morphology defects and affected ventral enclosure

E, F: Larvae with defective body shape

Scale bar: 20 μm

The proportion of affected progeny oscillated in some experiments between 10-20%. In the other experiments, the effect was almost missing suggesting that the inhibition of ACBP has to reach certain level to induce morphological phenotypes.

Many embryos arrested in 2-fold stage seemed to have non affected movement as well as body wall muscle cells. They were vigorously moving inside the eggshell.

Three other *C. elegans* genes (*acbp-2*, *acbp-3*, and *acbp-4*) have sequences highly similar to *acbp-1*. To make sure that *acbp-1* RNAi led to only *acbp-1* gene specific inhibition, we followed also worms after *acbp-2*, *acbp-3* and *acbp-4* RNAi. The inhibition was done using microinjections of *acbp-2* dsRNA, *acbp-3* dsRNA, and *acbp-4* dsRNA into wild type worms. Inhibition of either ACBP-2, ACBP-3 or ACBP-4 did not induce the same not even similar phenotype as inhibition of ACBP-1.

### 5.11. Inhibition of *acbp-1* affects the development of seam cells

We performed *acbp-1* RNAi (by microinjecting of *acbp-1* dsRNA) also on JR667 worms. Inhibition of ACBP-1 function in worms expressing seam cell GFP marker revealed reduction and rearrangement of seam cells indistinguishable from the effect of inhibition of NHR-60 function (Fig. 28).

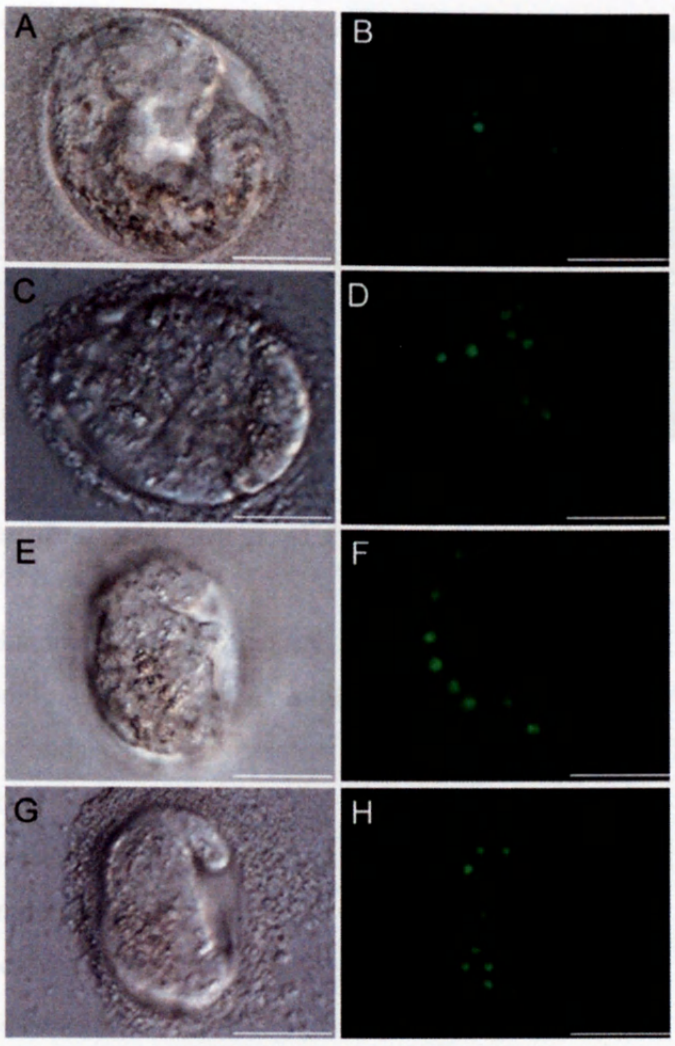


**Fig. 28: *acbp-1* RNAi induces defects of seam cell development**

Inhibition of ACBP-1 expression on JR667 worms revealed reduction and rearrangement of seam cells. Fluorescent images (GFP) of affectcd embryos are shown in pictures B, D, F, and H. The same embryos are shown also in Nomarski optics (A, C, E, and G).

