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Functional genomics of nuclear hormone receptors and their cofactors:

Connection between metabolism and development by diversified nematode nuclear hormone receptors

PhD thesis summary

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1 Introduction

1.1 General description of *Caenorhabditis elegans*

Caenorhabditis elegans is one of the model organisms used in molecular biology for a variety of reasons. The main advantage of the model organism is its multicellular eucaryotic character. The next advantage of the model organism is its transparent cuticle, thus all tissues and individual cells can be nicely observed (Julian and Davies, 2003). *Caenorhabditis elegans* is a small organism that is free-living and can be found across (most of) the world. The generation time is about 3.5 days and the nematode grows to a length of 1.3 mm with a diameter of 80 µm if there is a sufficient supply of food. The nematode feeds on bacteria.

C. elegans exists in two genders, hermaphrodite (XX) and male (XO) although the incidence of males is 1:500. Approximately 300 eggs of *C. elegans* are laid by the hermaphrodite. After hatching, the animals pass through four larval stages before reaching adulthood: Ll, L2, L3 and L4 (each punctuated by molt) (WormBase web site). In unfavorable environmental factors, a specific larval stage (dauer larva) develops instead of the normal L3 larva. Dauer larvae do not feed and survive several months. When food becomes available, dauer larvae molt to be normal L4 larvae (Hope, 1999).

C. elegans anatomy is simple. The body is tubular. The mouth is connected with the tubular intestine via the bi-lobed muscular pharynx. Pharynx pumps food through the intestine to the rectum and anus. Body wall muscle cells form four longitudinal rows along the intestine. Beyond the body wall muscle cells is the epidermal body wall covered with an external cuticle. *C. elegans* nervous system consists of dorsal and ventral nerve cords, a circumpharyngeal nerve ring and sensory receptors and ganglia. The reproductive system of adult males consists of a single testis connected with the rectum via vas deferens. Vas deferens and the rectum join up posteriorly and form a cloaca. Sexual structures are in the male tail. The reproductive system of adult hermaphrodites is formed by two gonadal arms. Each arm contains an ovary, oviduct, spermatheca and leads into the uterus. Gonads terminate at the vulva (Sulston and Horvitz, 1977).

1.2 Nuclear hormone receptors

Nuclear hormone receptors (NHRs) form a large super-family of transcription factors of the Metazoan species and work in content with a set of co-activators and co-repressors to activate or repress a specific target gene expression (Beato et al., 1995; Horwitz et al., 1996; Robyr et al.,

2000). The large NHR super-family is subdivided according to the sequence alignment and phylogenetic tree into six major subfamilies NR1-NR6 (Laudet, 1997; Gissendanner et al., 2004; Robinson-Rechavi et al., 2005; Germain et al., 2006). NHRs are important for many physiological functions such as cellular differentiation, development, metabolism, and defense against xenobiotics.

The NHR family includes proteins that have been identified as intracellular receptors for steroid and thyroid hormones, and small hydrophobic molecules such as retinoids, farnesoids, sterols and related components (Chawla et al., 2001). However, most members of the NHR family have unknown ligands and are called orphan receptors.

NHRs are defined by the highly conserved DNA binding domain (DBD) and a ligand binding domain (LBD) (Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995).

1.2.1 Regulation of transcription by nuclear hormone receptors

Regulation of gene transcription is critical for proper development, growth, tissue maintenance and metabolism. One of the regulatory levels is binding of NHRs to target genes and their gene expression activation or repression. The hormone response elements of the target genes are located in regulatory sequences normally present in the 5´-flanking region. Although the HREs are often found relatively close to the core promoter, in some cases they are present in enhancer region several kilobases upstream of the transcriptional initiation site (Aranda and Pascaul, 2001). Two consensus motifs have been identified: the sequence AGAACA is preferentially recognized by NHRs with CxGCKGFFxR “P box” and the sequence AGGTCA is recognized by mineralocorticoids, glucocorticoids, progesterone and androgen receptors (Umesono and Evans, 1989; Beato et al., 1995; Giguere, 1999). Although some monomeric receptors can bind to a single hexameric motif, most receptors bind as homo- or heterodimers to HREs, composed typically of two core hexameric motifs. For dimeric HREs, the half-sites can be configured as palindromes (Pal), inverted palindromes (IPs), or direct repeats (DRs) (Aranda and Pascaul, 2001).

1.2.2 Ligands

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

1.2.3 Orphan receptors

The nuclear hormone receptor family includes members with unknown ligands. They are called orphan receptors. Orphan receptors represent a diverse and ancient component of the nuclear receptor super-family, being found in nearly all animal species examined. Orphan nuclear receptors provide a unique and, until recently, largely untapped resource to uncover regulatory systems that impact on both health and human disease (Blumberg and Evans, 1998).

Recently, natural and synthetic ligands have been identified for several orphan receptors: xenobiotics (steroids, antibiotics) for PXR (Kliewer et al., 2002), prostaglandin derivates, fatty acids and leukotriens for PPAR (Desvergne and Wahli, 1999; Emery et al., 2001). Several subfamilies of orphan receptors have been found in metazoan organisms (Baniahmad and Tsai, 1993; Giguere, 1999).

1.2.4 Nuclear hormone receptors in *Caenorhabditis elegans*

The number of NHRs found in genomes of metazoan species varies from 48 in man, and 18 in *Drosophila melanogaster*, to more than 280 in the genome of *Caenorhabditis elegans* (Enmark and Gustafsson, 2001; Maglich et al., 2001; Van Gilst et al., 2002; Robinson-Rechavi et al., 2003; Ruau et al., 2004; Gissendanner et al., 2004; King-Jones and Thummel, 2005; Antebi, 2006). Among the predicted NHRs in *C. elegans*, 15 are orthologs of vertebrate and *Drosophila* genes. Most of these conserved receptors have regulatory functions in development (molting, dauer larva formation, epidermal cell development and differentiation, toxin resistance, neuronal development and sex determination); summarized in Sluder and Maina, 2001; Gissendanner et al., 2004; Antebi, 2006. The remaining 269 NHRs are now classified as supplementary nuclear receptors (supnrs). These receptors seem to be specific for nematode species and are distantly related to hepatocyte nuclear factor 4 (HNF-4) (Robinson-Rechavi et al., 2005). Vertebrate HNF-4 ((NR2A) in the Unified Nomenclature System for the Nuclear Hormone Receptors superfamily (http://www.ens-lyon.fr/LBMC/laudet/nurebase/nurebase.html (Duarte et al., 2002)) regulates lipid and glucose metabolism and differentiation of hepatocytes and enterocytes (Li et al., 2000; Hayhurst et al., 2001; Watt et al., 2003; Stegmann et al., 2006).

The function of most *C. elegans* supnrs is unknown. Nevertheless, the list of supnrs that have regulatory functions is growing. NHR-49 regulates genes involved in nutritional response and fatty acid beta-oxidation (Van Gilst et al., 2005 a, b; Atherton et al., 2008). A group of supnrs related to ODR-7 (Sengupta et al., 1994) called divergent NR genes (Miyabayashi et al., 1999) (or class III of *C. elegans* NHRs (Van Gilst et al., 2002)) were functionally studied and three of the 28

genes showed developmental phenotypes if overexpressed as GFP fusion genes (Miyabayashi et al., 1999).

In experiments on *C. elegans* a variety of processes, such as molting, dauer formation, epidermal cell development and differentiation, ovulation, vulval development and function and toxin resistance, are affected by the inhibition of these NHRs by RNA interference. Defects in neuronal development, sex determination and dauer formation, are seen on mutants in four of the conserved NHRs. A variety of developmental processes are influenced by the conserved *C. elegans* NHRs.

