Charles University

1st Faculty of Medicine

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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

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Prague 2009

ACKNOWLEDGEMENTS

I would like to thank mainly my supervisor MUDr. Zdeněk Kostrouch, CSc. for his scientific approach and understanding throughout my whole study.

I am grateful to prof. MUDr. Milan Elleder, DrSc. the head of the Institute of Inherited Metabolic Disorders for giving me the opportunity to work on this thesis.

I would like to thank MUDr. Marta Kostrouchová, CSc. and Dr. Michael Krause for their support and advice all along my work on the topic.

I thank all my colleagues at the Institute, namely Mgr. Kateřina Šimečková, Ph.D., Bc. Petr Yilma, Mgr. Eva Brožová, Ph.D., Ing. Jaroslav Vohánka, Veronika Kostrouchová, MUDr. Jakub Sikora, Ph.D., Filip Linx and David Yilma for their help and friendship.

Finally, I would like to thank Mgr. Petr Novák, Ph.D. and Mgr. Petr Přikryl for their advice and work on mass spectrometry.

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 Grants 301/05/0859, 304/08/0970, and 304/07/0529 awarded by the Czech Science Foundation, 0021620806 from the Ministry of Education, Youth and Sports of the Czech Republic, and (in part) by the Intramural Research Program of the NIH, NIDDK. IMIC institutional research concept (AVOZ50200510), and the Ministry of Education, Youth and Sports of the Czech Republic (LC 7017) are gratefully acknowledged for financial support as well.

Content:

1 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.

1.1 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).

2 Abbreviations

AF-1	activation function-1
AF-2	activation function-2
CBP/p300	CREB - binding protein
cDNA	complementary DNA
C. elegans	*Caenorhabditis elegans*
CGC	Caenorhabditis Genetics Center
COUP-TF	chicken ovalbumin upstream promoter-transcription factor
DBD	DNA binding domain
DNA	deoxyribonucleic acid
DTT	dithiotreithol
dNTP	deoxynucleotide triphosphate
DR	direct repeats
dsRNA	double stranded RNA
EDTA	ethylenediaminetetra-acetic acid
etc.	Et cetera
E. coli	*Escherichia coli*
e.g.	exempli gratia
Fig.	figure
gDNA	genomic DNA
GO	gene ontology
GFP	green fluorescent protein
HDAC	histone deacetylase
HNF4	hepatocyte nuclear factor 4
HPCF	Chromatofocusing HPLC
HPLC	high performance liquid chromatography
HPRP	High Performance Reversed Phase 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
Mb	Megabase
mRNA	messenger RNA
MS	mass spectrometry
MS/MS	tandem of mass spectrometry
N2	wild type strain of *C. elegans*
NaOAc	sodium acetate
NCoR	nuclear hormone receptor corepressor
NGM	nematode growth medium
NHR	nuclear hormone receptor
NR	nuclear receptor
P/CAF	p300/CBP – associated factor
Pal	palindromes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PF 2D	ProteomeLab™ PF 2D Protein Fractionation System
PPAR	peroxisome proliferator-activated receptor
PXR	Pregnane X receptor
qPCR	quantitative PCR
RAR	retinoic acid receptor
RB840	a worm strain harboring the *nhr-40 (ok667)* deletion
RXR	retinoid X receptor
RNA	ribonucleic acid
rNTP	ribonucleotide triphosphate
RPM	rotation per minute
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SDS	sodium dodecyl-sulfate
SMRT	silencing mediator of retinoid and thyroid receptors
SRC-1	steroid receptor coactivator-1
ssRNA	single stranded RNA
supnr	supplementary nuclear hormone receptor
T-PBS	Tween 20 in phosphate buffered saline

TFA	trifluoroacetic acid
TR	thyroid receptor
tRNA	transfer RNA
TTBS	tris tween buffered saline
UV	ultra violet
VDR	vitamin D receptor

3 Introduction

3.1 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* can be easily cultured under laboratory conditions and for its simplicity *C. elegans* became a useful model organism for genetic and developmental studies (Brenner, 1974). The described characteristics make the animal very amenable to genetic analyses (Brenner, 1974). Available on the internet is an online database (www.wormbase.org) focused on *Caenorhabditis elegans* research that is actively updated by scientists working in this field.