Scale bar: 20μm

### 5.12. NHR-60 and ACBP-1 co-localize at the nuclear periphery

ACBP-1::GFP expressing worms were used to assay a possible co-localization with NHR-60 in vivo. NHR-60 was detected by immunocytochemistry using #4529 primary and Alexa Fluor 568 secondary antibody. Co-localization maps (using confocal microscopy sections, kindly designed by Jakub Sikora (Institute of Inherited Metabolic Disorders, Prague)) revealed that these two proteins co-localize in the

peripheral regions of nuclei in embryos and in intestinal cells of larvae (Fig. 29). Co-localization in some epidermal cells was also observed but with lower intensity.

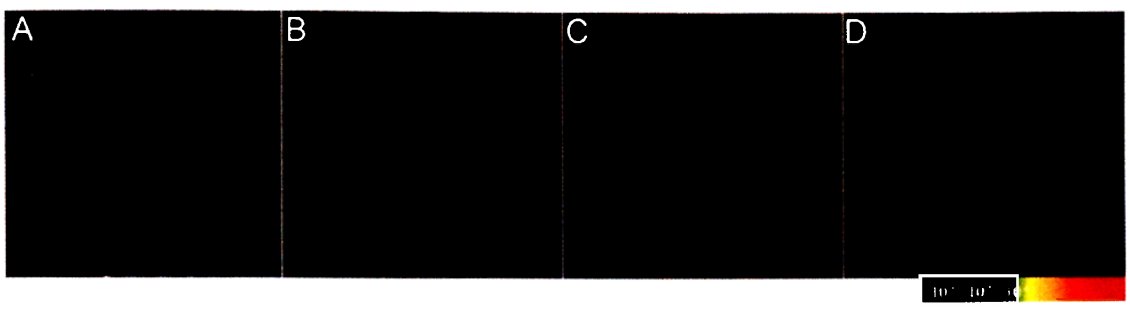


**Fig. 29: The dual-color image of confocal section from fixed ACBP-1::GFP worms**

The dual-color image of confocal section from fixed ACBP-1::GFP worms stained with polyclonal rabbit IgG primary antibody against *C. elegans* NHR-60 and goat anti rabbit IgG secondary antibody marked with rhodamine (Alexa Fluor 568) was split into two parts:

A: Red component (NHR-60 labeled with AF 568)

B: Green component (ACBP-1::GFP)

C: An unprocessed confocal microscopy dual-color image

D: Co-localization map (overlap coefficient 0.3)

### 5.13. NHR-60 and ACBP-1 interaction in vivo

Co-immuno precipitation experiments were performed to detect a possible interaction in vivo between NHR-60 and ACBP-1. The protein extract from ACBP-1::GFP transgenic worms as the experimental sample and the extract from N2 worms as a control were used. #4529 antibody against NHR-60 was bound to agarose beads and incubated with protein extracts. After wash, beads were pelleted and proteins were released and used for the subsequent Western blot analysis. The ACBP-1::GFP fusion protein was detected with antibody against GFP. No protein was detected in the control (Fig. 30). This result suggests an interaction between NHR-60 and ACBP-1.