The inhibition of these NHRs by RNA interference in experiments on *C. elegans* affects a variety of processes including molting (*nhr-23* (NR1F4)), *nhr-25*(NR5A3), *nhr-67*(NR2E2)) (Kostrouchova et al., 1998; Asahina et al., 2000; Gissendanner and Sluder, 2000; Kostrouchova et al., 2001; Duarte et al., 2002; Ruau et al., 2004; Gissendanner et al., 2004), dauer formation (*nhr-41*(NR2D1), *nhr-85*(NR1D)), ovulation (*nhr-6*(NR4A4)) (Gissendanner et al., 2004), epidermal cell development and differentiation (*nhr-25*(NR5A3)) (Chen et al., 2004 and Silhankova et al., 2005), vulval development and function (*nhr-67*(NR2E2), *nhr-85*(NR1D)), and toxin resistance (*nhr-8*(NR1I/J)) (Gissendanner et al., 2004). Mutants in four of the conserved NHRs show defects in neuronal development (*fax-1*(NR2E5), *unc-55*(NR2F7)), sex determination (*sex-1*(NR1G1)), and dauer formation (*daf-12*(NR1J)) (Carmi et al., 1998; Zhou and Walthall, 1998; Antebi et al., 2000; Much et al., 2000). The conserved *C. elegans* NHRs play critical roles in a variety of developmental processes similarly as in other animals studied so far.

We focused our studies on a subgroup of *C. elegans* NHRs which belong to the class I receptors according to the P box sequence classification scheme (Van Gilst et al., 2002). The P box sequence (CNGCKT) of the class I is the most similar to that of the vertebrate nuclear hormone receptors classified in unified classification of nuclear hormone receptors (Duarte at al., 2002; Ruau et al., 2004) as subfamily NR2 (that includes group A (HNF4), group B (including RXRs), group F (including COUP-TF), and subfamily NR1 (that includes group B (retinoic acid receptors) and group I (vitamin D receptor, VDR)). The receptors classified as NR1 and NR2 have the P box sequence CEGCKG, highly similar to CNGCKT, although the asparagine substitution for glutamic acid removes the negative charge (Brozova et al., 2006). A subgroup 8 of *C. elegans* nuclear hormone receptors with CNGCKT P box contains 18 members (NHR-60, NHR-116, NHR-168, NHR-141, NHR-129, NHR-40, NHR-1, NHR-47, NHR-10, NHR-17, NHR-68, NHR-101, NHR-136, NHR-153, NHR-154, NHR-209, NHR-120, NHR-137).

2 The aim of the study

The aim of the study was to contribute to the understanding of regulatory functions of nuclear hormone receptors by studies focused on a group of multiplied nuclear receptors present in the genome of *Caenorhabditis elegans*, specifically *nhr-40* and *nhr-60*.

The work presented in this thesis was a part of an effort to characterize the selected nuclear receptors systematically by means of *Caenorhabditis elegans* functional genetics and genomics. Studies constituting this thesis were meant as an application of proteomics in the functional analysis of the selected nuclear receptors in the frame of the complex biology of *Caenorhabditis elegans*.

The proteomic scope of this thesis was aimed at understanding of the selected receptors at the level of the protein, the protein nature, localization and dynamics as well as the proteomic characterization of the consequences of the protein loss of function.

2.1. The research strategy

We have studied several NHRs, including NHR-40 and NHR-60, that are supnrs belonging to a subgroup of 18 *C. elegans* NHRs characterized by the DNA binding sequence CNGCKT (Brozova et al., 2006; Simeckova et al., 2007).

Previously, Brozova et al. showed that inhibition of NHR-40 function by an apparent hypomorphic mutation in the gene or RNA interference (RNAi) induces a spectrum of defects including embryonic arrest, irregular body shape, defective muscle development and abnormal locomotion.

NHR-40 exemplifies the challenge in understanding the function of this large family of related supnrs. In order to extract more information from genetic knockdowns, we explored proteomic methods that might give us an additional insight into supnr function. In this work, we used comparative two-dimensional chromatography and mass spectrometry to characterize further the *nhr-40* (*ok667*) mutant phenotype at the proteome level. Using synchronized larval cultures of wild type worms and homozygous *nhr-40* (*ok667*) mutants, we were able to identify altered protein chromatographic profiles. The majority of the proteins with altered profiles were classified by gene ontology terms as being related to muscle and metabolism. These results are consistent with the previous phenotypic characterization of NHR-40 function and extend our insight by revealing a novel link to metabolism. Changes in the metabolic proteome led us to uncover an unexpected effect of temperature and caloric intake on the penetrance of the mutant phenotype. Comparative two-dimensional chromatography represents a relatively rapid and simple method to explore

metabolic phenotypes in any viable mutants and should be particularly useful in probing the function of the large family of supnrs in nematodes.

We focused also on NHR-60, another member of the subgroup of class I NHRs characterized by the P box sequence CNGCKT. NHR-60 is expressed ubiquitously, with a higher level in seam cells. Interestingly, it has a predominant localization on the periphery of nuclei. Its inhibition by RNAi or by expression of its C-terminally deleted mutant induces embryonic and early larval arrest with gross developmental defect including misposition and missing seam cells (Simeckova et al., 2007).

3 Material and Methods

Strains Wild type *C. elegans* N2 worms and the RB840 strain harboring the *nhr-40* (*ok667)* deletion were generously supplied by the Caenorhabditis Genetic Center (CGC) (Caenorhabditis Genetics Center, University of Minnesota, Minneapolis, MN, USA). Worms were cultured on Nematode Growth Medium plates and fed *Escherichia coli* OP50 as described (Brenner, 1974).

Synchronization and cultures Synchronized L1 larvae of wild type and mutant animals were prepared by bleaching gravid adult hermaphrodites and hatching embryos overnight in liquid without food. Synchronized and arrested L1 larvae were seeded on plates with a bacterial lawn grown overnight at room temperature. To segregate *nhr-40 (ok667)* mutant larvae with obvious movement defects from unaffected siblings, hatched L1s were placed outside the area covered by bacteria and animals able to move to food were collected independently of those that could not. In selected experiments, mutant embryos or larvae were inoculated directly to the bacterial lawn in order to eliminate the effect of starvation of non-moving larvae. Cultures were incubated at either 16 °C, 20 °C and 24 °C. These cultures were collected and stored at -80 °C in aliquots that were later used for proteomic analyses.

Chromatography Two-dimensional chromatographic protein separation was performed using a commercially available system ProteomeLab™ PF 2D Protein Fractionation System (Beckman Coulter, Inc. Fullerton, CA). Two mg of total protein in 2 ml volume was separated by chromatofocusing and then the first dimension fractions were separated by reversed phase chromatography.

Mass spectrometric analysis and data processing Fractions chosen for mass spectrometric analysis were dried down, the pellets were dissolved in 15 µl of cleavage buffer containing 0.01 % 2-mercaptoethanol (Sigma Aldrich, St. Louis, MO), 0.05 M 4-ethylmorpholine acetate pH 8.1 (Fluka, Buchs, Switzerland), 5 % MeCN (Merck, Darmstadt, Germany), and 10 ng/µl sequencing grade trypsin (Promega, Madison, WI). Digestion was carried out overnight at 37 °C and the resulting peptides were subjected to mass spectrometric analysis.