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. Males produce sperms only and can fertilize hermaphrodites. Hermaphrodites produce both (sperms and oocytes) and are capable of self-fertilization but cannot fertilize each other. The genders have a constant number of somatic cells. Adult hermaphrodites have 959 somatic nuclei, adult males 1031. Full cell lineage is precisly described on the WormBase web site (Corsi, 2006).

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) (Fig. 1). 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).

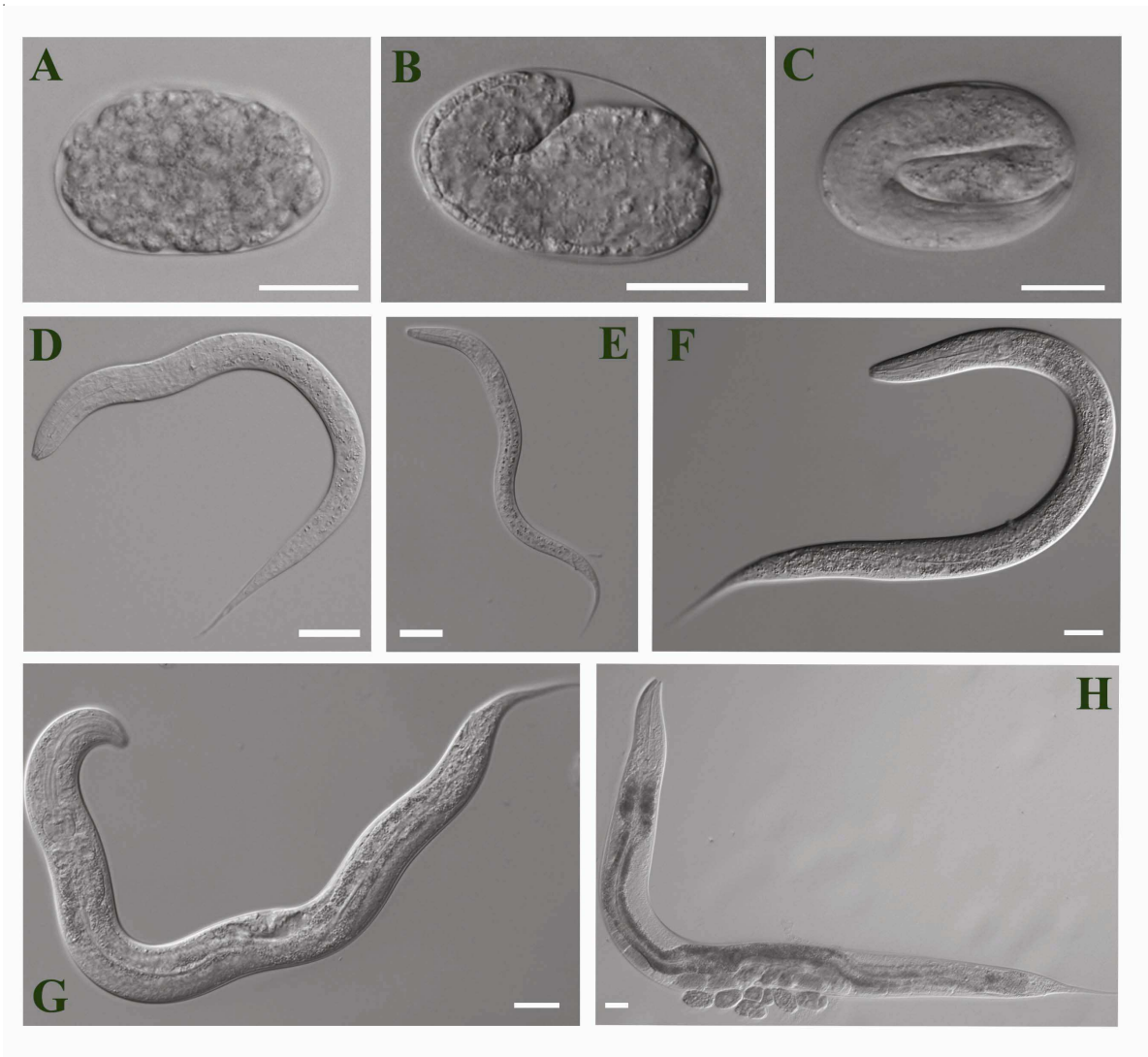

Fig. 1: *Caenorhabditis elegans* developmental stages

C. elegans embryonic (comma (A), 1.5-fold (B) and 3-fold (C)) and larval (L1 larva (D), L2 larva (E), L3 larva (F) and L4 larva (G)) stages. Panel H shows an adult hermaphrodite with its progeny.

Scale bar: 20 µm.

C. elegans anatomy is simple. The body is tubular. The basic anatomy of *C. elegans* includes a mouth, pharynx, intestine, gonads and a collagenous cuticle. 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).

The first sequenced genome of a multicellular organism was of *C. elegans*. The finished genome sequence was published in 1998 (The *C. elegans* Sequencing Consortium, 1998), although a number of small gaps were present. The genome has a relatively small size (approximately 97 Mb) that represents one-thirtieth of the size of a mammalian genome. Wild-type *C. elegans* hermaphrodites contain five pairs of autosomes and one pair of X chromosome. On the contrary, males contain five pairs of autosomes and only a single X chromosome.