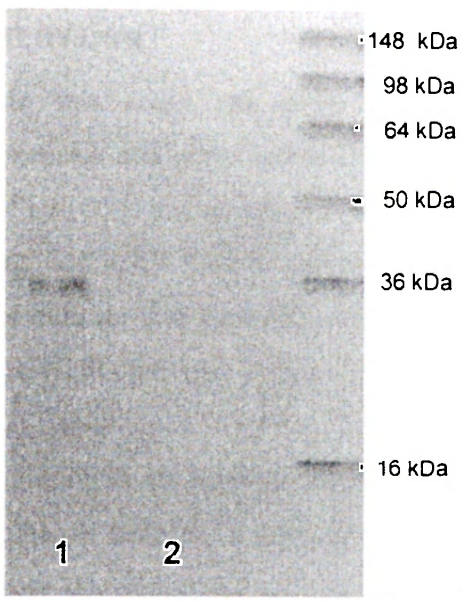


**Fig. 30: NHR-60/ACBP-1::GFP co-immuno precipitation**

Protein extracts after NHR-60/ACBP-1::GFP co-immuno precipitation were analyzed by Western blot using primary mouse monoclonal IgG antibody against GFP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and secondary anti mouse IgG antibody conjugated with horseradish peroxidase. Protein migrating at expected size 36 kDa (10 kDa for ACBP-1 + 26 kDa for GFP) was detected in extract released from agarose beads after NHR-60/ACBP-1::GFP co-immuno precipitation in experimental sample (ACBP-1::GFP expressing worms, lane 1). No band was detected using antibody against GFP in control (N2 wild type worms, lane 2).

Similarly, we tried to confirm the co-immuno precipitation between NHR-60 and ACBP-1 also using antibodies against NHR-60 (#4529) and ACBP-1 (a kind gift from Dr. Nils Færgeman (BMB, University of Denmark)). However, we have not obtained positive results. We have not detected any protein migrating at the expected size after Western blot analysis. It is necessary to use some another technique to confirm the possible in vivo interaction between NHR-60 and ACBP-1.

## 6. Discussion

In this study, we show that NHR-60 is required during *C. elegans* embryogenesis and early larval development. Following NHR-40 (Brozova *et al.*, 2006), NHR-60 is the second member of the small subgroup of supnrs with the P-box sequence CNGCKT that is shown to have an important developmental role. Our findings indicate that members of the 269 supnrs are likely to join the majority of canonical NHRs that play critical developmental roles.

The NHR-60 function, based on ubiquitous expression and the character of developmental defects, is probably more general. Inhibition of NHR-60 results in both gross developmental defects and cell specific regulatory events. The former function is visualized as arrested embryos and severe dysmorphogenesis that are accompanied by miss-position and missing seam cells. Keeping with such function, NHR-60 is strongly expressed in germ line and its inhibition is connected with decreased number of progeny. However, the cell specific regulatory roles are supported by enhanced expression in specific cell types.

One type of cells that seems at least particularly dependent on NHR-60 function is the seam cells. These cells show high level of *nhr-60* reporter genes expression. They accumulate higher amount of NHR-60 protein and are affected in embryos and larvae subjected to *nhr-60* RNAi. The role of NHR-60 in seam cells appears to be after their specification, given the onset of the up-regulation in these cells and the persistence of clearly defined seam cells following *nhr-60* RNAi. The requirement of NHR-60 for seam cell development is suggested by defects of seam cell separation and positioning in *nhr-60* inhibited larvae.

An alignment of NHR-60 with NHRs with known crystal structure indicates that NHR-60 C-terminal region has similarity to AF-2 region capable of interactions with coactivators. In accordance with that, expression of the C-terminally deleted NHR-60 AF-2- isoform induces a phenotype similar to NHR-60 inhibition. This is also supported by overexpression of full length NHR-60. Such overexpression did not induce any observable developmental phenotype and was joined with enhanced nuclear staining by antibody against NHR-60.

Interestingly, we found that *nhr-60*::GFP expression in seam cells is downstream of *nhr-23*. NHR-23 (CHR-3) is strongly required for development and molting (Kostrouchova *et al.*, 1998; Kostrouchova *et al.*, 2001). The role of NHR-23 seems to be more superior, probably dictating specific conditions for NHR-60 seam cell expression since NHR-60 expression in other cell types is not visibly dependent on NHR-23.