Five microliters of tryptic peptide mixture was applied on the Magic-C18 column. The column was connected to a LCQDECA ion trap mass spectrometer (ThermoQuest, San Jose, CA) equipped with a nanoelectrospray ion source. Spectra were searched with the SEQUESTTM software against the SwissProt database.

Meta-data analysis Gene ontology analysis was done as described (Boyle et al., 2004). Genes corresponding to proteins that showed altered chromatographic profiles in *nhr-40(ok667)* and control worms were analyzed using the *C. elegans* database (http://www.wormbase.org/ WormBase Release 190).

Western blot Western blots were performed using a standard protocol. Protein concentration was estimated using BCA Protein analysis kit (Pierce, Rockford, IL) as recommended and 80 μg of proteins were loaded for each sample. The primary rabbit polyclonal IgG anti-NHR-60 (Simeckova et al., 2007) and mouse monoclonal IgG anti-MYO-3 (Miller et al, 1983; Miller et al., 1986) antibodies were used. For densitometric analysis, the films were scanned and analyzed by Image J program available at http://rsb.info.nih.gov/ij/.

Immunocytochemistry Immunocytochemistry and light microscopy were done as described (Brozova et al., 2006; Simeckova et al., 2007).

Molecular biology methods All molecular biology methods as total RNA isolation, PCR, reverse transcription PCR (RT-PCR), quantitative real-time PCR (qPCR), cloning, transformation, in vitro transcription as well as RNA interference were performed according to standard protocols (www.wormbase.org).

4 Results

4.1 Proteomic analysis uncovers a metabolic phenotype in *C. elegans* after *nhr-40* reduction of function

4.1.1 Proteome characterization by differential two-dimensional liquid chromatography

To detect differences in proteome composition between wild type and mutant larvae, we prepared total protein from synchronized, starved L1s of N2 and homozygous *nhr-40 (ok667)* animals and then separated proteins using chromatofocusing. First dimension chromatography separated all proteins into 37 fractions based on the isoelectric point. Each fraction was then automatically separated into an additional 35 fractions by the second dimension, reversed phase HPLC based on surface hydrophobicity. Results from these fractionation methods were very robust and reproducible. Second dimension chromatograms of repeated first dimension fractions yielded almost identical patterns as did comparable fractions from independently prepared material. For example, we tracked the elution profile of NHR-60, another supnr for which we have reliable antibodies and experience; this relatively low abundant transcription factor can be easily detected by Western blot analysis. NHR-60 was eluted repeatedly at the same time in both dimensions indicating high resolution separation and reproducibility for non-abundant proteins.

4.1.2 Identification of altered chromatographic pattern of muscle and metabolic proteins in *nhr-40* (*ok667*) larvae

Comparison of paired chromatograms obtained from *nhr-40* (*ok667*) and control larvae showed similarities in some major peaks and in the trend of curves, but many major peaks were clearly different. It was possible to manually overlay chromatograms using the computer program 32Karat provided with the apparatus and to identify grossly different peaks and corresponding fractions.

The same result was obtained using the computer programs ProteoVue and DeltaVue which graphically represent the chromatographic peaks as artificial electrophoretic bands shown in green or red color for each compared sample separately. The overlaid picture then highlights the chromatographic peaks containing more protein estimated by absorbance at 214 nm. The baseline of

chromatograms was apparently affected by mobile phase gradient and differed in individual chromatograms. In the majority of cases, the baseline shape was almost identical in paired chromatograms but in some cases one of the chromatograms had a higher baseline compared to the corresponding chromatogram and this resulted in green or red coloration of the fraction profile. Nevertheless, grossly different peaks were recognized by the ProteoVue and DeltaVue computer programs and were always controlled on classical chromatogram curves in 32Karat software. The comparison of complete chromatograms identified approximately 50 major peaks recognized by the DeltaVue program as more abundantly expressed in control fractions and 10 as more abundant in the *nhr-40 (ok667*) worms (Fig. 1). Two complete analyses and one analysis with only selected second dimension separations were done.

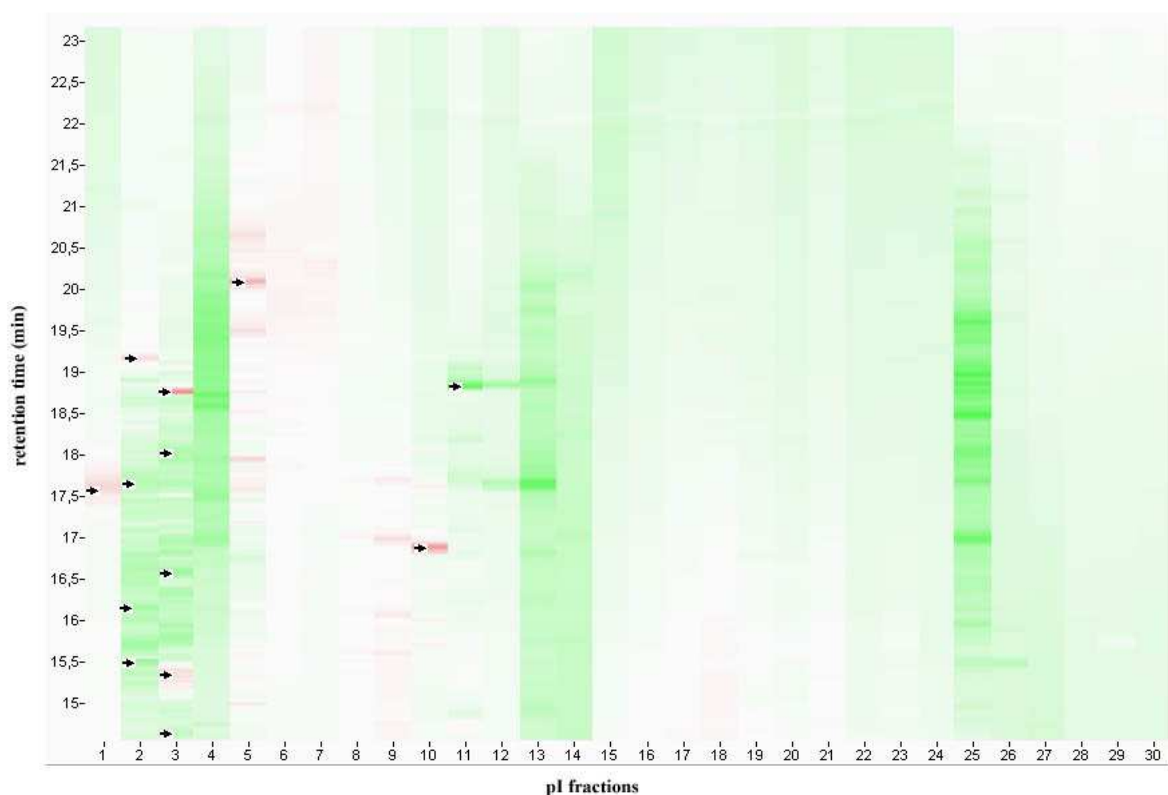

Fig. 1: Representative part of the whole proteome differential display of second dimension chromatograms prepared using the program DeltaVue

Green bands represent fractions with greater absorbance at 214 nm in *nhr-40 (ok667)* worms whereas red bands were higher in control animals. The tendency of a single dominant color in individual lines reflects a higher baseline in the particular chromatogram. Fractions corresponding to bands marked by arrows were analyzed by mass spectrometry.

