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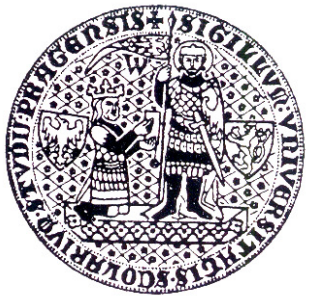

Nuclear hormone receptors in *Caenorhabditis elegans*: NHR-60 regulates embryonic development

PhD thesis summary

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Prague 2007

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PhD thesis sent:

Defense of PhD thesis:

Place of defense:

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This PhD thesis was elaborated within the PhD program for studies in Biomedicine at the Institute of Inherited Metabolic Disorders, 1st Faculty of Medicine, Charles University, Prague.

This work was supported by the grant projects 303/03/1115 and 301/05/0859 from Czech Science Foundation and by grant 0021620806 from Ministry of Education and Youth of the Czech Republic.

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1. Introduction

1.1. *Caenorhabditis elegans* - a model organism

Caenorhabditis elegans is a small, free-living, soil nematode that lives across most of the world. *C. elegans* feeds on bacteria. It has a constant number of somatic cells. *C. elegans* has two genders – males and hermaphrodites. Adult hermaphrodites have 959 somatic nuclei, adult males 1031. Cell lineages are well characterized. *C. elegans* has a short life cycle, has small size and can be easily cultivated under laboratory conditions. 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. Adult animals are approximately 1 mm long. In the case of unfavorable environmental factors, a specific larval stage (dauer larvae) develop instead of the normal L3 larvae. Dauer larvae do not feed and survive several months. When food becomes available, dauer larvae molt to the normal L4 larvae (Hope, 1999).

C. elegans embryogenesis has two distinct periods (Sulston *et al*., 1983). The first one comprises of mitotic cell divisions generating founder cells cells. Initial cell divisions generate six cells of unequal size called founder-cells: AB, E, MS, C, D, and P_4. Each of stem cell lineages has its own internal clock and 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*., 1978). 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.

During post-embryonic development, germ line proliferates. Mature gonads are formed during the L4 stage. 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).

1.1.1. Genetics

Wild-type *C. elegans* hermaphrodites contain five pairs of autosomes and one pair of X chromosomes. Males contain five pairs of autosomes and a single X chromosome. The condensed chromosomes are cytogenetically indistinguishable. The *C. elegans* chromosomes have diffuse kinetochores (Albertson and Thomson, 1982).

The genome has a relatively small size - approximately 97 Mb. The *C. elegans* Genome Sequencing Consortium was established to determine the entire *C. elegans* DNA sequence. The essentially complete sequence was published in Science in December 1998. 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/], WORM ATLAS [http://www.wormatlas.org/] and many others.

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 and trans-spliced. During the *trans*-splicing, a short RNA leader is attached to the 5´end of processed mRNA. Two types of splice leaders were described in *C. elegans* – SL1, which is *trans*-spliced to 5´ends of monocistronic genes and the first genes in operons and SL2, which is *trans*-spliced as second and additional genes in operons.

Protein-coding genes are distributed over the whole genome. The genome encodes approximately 22,000 genes. Alternative splicing is observed. The number of splicing isoforms per gene is low (Spieth and Lawson, 2006). Two types of pseudogenes could be found in *C. elegans* genome. Processed pseudogenes (sporadic in *C. elegans*) are created by reverse transcription of mRNA into cDNA and its subsequent insertion into gDNA. Unprocessed pseudogenes arise by gene duplication and subsequent disablement. They usually have frameshifts, premature STOP codons, insertions, etc. (Harrison *et al*., 2001). The number of pseudogenes in *C. elegans* (561 annotated) is still not well estimated.

1.1.2. 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 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 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).

1.1.3. 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.

1.1.4. 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).

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).

1.1.4.1. 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 a 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).

1.1.4.2. 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). 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).

1.1.4.3. 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. Actin filaments and microtubules were shown to be required for successful 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 (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 (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).

1.1.4.4. 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). 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.