Alternatively, NHR-60 function may be linked to the growth and proliferation of seam cells because of disappearance of NHR-60 at the time of seam cell fusion. To fully explore the possible links between NHR-60 and NHR-23, next studies are necessary.

As epidermis, seam cells must participate in the coordination of larval molts among all hypodermal cells of the animal. Keeping with the critical role of seam cells in regulation of larval transitions and integration of developmental regulatory events, this particular cell type shows involvement of several NHRs.

NHR-25 is required for proper seam cells elongation and formation of their processes and for subsequent seam cell fusions (Silhankova *et al.*, 2005). Several additional receptors are likely to be involved in regulation of transcription in seam cells: NHR-72, NHR-73, NHR-74, NHR-75, NHR-77, NHR-81, NHR-82, and NHR-89. Although RNA interference did not reveal any developmental phenotype, three genes (*nhr-77*, *nhr-81*, and *nhr-82*) showed severe developmental phenotype connected with altered number of seam cells induced by expression of GFP transgenes in seam cells (Miyabayashi *et al.*, 1999). However, the potential for redundancy of function among the supnrs is a real possibility suggesting a caution in interpretations.

An unusual feature of NHR-60 biology is its localization. Localization at the nuclear periphery is not typical for NHRs. Most of NHRs are known to be shuttling between the cytoplasm and the nucleus or are constitutively nuclear. In both cases, NHRs show a patchy or diffuse pattern (Ktistaki *et al.*, 1995). The peripheral localization has been reported for several transcription factors. For these factors, the

pattern was shown to be related to interactions with the nuclear lamina and lamins. This interaction was shown to be linked to the inhibition of transcription.

Although we cannot exclude the possibility that NHR-60 localization at the nuclear periphery is also linked to the transcription inhibition or NHR-60 functional withdrawal, we prefer another explanation. NHR-60 localization at the nuclear periphery can reflect NHR-60 interactions with metabolic or other regulatory proteins. Keeping with this, NHR-60 overexpression led to increased peripheral presence of NHR-60 and did not induce any observable affected phenotype that can be expected for a transcription inhibitory function.

Another possibility for a functional link between the peripheral nuclear localization of NHR-60 is its hypothetical interaction with metabolic proteins known to reside in this region. Hepatocyte nuclear factor 4 (HNF4) that is important for development and liver specific gene expression in vertebrates (Jiang *et al.*, 1995; Sladek, 1993; Li *et al.*, 2000) interacts functionally and physically with acyl-coenzyme A binding protein (Petrescu *et al.*, 2003).

Acyl-coenzyme A binding proteins (ACBPs) are highly conserved proteins that bind long chain acyl-CoA esters. Long chain acyl-CoA esters are intermediates in fatty lipid synthesis and function in the gene regulation (Knudsen *et al.*, 2000). ACBP was expected to be cytosolic but detailed analysis of ACBP distribution in rat hepatic and hepatoma cells localized ACBP also into the nucleus (Elholm *et al.*, 2000). *C. elegans* genome contains four ACBP orthologs called ACBP-1 to 4. ACBP-1 is the most conserved protein in this group. HNF4α was detected inside the nucleus but the area of its co-localization with ACBP (Petrescu *et al.*, 2003) resembles the localization that we report here for NHR-60 nuclear localization and co-localization with ACBP-1. It is possible that the peripheral nuclear zone has broader function for interactions of NHRs with metabolic or regulatory proteins. It seems likely that the zone of co-localization of NHR-60 with ACBP-1 could be the place, where NHR-60 and ACBP-1 interact. The fact that ACBP-1 inhibition leads to similar developmental phenotype as inhibition of NHR-60 supports the functional interaction between NHR-60 and ACBP-1.