We performed mass spectrometric analysis on twenty-six paired fractions that were different between strains to identify the protein components represented in these fractions. To control for slight differences in elution profiles, we also searched if proteins that were reduced or missing in a given fraction from one strain were actually eluted in a neighboring fraction in either the first or the second dimension of chromatography. Elution of the missing protein in neighboring fractions was not observed in eight neighboring fractions analyzed. Chromatographic fractions that corresponded to identified peaks of paired fractions were prepared and analyzed using liquid chromatography - tandem mass spectrometry (LC/MS/MS) for identification of present proteins by peptide microsequencing to derive sequence of individual peptides. Using these approaches we were able to identify a discrete set of proteins that had significantly altered chromatographic profiles, presumably due to alterations in post-translational modifications, turnover rates, or alterations in gene expression between wild type and mutant animals.

Gene Ontology (GO) analysis for the terms “cellular distribution” and “function” classified most proteins that were missing in *nhr-40* (*ok667*) fractions as muscle and metabolism related (Fig. 2). In contrast, GO classification of proteins found to be differentially present in *nhr-40* (*ok667*) larvae indicated proteins related to oxidative stress over-represented (SOD-1, SOD-5) and proteins related to elevated translation (RPS-28, RPL-7, ELT-4).

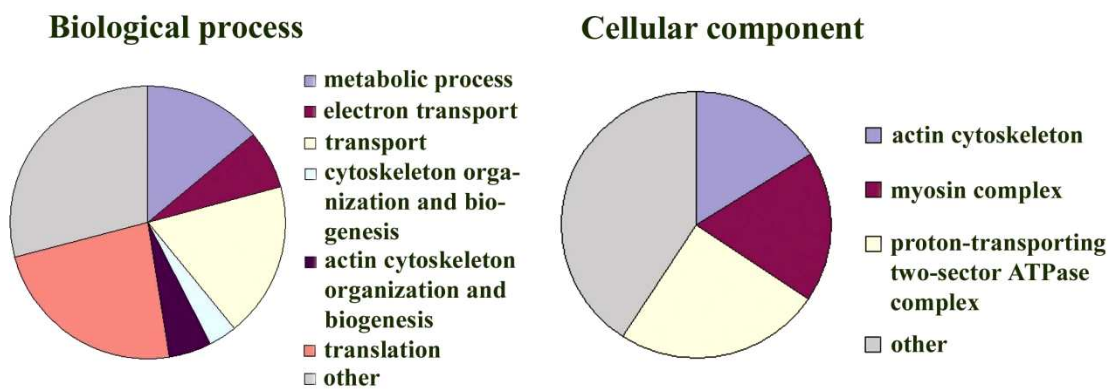

Fig. 2: Gene Ontology analysis of differentially eluted proteins

Proteins identified as decreased or absent in *nhr-40 (ok667)* worms were linked to GO categories (Boyle et al., 2004) of biological process or cellular component.

4.1.3 Proteomic analysis uncovers a muscle phenotype

Our observation that proteins related to the muscle function have abnormal chromatographic patterns in *nhr-40 (ok667)* mutants was consistent with phenotypic characterizations that

demonstrated defects in myogenesis and locomotion in these mutants. Keeping with the published results that employed visualization of muscle fiber formation by phalloidin staining (Brozova et al, 2006), immunocytohemistry showed irregular structure of myosin fibers in morphologically affected animals but not visible differences in mutant larvae that were able to overcome the body shape defect (Fig. 3).

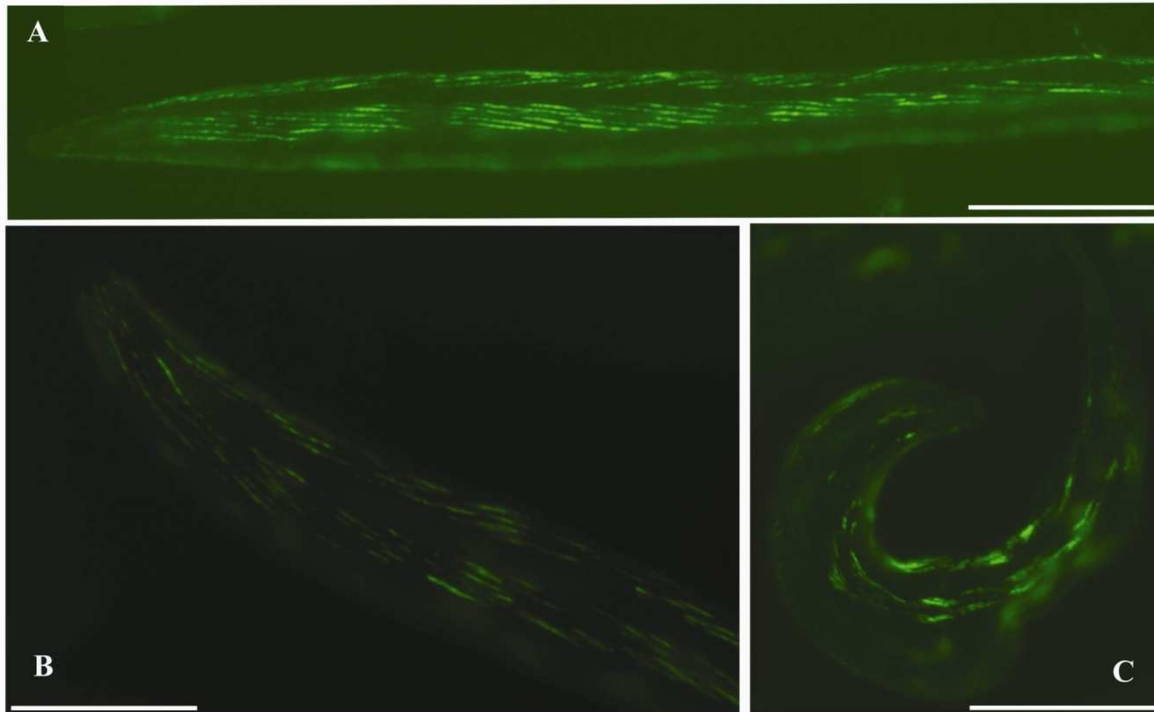

Fig. 3: Detection of MYO-3 by immunocytochemistry

Detection of MYO-3 by immunocytochemistry in L1 wild type (A) and *nhr-40 (ok667)* mutant larvae (B and C). Panel B shows a mutant larva that was able to escape the mutant muscle phenotype with well organized myofibrils whereas the larva shown in panel C (representing an animal with locomotion defects) has severely disrupted myosin fibrils. Scale bar: 20 µm.

One of the altered muscle protein profiles identified in the current proteomic analysis was for MYO-3. MYO-3 is the minor myosin heavy chain isoform of body wall muscle that forms the central component of the muscle thick filament and nucleates filament assembly (Miller et al., 1983; Miller et al., 1986; Waterston, 1989; Moerman and Williams, 2006). Body wall muscles comprised more than 10 % of the body mass in *C. elegans* (Francis and Waterston, 1985) making MYO-3 a very abundant protein.

To explore the altered MYO-3 profile in more detail, we analyzed *myo-3* gene expression and MYO-3 protein levels in *nhr-40 (ok667)* mutants compared to wild type animals. Using

quantitative reverse transcription polymerase chain reaction (qPCR) analysis, we found that *myo-3* is expressed at similar levels in wild type and homozygous *nhr-40 (ok667)* mutants, as were several other muscle structural genes we assayed (Fig. 4). We did find that several muscle-related genes were slightly decreased in expression in the mutant, including *act-1*, *unc-52* and *myo-2* although none of the protein products of these genes were identified in our proteomic analysis. These results suggest that the change in the MYO-3 elution profile was not due to changes in gene expression between wild type and *nhr-40 (ok667)* mutants.