1.1.4.5. 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 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. 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 male sensory rays. Seam cells originating from divisions of these three seam cells in males do not fuse and do not form alae (Sulston and Horvitz, 1977). At the end of the L4 stage, seam cells fuse.

1.2. Nuclear hormone receptors (NHRs)

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).

1.2.1. Structure of NHRs

All NHRs have conserved structure, which consists of 5 domains. The first N-terminal domain (A/B) is not conserved. 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).

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. 1). 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 HRE. 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.

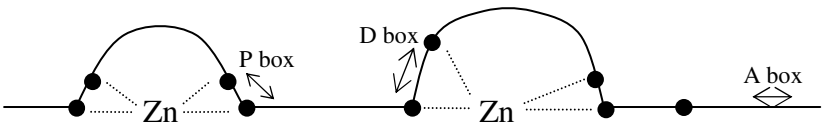

Fig. 1: DNA binding domain of nuclear receptors

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

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

A ligand binding domain (LBD, E domain) is conserved. 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).

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

1.2.2. 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).

1.2.3. 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). Such diversity appears to be a characteristic of nematodes 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).

2. Materials and methods

2.1. Materials

Following *C. elegans* strains were used: **N2** wild type animals (var. Bristol), **JR667** (expressing seam cell GFP marker), **SU93** (expressing *ajm-1*::GFP marker), **PD7963** (expressing *hlh-1*::GFP marker) **#4991** (*chr3::gfp* (*nhr-23::gfp*) 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.

Following vectors were used: **pRF4** (containing the *rol-6(su1006)* mutant collagen gene), **L4440** (containing two convergent T7 polymerase promoters), **pCR®4-TOPO® Cloning Vector** (Invitrogen) for the direct insertion of *Taq* polymerase-amplified PCR products and containing T3 and T7 polymerase promoters, **pPD95.67** and **pPD95.75** (promoterless GFP vectors), **pPD49.83** carrying the hsp16-41 promoter sequence. Vectors **L4440**, **pPD95.67**, **pPD95.75** and **pPD49.83** were kindly afforded by Dr. Andrew Fire (Stanford University School of Medicine, Stanford CA, USA).

2.2. Methods

2.2.1. Molecular biology

All molecular biology methods as gDNA and total RNA isolation, PCR, single worm PCR, reverse transcription PCR (RT-PCR), quantitative real-time PCR (qPCR), cloning, transformation, in vitro transcription as well as western blot analysis, immunocytochemistry, co-immuno precipitation etc. were performed according to standard protocols.

2.2.2. Maintenance of *C. elegans*

2.2.2.1. Worm cultivation and synchronization

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. Worms could be transferred on a new plate using a chunk of agar from an old plate or picked individually using a platinum wire with a hook.

Synchronization: Worms (*C. elegans* gravid hermaphrodites) were collected in 12 ml centrifuge tubes and washed. 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 till worms disappeared (only embryos stay visible). After that, embryos were washed with water several times and were incubated overnight at room temperature, shaking. Next day, the worm culture was synchronized in L1 stage.

2.2.2.2. Preparation of transgenic *C. elegans* strains

The plasmid DNA of construct was co-injected with pRF4 plasmid 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. Transgenic worms carrying specific GFP fusion constructs were observed using Olympus SZX12 Stereomicroscope System or Olympus BX60 System Microscope both equipped with a light fluorescence attachment.

2.2.2.3. RNA interference

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 et room temperature. Worms were placed on ordinary NGM plates with OP50 and followed for phenotypical or behavioral changes throughout their development.

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 (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.

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.

2.2.2.4. 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.

2.2.2.5. 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). Then standard immunostaining followed.

3. 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

4. Results

4.1. Characterization of the *nhr-60* gene

NHR-60 belongs to the Class I of nuclear receptors. *nhr-60* is a member of subgroup of 18 *C. elegans* supnrs that have the P-box sequence CNGCKT. The *nhr-60* gene is localized on chromosome V (cosmid F57A10.5) and consists of seven exons spanning approximately 2.7 kb (Fig. 2). The gene prediction is shown on WormBase web (www.wormbase.org, release WS150, Nov. 30, 2005).