Several NHR-60/ACBP-1 co-immuno precipitation experiments indicated the possibility of physical interaction between these two proteins but many experiments failed. It is possible that antibodies bind targeted proteins in regions that are closely positioned to the interacting interface of NHR-60 and ACBP-1. Additional methods for visualization of NHR-60 and ACBP-1 physical interaction are necessary. It is also possible that the interaction of NHR-60 and ACBP-1 requires additional factors that were not controlled in our experiments, the charging of ACBP-1 with acyl-coenzyme A being in the first place. This indicates the need for employment of specific in-vivo interaction methods that may visualize a subpopulation of interacting molecules.

## 7. Conclusions

In this study, we have shown that *C. elegans* nuclear hormone receptor NHR-60 is a functional gene that has function important for a proper embryonic and early larval development of *C. elegans*.

We found that *nhr-60* is likely to be expressed as a single isoform in all developmental stages.

NHR-60 expression is ubiquitous in embryos and larval stages. The expression is enhanced in epidermal seam cells and germ line in older stages. The level of expression is almost constant during development but decreases in late L4 and adult stage.

NHR-60 functions during embryogenesis and in seam cells is downstream of NHR-23. Overexpression of full length NHR-60 does not induce any visible developmental phenotype.

The function of NHR-60 may be spatially localized at the nuclear periphery and is dependent on AF-2 domain.

Based on co-localization studies and similar phenotypes of NHR-60 and ACBP-1 inhibition, NHR-60 is likely to be functionally linked with ACBP-1.

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# 9. Appendix

## 9.1. The list of primers

| name | sequence | restriction site |
|---|---|---|
| #4520 | acgcgtcgacgtcggaatgaacccccttgc | Sal I |
| #4521 | cgggatcccggatcaacggtgcaacagac | Bam HI |
| #4535 | acgcgtcgactggccgaggcatactgtccacg | Sal I |
| #4536 | cgggatcccaaatcaggcattcagttgggcg | Bam HI |
| #4566 | gatccgtcaggctcaatgataacc | - |
| #4684 | ttccaagcgccgctgcgcattgtctc | - |
| #4685 | cagaatttccagcactcgaggagcgga | - |
| #5156 | atgcttcaaccaacagaatcaccc | - |
| #5157 | tgtccatgatatcttcaataacccg | - |
| #5169 | atgaccctctcgttcgacgacgctg | - |
| #5170 | cgatgagctcctcaacaagagcaac | - |
| #5293 | atgtgtgacgacgaggttgccgc | - |
| #5294 | gctcattgtagaaggtgtgatgcc | - |
| #6014 | cggatccgtccatatgattcaatccagttc | Bam HI |
| #6015 | cccatggtatgtacatttcccatttattcac | Nco I |
| #6016 | cccatggtttcggagccacgagaagtagatg | Nco I |
| #6045 | acgcgtcgacccctgatcaacaccagtccatttcc | Sal I |
| #6046 | acgcgtcgacgctcacatttctgataccccg | Sal I |
| #6051 | catgccatggcttcaacttgttcaccaccgtag | Nco I |
| #6052 | acgcgtcgacgacactttggagcatgagccaaag | Sal I |
| #6060 | cgggatccgcacacgcctgaccttgaaaagatgg | Bam HI |
| #6063 | acgcgtcgacccatcttttcaagctcaggcgtgtc | Sal I |
| #6165 | taggtccgccaagactaccgaaac | - |
| #7000 | agctctagaagcacctcaatcactgtgattactg | Xba I |
| #7001 | tttctgcaggaagcttctccttgtgcagagaacg | Pst I |
| #7002 | agctctagatggaaagagctcgaaggagtgtctc | Xba I |
| #7003 | tttctgcagtccaatgagagcgaggttggtt | Pst I |

| | | |
|---|---|---|
| #7004 | agctctagattgagcagcgaatgaaatggaatg | Xba I |
| #7005 | tttctgcagctccattgatctcaacagtctctc | Pst I |
| #7013 | aaactgcagttctctacgtgtctaagtttctttc | Pst I |
| #7014 | aaaggattcgctccgtacttggcgatgagctcc | Bam HI |
| #7016 | aaactgcagaccttgcagctacagtacccaccagc | Pst I |
| SL1 | ggtttaattacccaagtttgag | - |
| SL2 | ggttttaacccagttactcaag | - |