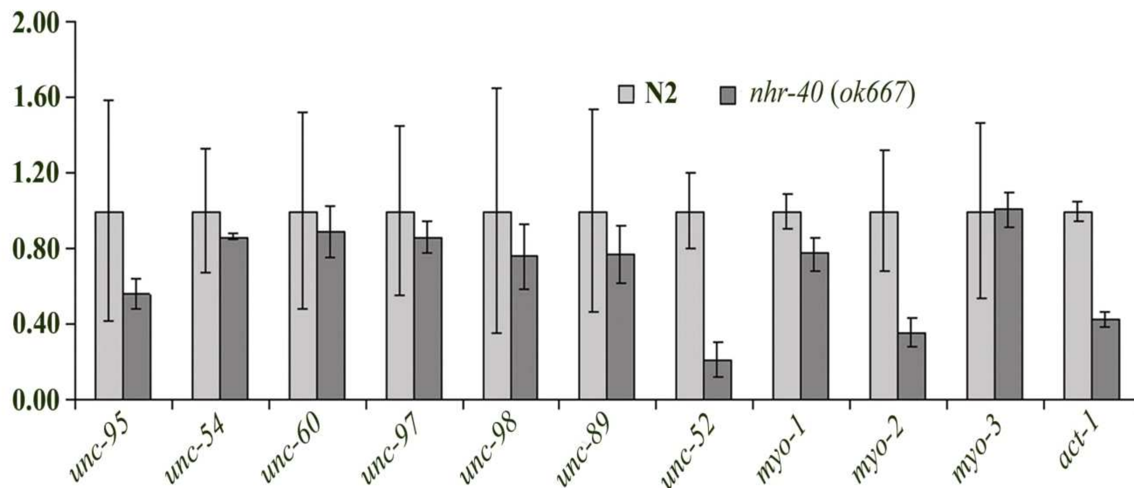

Fig. 4: The expression of selected muscle genes using qPCR

The expression of selected muscle genes in wild type and mutant L1 larvae under normal conditions from two independent experiments was essayed by quantitative PCR. cDNA was prepared from total RNA extracts of the control and the experimental samples. Relative levels of the expression were determined from calibrated experiments normalized to *ama-1* expression. The results were given from two independent experiments done in triplicate and standard deviation bars are indicated (in arbitrary units).

We also assayed MYO-3 protein levels by Western blot in wild type and *nhr-40 (ok667)* mutants using a specific antibody kindly provided by D. Miller (Miller et al., 1983; Miller et al., 1986). We were surprised to see that MYO-3 levels, as detected by this antibody, were much more abundant in the mutant animals compared to wild type animals (Fig. 5). This was in contrast with our proteomic analysis in which MYO-3 was decreased or eliminated from its normal chromatographic migration. Given that the level of *myo-3* expression is nearly identical between N2 and *nhr-40 (ok667)* animals, we interpret our proteomic and Western blot results to suggest that MYO-3 undergoes a different modification in the mutant animals, perhaps due to miss-assembly of the myofibril lattice resulting in aggregation. The size of MYO-3, about 229 kDa, precludes us from

detecting typical post-translational modifications (e.g. phosphorylation, ubiquitination, etc.) by size alterations between wild type and mutant animals that might underlie the altered profile. We were also unable to determine by Western blot which *nhr-40* mutant fractions contained the altered MYO-3 because of the low amount of protein in each second dimension fraction and relatively insensitive antibody. Therefore, although we observe that the MYO-3 elution profile is altered in *nhr-40* mutants, we have no insight into the nature of the changes to the altered MYO-3 characteristics.

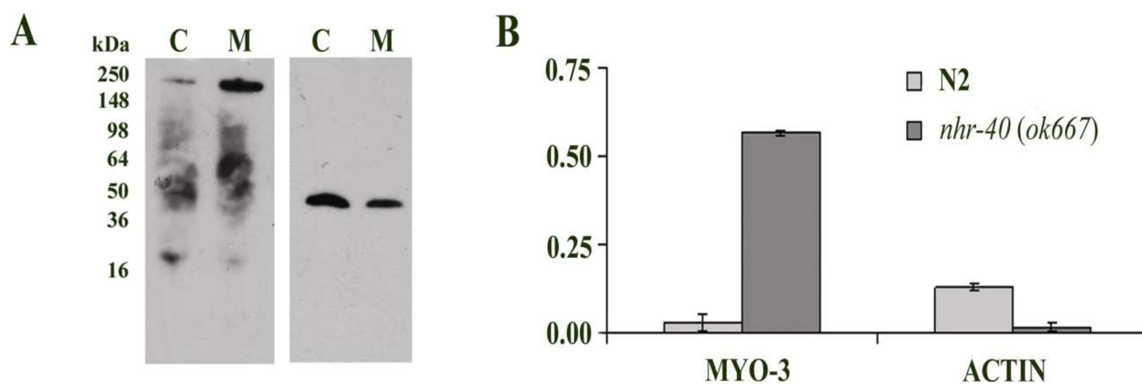

Fig. 5: Detection of MYO-3 and actin in wild type and *nhr-40 (ok667)* larvae by Western blot
A. Levels of myosin heavy chain MYO-3 (left panel) and actin (right panel) in wild type (C) and *nhr-40 (ok667)* (M) larvae detected by Western blot. MYO-3 levels are elevated in mutant larvae relative to actin on the same, reprobed blot. B. Densitometric analysis of MYO-3 and actin Western blot signals. The results were given from three independent experiments and standard deviation bars are indicated (in arbitrary units).

4.1.4 Proteomic analysis uncovers a metabolic phenotype

The identification of metabolic-related genes in the proteomic analysis of wild type and mutant animals led us to investigate the phenotypic effects of different growth conditions. We assayed the locomotion phenotype in two conditions that are known to effect growth rate in *C. elegans*: 1) temperature and 2) caloric intake (feeding).

4.1.4.1 Decreased culture temperature and starvation elevates the penetrance of muscle phenotype in *nhr-40 (ok667)* larvae

Wild type and homozygous mutant animals were synchronized by bleaching gravid adults and the resulting hatched L1s were reared at one of three different temperatures (16 °C, 20 °C, 24 °C). Larvae were placed outside the normal bacterial lawn on plates and incubated at one of these temperatures for 24 hrs after which time the proportion of animals with severe locomotion defects were scored. We found that growth temperature affected the penetrance of the mutant locomotion phenotype. Surprisingly, animals reared at low temperature (16 °C) had a significantly higher penetrance of the locomotion defect than seen at normal (20 °C) or elevated (24 °C) temperatures (Fig. 6).

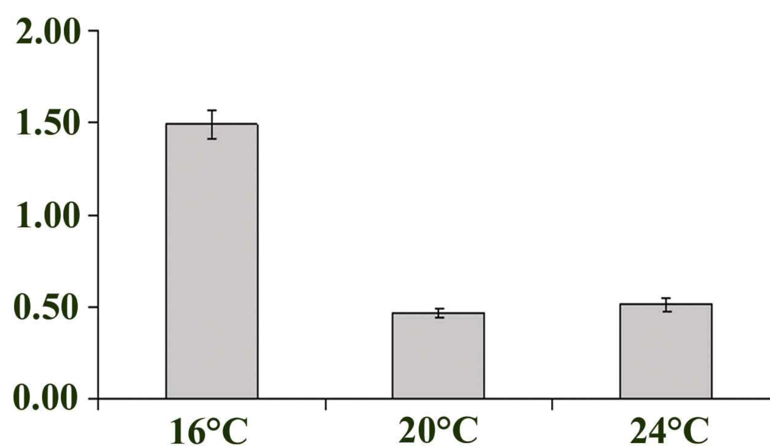

Fig. 6: The influence of culture temperature on the penetrance of *nhr-40 (ok667)* locomotion phenotype

Low culture temperature dramatically increases the proportion of severely affected larvae (n=4,291 for *nhr-40 (ok667*). Y-axis shows ratio of severely affected non-moving larvae and larvae with milder phenotype able to reach food.