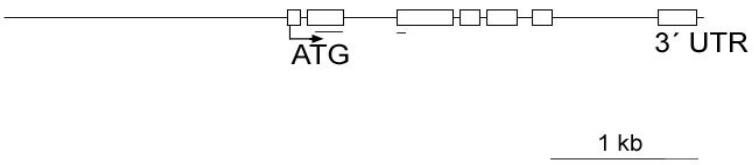

Fig. 2: Schematic representation of *nhr-60* genomic organization
nhr-60 exons are marked as open rectangles. DBD domain is underlined.

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.

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 and sense primer SL1 or SL2 respectively was performed. Amplified DNA fragment from PCR with SL1 was purified from the agarose gel and sequenced. PCR with SL2 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 confirmed the gene structure prediction with a single prominent splicing isoform.

4.2. *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. 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. 3). We suggest to name CBG22907 as *Cbnhr-60*.

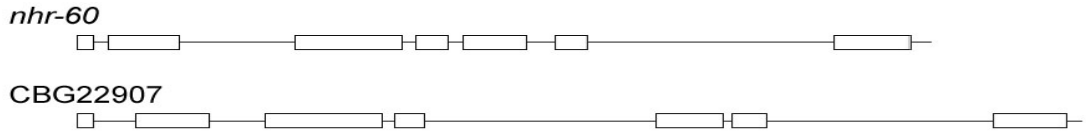

Fig. 3: A comparison of genomic organization of *C. elegans nhr-60* and *C. briggsae* CBG22907
Exons are marked as open rectangles. Note their conserved number and length.

4.3. *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 *ama-1* DNA fragments 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 results revealed that *nhr-60* is expressed in all developmental stages and the expression decreases in L4 and adult stage (Fig. 4A).

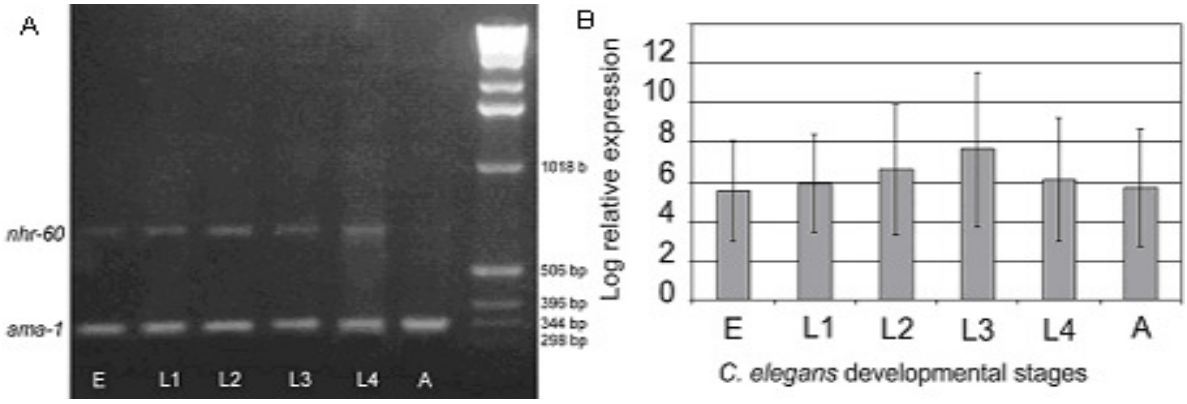

Fig. 4: *nhr-60* expression profile using semiquantitative (A) and quantitative (B) PCR

For quantitative PCR obtained results were normalized to *ama-1* or *act-1*. *nhr-60* expression levels during individual developmental stages were calculated from calibration curves. Using qPCR we confirmed that *nhr-60* is expressed in all *C. elegans* developmental stages. *nhr-60* expression is increased in L3 and then decreases in L4 and adults (Fig. 4B).