## 9.2. The list of figures

## 9.3. The list of author's publications and presentations

Publications:

**Simeckova, K.**, Brozova, E., Vohanka, J., Pohludka, M., Kostrouch, Z., Krause, M. W., Rall, J. E. and Kostrouchova, M. (2007): Supplementary nuclear receptor NHR-60 is required for normal embryonic and early larval development of *Caenorhabditis elegans*. *Folia Biologica* (Praha), in press.

Brozova, E., **Simeckova, K.**, Kostrouch, Z., Rall, J. E., and Kostrouchova, M. (2006): NHR-40, a *Caenorhabditis elegans* supplementary nuclear receptor, regulates embryonic and early larval development. *Mech Dev.* 123, 689-701

Presentations:

**Šimečková, K.**, Brožová, E., Kostrouch, Z., Kostrouchová, M. (2004): NHR-60 reveals a new site of localization for nuclear hormone receptors – the nuclear lamina. Poster presentation. *European worm meeting*, Interlaken, Switzerland, May 22 – 25, 2004.

Brožová, E., **Šimečková, K.**, Kostrouch, Z., 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, May 22 – 25, 2004.

**Šimečková, K.** (2004): Nuclear hormone receptor 60 is involved in *Caenorhabditis elegans* embryonic development and is localized in nuclear periphery. Oral presentation. *2nd Meeting of the Doctoral Schools of the Charles University (Prague) – University Louis Pasteur (Strasbourg)*, Prague, the Czech republic, October 24 – 27, 2004.

**Šimečková, K.** (2005): Nuclear hormone receptor 60 is involved in *Caenorhabditis elegans* embryonic development and is localized in nuclear periphery. Oral presentation. 6th Student Conference, 1st Faculty of Medicine, Prague, the Czech Republic, May 23, 2005.

**Šimečková, K.**, Brožová, E., Vohánka, J., Pohludka, M., Kostrouch, Z., Kostrouchová, M. (2005): NHR-60, a *Caenorhabditis elegans* supplementary nuclear receptor residing at the nuclear periphery, regulates embryonic development in connection with acyl-coenzyme A binding protein. Poster presentation, *15th International Worm Meeting*, Los Angeles, USA, June 25 – 29, 2005.

Brožová, E., **Šimečková, K.**, Kostrouch, Z., Kostrouchová, M. (2005): NHR-40, a *Caenorhabditis elegans* nuclear hormone receptor is important for late embryonic and larval development. Poster presentation, *15th International Worm Meeting*, Los Angeles, USA, June 25 – 29, 2005.

**Simeckova, K.** (2005): NHR-60, a supplementary nuclear receptor residing at the nuclear periphery, regulates embryonic development of *C. elegans*. Oral presentation. *3rd Meeting of the Doctoral Schools of the Charles University (Prague) & Louis Pasteur University (Strasbourg)*, Strasbourg, France, December 11 – 14, 2005.

**Šimečková, K.**, Brožová, E., Vohánka, J., Pohludka, M., Kostrouch, Z., Kostrouchová, M. (2006): NHR-60, a *Caenorhabditis elegans* supplementary nuclear receptor residing at the nuclear periphery, regulates embryonic development in connection with acyl-coenzyme A binding protein. Poster presentation. Hersonissos, Greece, April 29 – May 3, 2006.

Brožová, E., **Šimečková, K.**, Kostrouch, Z., Kostrouchová, M. (2006): NHR-40, a *Caenorhabditis elegans* supplementary nuclear receptor regulates the development of embryos and L1 larvae. Poster presentation. Hersonissos, Greece, April 29 – May 3, 2006.