As a second perturbation of metabolism, we assayed the penetrance of the *nhr-40 (ok667)* locomotion defects in larvae exposed to different levels of food (bacteria), ranging from starvation to normal feeding. Intermediate levels of bacterial growth were achieved by seeding different concentrations of bacteria on plates and then irradiating the plates to kill most bacteria prior to placing the animals to be assayed on. Decreased availability of bacterial food led to a significantly higher penetrance of the locomotion defect compared to their well-fed siblings (Fig. 7). No similar effect was observed on N2 control larvae.

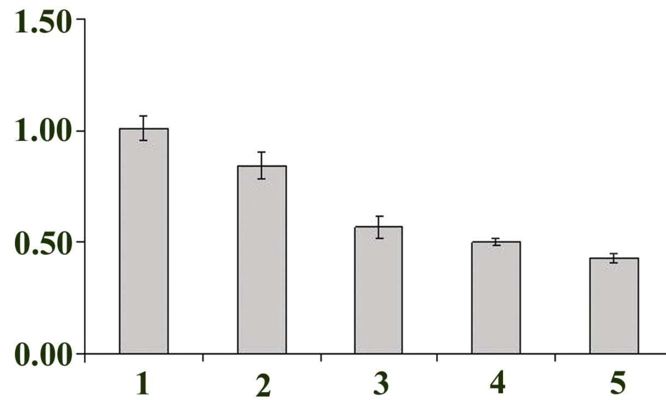

Fig. 7: The effect of food restriction on the *nhr-40 (ok667)* mutant phenotypes

nhr-40 (ok667) larvae cultured on plates with no food, 3.33 x 10^5, 3.33 x 10^6, 3.33 x 10^7, 1.65 x 10^9 bacteria (numbered 1 to 5, respectively) were scored for severity and penetrance of phenotypes; results of three independent experiments. The results show a progressively elevated penetrance of phenotypes as the food supply is restricted (n= 3,044; $P<0.05$ for all food restricted cultures 1, 2 and 3). Y-axis shows ratio of severely affected non-moving larvae and larvae with milder phenotype able to reach food.

Taken together, these results demonstrate that conditions that slow down metabolism and development, low temperature and caloric restriction, exacerbate the locomotion defects. This observation is in strong contrast with most temperature sensitive mutants that have more normal phenotype at lower temperatures (O`Connell et al., 1998; Clark et al., 1997; Hosono et al., 1985) and that favor correct protein folding. Food restriction causes an extension of lifespan in *C. elegans* (Houthoofd et al., 2004; Walker et al., 2005) and can decrease the penetrance of the mutant phenotype of *unc-103* (ERG like K+ channel) (LeBoeuf et al., 2007).

4.2 Proteomic characterization of NHR-60

4.2.1 *nhr-60* is highly expressed in all developmental stages

First of all, we focused on the *nhr-60* expression on the level of transcription. To determine the developmental pattern of *nhr-60* expression in *C. elegans*, we prepared cDNA from embryos, all larval stages and adults. Using *nhr-60* specific primers we analyzed the expression by real-time PCR. The *nhr-60* expression was normalized to *ama-1* and *act-1* expressions.

We found that *nhr-60* is highly expressed in all developmental stages and the maximum of the expression has been found in the L3 stage (Fig. 8).

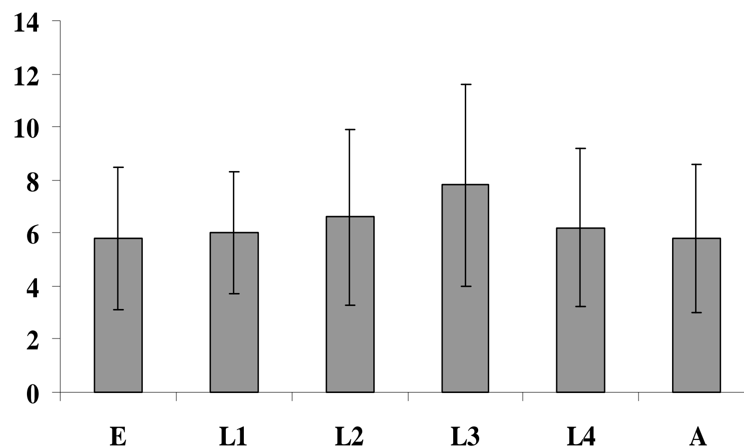

Fig. 8: The analysis of *nhr-60* mRNA expression during *C. elegans* development.
The graph shows average and standard deviations from three independent experiments. The Y-axis represents log of number of copies per 250 ng of total RNA that were normalized to *ama-1* expression. In this case, the expression was normalized to *act-1* expression, there is a very similar pattern. Total RNA was prepared from synchronized *C. elegans* cultures harvested as embryos obtained by lysis of gravid adult hermaphrodite cultures (E), or grown as synchronized larval stages L1 to L4 and young adult animals (A).

4.2.2 Expression of NHR-60

Although the expression of *nhr-60* is high on the level of RNA we searched the expression on the level of proteins. Using Western blot analysis for individual developmental stages we surprisingly detected a band migrated at 64 kD in embryos, all larval stages and adult animals as well (Fig. 9). Because the expected size of NHR-60 protein is approximately 50 kD we suppose NHR-60 to be posttranslationally modified.

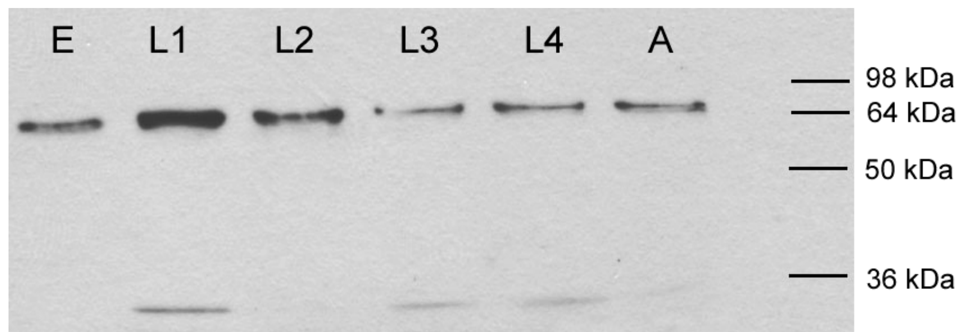

Fig. 9: Detection of NHR-60 in *C. elegans* developmental stages using Western blot
60 µg of protein lysates from developmental stages of *C. elegans* were used for Western blot detection of NHR-60 using rabbit polyclonal antibody against NHR-60. NHR-60 is expressed in all stages. The detection of 36 kDa protein is the consequence of contamination with bacteria.

We were interested in the protein forms. Western blot analysis of *C. elegans* protein extracts from mixed populations revealed the presence of two NHR-60 specific bands. The prominent band is migrating at 64 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. 10). 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* were fed on OP50 and their guts were filled with the bacteria.