4.4. 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 of N2 worms were performed. The analysis revealed the presence of two NHR-60 specific bands: the prominent band migrating at 65 kDa and the minor band migrating at 50 kDa (in case when high quantities of protein lysates were used for the analysis). The minor band has up to approximately 10% of the intensity of the prominent band. An additional band (migrating at 36 kDa) was detected. This protein comes from contamination with OP50 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. 5).

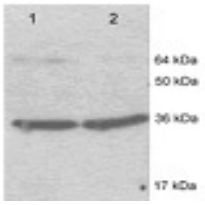

Fig 5: Western blot analysis of NHR-60 in wild type embryos and in embryos following *nhr-60* RNAi. Protein lysates from wild type embryos (lane 1) and embryos subjected to *nhr-60* RNAi (lane 2) were analyzed. *nhr-60* RNAi resulted in decreased detection of protein migrating at 64 kDa. The 36 kDa protein 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 nuclei. NHR-60 was detectable from the 1-cell stage of development till 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. 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 heat-shock driven promoter

4.5. *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 included either 1,950 bp, 580 bp or 340 bp upstream of the predicted start of translation and gDNA up to the part of the second exon (Fig. 6).

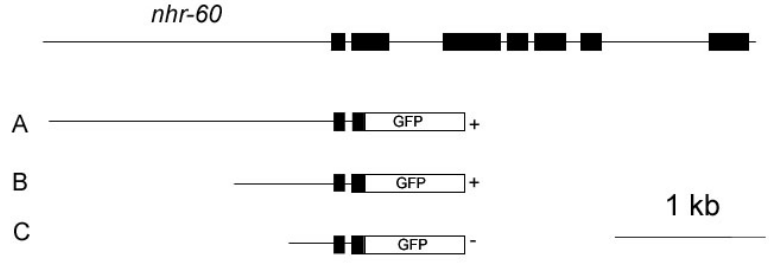

Fig. 6: A schematic representation of *nhr-60*::GFP transcriptional fusion constructs

Constructs *nhr-60*::GFP A and B are expressed in many different cell types and reveal the same expression pattern for both constructs. Construct *nhr-60*::GFP C did not show any expression. Such lack of expression suggest 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 our 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 a whole larval development. The GFP signal in seam cells disappeared in adult animals after seam cells undergo homotypic cell fusion. We also 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 of the mature tail in addition.

4.6. *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* (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. In contrast, *nhr-60* RNAi had no effect on the expression of *nhr-23*::GFP although *nhr-60* RNAi related affected phenotype was observed.

4.7. 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, 1998, Voinnet, 2005). dsRNA was introduced into worms either by soaking, feeding or by microinjecting. Natural function of *nhr-60* was also blocked and studied 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 heat-shock promoter.

4.7.1. RNA mediated interference of *nhr-60*

nhr-60 specific double stranded RNA was prepared. A part of *nhr-60* cDNA, 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 *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 failed to hatch. The majority of the affected embryos were arrested at the 2-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 atypical spherical shape instead ovoid. Defects were also observed in a small fraction of L1 progeny of hermaphrodites following *nhr-60* RNAi. Affected larvae developed from affected embryos, that 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.

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).

To exclude the possible redundancy in *nhr-60* and *nhr-116* function we blocked also the function of *nhr-116* by RNAi. We did not observe any statistically important effect on progeny following *nhr-116* RNAi.

4.7.2. *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. The average number of observable seam cells in JR667 embryos after *nhr-60* RNAi decreased to 9.8 seam cells per an animal. In control animals (embryos of non-treated JR667 hermaphrodites) 19.1 seam cells per an animal 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.

4.7.3. Expression of NHR-60 dominant –negative isoform

NHRs lacking transcriptional activation domains can function as dominant negative factors (Liu *et al.*, 2002). 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 hours at 31°C) eventually 36% of affected progeny (30 min at 34°C). 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 either at 31°C or at 34°C had no unfavorable 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

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.

4.8. *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.

4.8.1. 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 *ama-1* DNA fragments were co-amplified as internal controls. The results showed that *C. elegans acbp-1* is expressed in all developmental stages. Using qPCR we confirmed *acbp-1* expression in *C. elegans* embryos, larval stages and adult animals as well.