C. elegans differs from the other eucaryotic animals in having operons. The genome encodes approximately 22,000 genes and approximately 15 % of *C. elegans* genes are organized in operons (Blumenthal et al., 2002). Genes contain usually short exon (median size 123 bases) and intron (median size 47 bases) sequences.

3.2 Nuclear hormone receptors

Nuclear hormone receptors (NHRs) form a large super-family of transcription factors of the Metazoan species and work in concert 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).

3.2.1 Functional domains of NHRs

NHRs have a similar domain organization: an A/B domain, a central DNA binding domain, a hinge region, and a carboxy-terminal LBD (Burris, 2008; Yen, 2001). Some receptors contain also a C-terminal region (F) of unknown function (not shown in Fig. 2) (Aranda and Pascaul, 2001). Each of these domains and regions may sub-serve multiple functions, and thus their names may only reflect the first function ascribed to them (Yen, 2001).

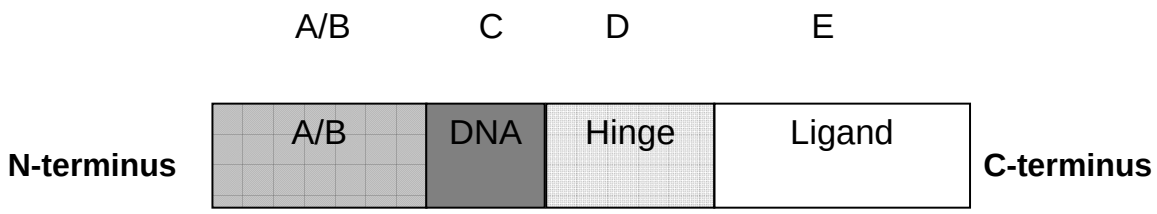

Fig. 2: Schematic structure of a nuclear hormone receptor

NHRs consist of an A/B domain (A/B), a central DNA binding domain (C), a hinge region (D), and a carboxy-terminal LBD (E).

3.2.1.1 Amino-terminal (A/B) domain

The amino-terminal regions have variable lengths and are not conserved among the members of the super-family. The role(s) of the amino-terminal domain is not understood in detail. The domain contains a specific AF-1 activation function that contributes to interactions with transcriptional machinery (Robyr et al., 2000). This region can also interact with cell-specific (Giguere, 1999) and promoter-specific (Tora et al., 1988) transcription.