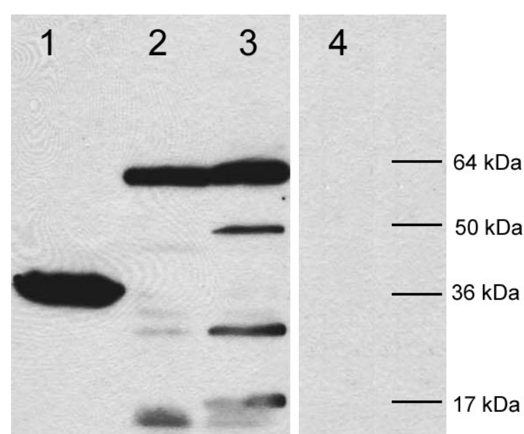

Fig. 10: Analysis of wild type NHR-60 by Western blot
Protein lysates from *E. coli* (lane 1) or *C. elegans* N2 worms (lanes 2 to 4) were analyzed by Western blot using rabbit polyclonal antibody against NHR-60 (lanes 1 to 3) or preimmune serum (lane 4). 40 µg of protein extract was loaded in lanes 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 an expected size of 50 kDa. A protein migrating at 36 kDa (lane 1) that is detected in some experiments by the anti-NHR-60 antibody is a non-specific interaction with some protein from bacterial lysate (all worms were fed on bacteria).

To be sure that the 64 kD protein recognized by the used antibody is modified NHR-60 protein we designed an experiment containing inhibition of *nhr-60* expression by RNAi and subsequent Western blot analysis. The signal of 64 kD protein was lost after the inhibition of *nhr-60* expression and the minor band was not detected (Fig. 11).

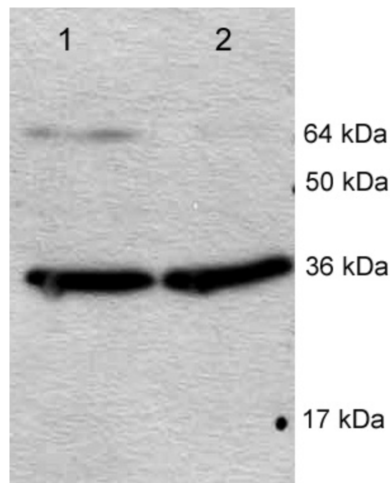

Fig. 11: Western blot analysis of NHR-60 in wild type embryos and in embryos following *nhr-60* RNAi

Protein lysates from wild type embryos (lane 1) and embryos subjected to *nhr-60* RNAi (lane 2) were analyzed by Western blot using the 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.

5 Discussion

5.1 Proteomic analysis uncovers a metabolic phenotype in *C. elegans* after *nhr-40* reduction of function

In this study, we used a commercially available system for analysis of synchronized cultures of *C. elegans* carrying a previously characterized mutation in *nhr-40*, a nuclear hormone receptor which regulates embryonic and larval development, with the most prominent phenotype of loss of function in muscle development (Brozova et al., 2006).

The standard protein fractionation protocol provided by the manufacturer identified a limited number of peaks present only in the control proteome or only in the mutant larvae proteome. More than 50 peaks had significantly higher values (more than twice) in one proteome compared to the other one. The peaks that differed in comparative analysis corresponded to fractions that contained a set of proteins detectable only in one paired material. Gene Ontology analysis (Boyle et al., 2004) classified one third of proteins as muscle related and proteins with metabolic functions. One fraction clearly absent in the mutant proteome contained MYO-3, the heavy chain myosin. MYO-3 is however expressed more efficiently in mutant worms in contrast to other myosins and actin. The search for the expression of selected muscle related genes on mRNA level showed only small differences between mutant and control cultures. Keeping with the published results that employed visualization of muscle fiber formation by phalloidin staining (Brozova et al., 2006), immunohistochemistry showed irregular structure of myosin fibers in morphologically affected animals but not visible differences in mutant larvae that were able to overcome the morphological defects.

The search for proteins expressed more abundantly in mutant worms in comparison to controls identified only 10 peaks clearly higher in mutants and a diverse spectrum of Gene Ontology annotations.

C. elegans development can be slowed down by food removal. The most widely used method for synchronization of *C. elegans* cultures is incubation of newly hatched larvae at 20 ^{o}C without food. This step usually does not disrupt the development and synchronized larvae develop normally when food is supplied. The normal development of *C. elegans* proceeds through four larval stages and is completed in 3 - 4 days at regular conditions. The timing of developmental steps is affected by food supply as well as by temperature. Although the normal temperature for *C. elegans* living in the wild is lower, the standard culture conditions for laboratory cultures are usually between 16 ^{o}C and 25 ^{o}C. While the lower temperature is used for prolongation of developmental stages, higher temperatures are used for speeding up the development.

We hypothesized that higher expression of *myo-3* may be connected with partial rescue of muscle formation in *nhr-40* (*ok667*) worms. This is likely to be affected by temperature and the feeding status. Food restriction prolongates *C. elegans* lifespan but reduces the body size (Tain et al., 2008). Keeping with the possible NHR-40 metabolic function judged from identification of metabolically active proteins decreased in *nhr-40* (*ok667*) larvae, the decreased culture temperature and dietary restriction dramatically increased the proportion of severely affected mutant worms. This indicates that the primary NHR-40 function may be a regulation of response to metabolic status and its connection to development.

We also hypothesized, that degradation of unincorporated myosin molecules may be the main mechanism of defective muscle fiber formation. Inhibition of proteasome dependent protein degradation, however, did not affect the proportion of severely affected mutant larvae and did not alleviated the defective movement (data not shown). It is therefore likely that NHR-40 regulates a not yet understood metabolic step in muscle development.

5.2 Proteomic analysis of NHR-60

NHR-60 is the second member of the nematode nuclear receptors characterized by the DNA-binding protein sequence CNGCKT that was studied in detail.

Its developmental functions are likely to be quite general or project through a common mechanism to widespread morphological defects. This is in agreement with its rather steady expression during the developmental stages found by quantitative PCR and the broad expression pattern that includes many cell types (Simeckova et al., 2007). NHR-60 is predominantly localized at the nuclear periphery. This is an unexpected intracellular localization (Simeckova et al., 2007) for a nuclear hormone receptor. This localization may reflect a specific place of action of nuclear hormone receptors in a broad sense accented in the case of NHR-60. Keeping with this, the proteomic characterization of NHR-60 indicates its very efficient posttranslational modification. The two forms of NHR-60 found in this study are consistent with the basic form of NHR-60 migrating at 50 kDa and a posttranslationally modified 64 kDa prominent form of NHR-60 constituting of approximately 90% of all detectable NHR-60 molecules. The difference in size is approximately 14 kDa and may correspond to NHR-60 SUMOylation or di-ubiquitination. It is tempting to speculate that the diversification of NHRs in nematodes is in the case of NHR-60 accompanied by a further diversification at protein level.

6 Conclusions

Our work indicates that NHR-40 connects metabolic signals with regulation of development of *C. elegans* body wall muscles. We show that NHR-40 affects muscle development by a not yet known mechanism that involves myosin MYO-3 at a post-transcriptional and post-translational level, most likely involving muscle fiber assembly in a cellular context.

We showed that lowered metabolism, exemplified by controlled restricted food intake and decrease of the culture temperature increases the penetrance of NHR-40 loss of function phenotype. Our work indicates that multiplication of *C. elegans* nuclear hormone receptors may reflect specific regulatory tasks important for synchronization of metabolic status and developmental programs. This, together with detailed analyses of other related nuclear hormone receptors, is likely to shed a new light at the evolutionary pressure involved in the multiplication of nematode nuclear receptors and the plasticity of the nuclear hormone receptors.