4.8.2. ACBP-1::GFP reporter genes expression

In order to find *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. 7).

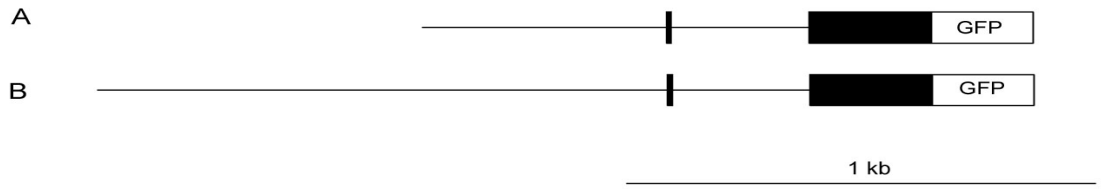

Fig. 7: A schema of ACBP-1::GFP translational fusion constructs. *acbp-1* exons are marked as black rectangles. A: ACBP-1::GFP S construct, B: ACBP-1::GFP L construct

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. Strong expression was observed in the intestinal cells. The expression started in embryos (comma stage) and continued until adulthood. Weak but detectable expression was found also in body wall muscle cells, some epidermal cells, seam cells, and in several neurons. 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.

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

acbp-1 expression was inhibited by RNAi using microinjections of in vitro synthesized *acbp-1* dsRNA into young gonads of 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. 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.

We performed *acbp-1* RNAi also on JR667 worms. Inhibition of ACBP-1 function in these worms revealed reduction and rearrangement of seam cells indistinguishable from the effect of inhibition of NHR-60 function.

Three other *C. elegans* genes (*acbp-2*, *acbp-3*, and *acbp-4*) have sequences highly similar to *acbp-1*. We followed also worms after *acbp-2*, *acbp-3* and *acbp-4* RNAi. Inhibition of either ACBP-2, ACBP-3 or ACBP-4 did not induce the same not even similar phenotype as inhibition of ACBP-1.

4.10. 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

(ACBP::GFP and NHR-60) co-localize in the peripheral regions of nuclei in embryos and in intestinal cells of larvae. Co-localization in some epidermal cells was also observed but with lower intensity.

4.11. 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 (the primary goat IgG anti-GFP antibody and the secondary anti-goat IgG antibody conjugated with horseradish peroxidase). No protein was detected in the control (Fig. 8). This result suggests an interaction between NHR-60 and ACBP-1.

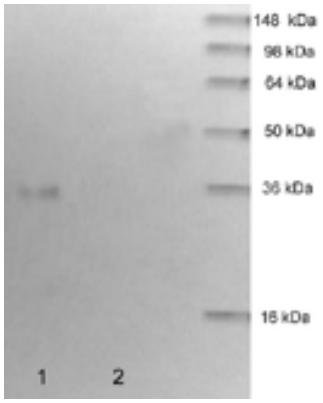

Fig. 8: NHR-60/ACBP-1::GFP co-immuno precipitation

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 the experimental sample (ACBP-1::GFP expressing worms, lane 1). In control (N2 wild type worms, lane 2) no band was detected using antibody against GFP.

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 misposition 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 fucntion.

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 ingteraction 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.

8. 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 *Caenorhabiditis 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.: 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.

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9. Abbreviations

ACBP	acyl-coenzyme A binding protein
AF-1	activation function-1
AF-2	activation function-2
C. elegans	*Caenorhabditis elegans*
cDNA	complementary DNA
CGC	Caenorhabditis Genetics Center
CTE	carboxy terminal extension
DBD	DNA binding domain
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
Fig	figure
gDNA	genomic DNA
GFP	green fluorescent protein
HNF4	hepatocyte nuclear factor 4
HRE	hormone response element
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
LBD	ligand binding domain
Mb	Megabase
mRNA	messenger RNA
NGM	nematode growth medium
NHR	nuclear hormone receptor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SL1	splice leader 1
SL2	splice leader 2
ssRNA	single stranded RNA
supnr	supplementary nuclear receptor
TTBS	Tris Tween buffered saline

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