The proteomic characterization of NHR-60 shows that NHR-60 is very efficiently posttranslationally modified and the predominant form of this receptor acquires a form that is further diversified on the protein level from the canonical molecular structure of members of nuclear hormone receptor family. This molecular form may represent a form existent transiently also in case of other NHRs. Rhabditidae may use this molecular form for fortification of a specific molecular function of NHR-60.

7 The list of author's publications and presentations

Publications:

M. Pohludka, K. Simeckova, J. Vohanka, P. Yilma, P. Novak, M. W. Krause, M. Kostrouchova, Z. Kostrouch: Proteomic analysis uncovers a metabolic phenotype in *C. elegans* after *nhr-40* reduction of function, *Biochem Biophys Res Commun*. 374 (1):49-54 (2008)

Simeckova K, Brozova E, Vohanka J, **Pohludka M**, Kostrouch Z, Krause MW, Rall JE, Kostrouchova M: Supplementary nuclear receptor NHR-60 is required for normal embryonic and early larval development of *Caenorhabditis elegans. Folia Biologica (Praha)* 53 (3):85-96 (2007)

P. Liby, M. Kostrouchova, **M. Pohludka**, P. Yilma, P. Hrabal, J. Sikora, E. Brozova, M. Kostrouchová, J. E. Rall, Z. Kostrouch: Elevated and Deregulated Expression of HDAC3 in Human Astrocytic Glial Tumours. *Folia Biologica (Praha).* 52, 21-33 (2006)

P. Liby, **M. Pohludka**, J. Vohanka, M. Kostrouchova, D. Kostrouch, M. Kostrouchova, E. J. Rall and Z. Kostrouch : BIR-1, the Homologue of Human Survivin Regulates Expression of Developmentally Active Collagen Genes in *C. elegans*. *Folia Biologica (Praha)* 52 (4): 101-108 (2006)

Presentations:

M. Pohludka, K. Simeckova, Z. Kostrouch, M. Kostrouchova: ACBP-1, the acyl coenzyme A-binding protein -1 is a cofactor of NHR-60. Poster presentation. *European Worm Meeting*, Sevilla, Spain, March 29 – April 2, 2008.

M. Pohludka, P. Yilma, M. Kostrouchová, Z. Kostrouch: Multidimensional characterization of RB840 proteome. Poster presentation. *European Worm Meeting*, Sevilla, Spain, March 29 – April 2, 2008.

D. Kostrouch, **M. Pohludka**, P. Liby, M. Kostrouchova, F. Behensky, M. Kostrouchova and Z. Kostrouch: Characterization of the proteome of *C. elegans* L1 larvae with forced expression of BIR-1. Poster presentation. *European Worm Meeting*, Sevilla, Spain, March 29 – April 2, 2008.

K. Simeckova, L. Mlejnkova, **M. Pohludka**, Z. Kostrouch, M. Kostrouchova: Functional analysis of *C. elegans* supplementary nuclear receptors characterized by the P box sequence CNGCKT. Poster presentation. *European Worm Meeting*, Sevilla, Spain, March 29 – April 2, 2008.

M. Pohludka, J.Vohanka, M. Kostrouchova, Z. Kostrouch: Analysis of the proteome phenotype of nhr-23 RNAi by two dimensional chromatography. Poster Presentaion. *16th International C. elegans Meeting*, Los Angeles, USA, June 27 - July 1, 2007.

E. Brozova, **M. Pohludka**, J. Vohanka, P. Yilma, M. Kostrouchova, Z. Kostrouch Characterization of *C. elegans* mutant L1 larvae (*nhr-40*, RB840) on the proteome level by comparative two dimensional chromatography: chromatographic focusing followed in line by reversed phase chromatography and mass spectroscopy. Poster Presentaion. *16th International C. elegans Meeting*, Los Angeles, USA, June 27 - July 1, 2007.

K. Simeckova, E. Brozova, J. Vohanka, **M. Pohludka**, Z. Kostrouch, M. Kostrouchova NHR-60 regulates development of seam cells downstream of NHR-23 (CHR3). Poster Presentaion. *16th International C. elegans Meeting*, Los Angeles, USA, June 27 - July 1, 2007.

D. Kostrouch, P. Liby, M. Kostrouchova, **M. Pohludka**, J. Vohanka, E. Brozova, M. Kostrouchova, Z. Kostrouch: Analysis of BIR-1 function using comparative DNA microarrays and 2D chromatography. Poster Presentation. *16th International C. elegans Meeting*, Los Angeles, USA, June 27 - July 1, 2007.

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J. Sikora, **M. Pohludka**, L. Dvořáková, Z. Vernerová, P. Přikryl, J. Vohánka, P. Novák, L. Stolnaja, I. Rychlík, J. Vlasák, Z. Kostrouch and M. Elleder: Is there a primary (hereditary) SAA amyloidosis? Clinical, histopathologic, proteomic and genetic study in a family with amyloidosis. Oral presentation. 3. *sympózium & workshop Molekulární patologie: proteomika, genomika,* Olomouc, Czech Republic, May 04 – May 05, 2007.

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M. Pohludka, J. Vohanka, M. Kostrouchova, Z. Kostrouch.: Analysis of CHR-3 (nhr-23) Loss of Function in L1 Larval Stage Using Whole Genome Microarrays and Comparative Two Dimensional Protein Chromatography. Poster presentation. *European Worm Meeting*, Hersonissos, Greece, April 29 – May 3, 2006.

K. Simeckova, E. Brozova, J. Vohanka, **M. Pohludka**, Z. Kostrouch, M. Kostrouchova.: 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. *European Worm Meeting*, Hersonissos, Greece, April 29 – May 3, 2006.

P. Liby, M. Kostrouchova, **M. Pohludka**, J. Vohanka, E. Brozova, M. Kostrouchova, Z. Kostrouch.: Inhibition of *bir-1*, the Homologue of Human Survivin, Induces Changes of Expression of Developmentally Active Collagen Genes in L1 Larval Stage. Poster presentation. *European Worm Meeting*, Hersonissos, Greece, April 29 – May 3, 2006.

8 Abbreviations

cDNA	complementary DNA
C. elegans	*Caenorhabditis elegan*
COUP-TF	chicken ovalbumin upstream promoter-transcription factor
DBD	DNA binding domain
DNA	deoxyribonucleic acid
DR	direct repeats
E. coli	*Escherichia coli*
e.g.	exempli gratia
Fig.	figure
GO	gene ontology
GFP	green fluorescent protein
HNF-4	hepatocyte nuclear factor 4
HPLC	high performance liquid chromatography
HRE	hormone response element
IPs	inverted palindromes
kb	kilobase
LBD	ligand binding domain
LC/MS/MS	sequence of liquid chromatogpraphy and tandem of mass spectrometry
mRNA	messenger RNA
MS	mass spectrometry
N2	wild type strain of *C. elegans*
NHR	nuclear hormone receptor
Pal	palindromes
PCR	polymerase chain reaction
PF 2D	ProteomeLab™ PF 2D Protein Fractionation System
PPAR	peroxisome proliferator-activated receptor
PXR	Pregnane X receptor
qPCR	quantitative PCR
RB840	a worm strain harboring the *nhr-40 (ok667)* deletion
RXR	retinoid X receptor
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
supnr	supplementary nuclear receptor
VDR	vitamin D receptor

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