3.2.1.2 DNA binding domain

The DNA binding domain (DBD) is located in the central portion of the NHR and consists of two zinc finger motifs, each containing four highly conserved cysteine molecules coordinating binding of a zinc atom (Yen, 2001). This results in a formation of tertial structure containing helixes that interact with DNA. Within the zinc finger, there is a "P box" that is crucial in a sequence-specific recognition of hormone response elements (HRE) by different members of the nuclear hormone super-family (Wahli and Martinez, 1991**)**. Additionally, there are other important regions within the minor groove of the HRE just downstream of the second zinc finger called "A box". Within the DBD, there is a region called "D box", that contributes to the dimerization properties of NHRs (Fig. 3).

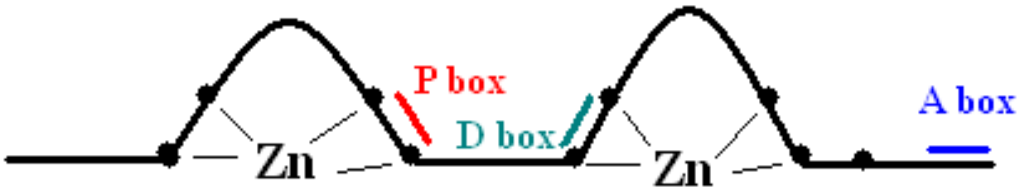

Fig. 3: DNA binding domain of nuclear hormone receptors

A schematic representation of two “zinc fingers” with depicted sub-domains. All cysteins of conformational importance are marked with black dots.

3.2.1.3 Hinge region

The hinge region located between the DBD and LBD contains an amino acid sequence that is associated with nuclear localization (Evans, 1988). The sequence allows DBD to rotate and enables some receptors to bind as dimer to both direct and inverted HREs (Glass, 1994; Giguere, 1999). The hinge region has additional properties. The hinge region also interacts with corepressors (Horwitz et al., 1996).

3.2.1.4 Ligand binding domain

A ligand binding domain is conserved among nuclear hormone receptors and is responsible for ligand binding, dimerization, interaction with heat-shock proteins and transactivation. The LBD of nuclear hormone receptors consists of a conserved arragement of helices (11-12 α-helices), sandwiching and participating in a ligand binding pocket (Yen, 2001). The LBD contains AF-2 (Activation function-2) site localized at the carboxy-terminus of the LBD. The region mediates ligand dependent transactivation (Giguere, 1999).

3.2.2 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).

Several orphan nuclear hormone receptors can bind DNA with high affinity as monomers (Giguere, 1999). Steroid hormone receptors almost exclusively bind to the HRE as homodimers. Two steroid hormone receptor monomers bind cooperatively to their response elements, and dimerization interfaces have been identified both in the LBD and in the DBD (Aranda and Pascaul, 2001). Although several nonsteroidal nuclear hormone receptors also bind DNA as homodimers, many nonsteroidal receptors bind to their HREs preferentially as heterodimers. In this case, the RXR is the promiscuous partner for different receptors (Bugge et al., 1992; Kliewer et al., 1992; Leid et al., 1992). Typical heterodimeric receptors such as TR, RAR, or VDR can bind to their response elements as homodimers, but heterodimerization with RXR strongly increases the efficiency of DNA binding and transcriptional activity (Aranda and Pascaul, 2001). Some monomeric receptors can also form heterodimers with RXR, and the heterodimers then recognize DRs rather than the monomeric extended sequence. And some homodimeric receptors can also form heterodimers with RXR (Giguere, 1999).

Theoretically, four different states of heterodimer occupancy can be predicted: both receptors unoccupied, only RXR occupied, only the partner receptor occupied, and both receptors occupied (Aranda and Pascaul, 2001). However, three types of heterodimeric complexes exist: unoccupied heterodimers, nonpermissive heterodimers that can be activated only by the partner's ligand but not by an RXR ligand alone (Kurokawa, 1994; Forman et al., 1995), and permissive heterodimers that can be activated by ligands of either RXR or its partner receptor and are synergistically activated in the presence of both ligands (Janowski et al., 1996; Kliewer et al., 1992; Willy, 1995). This demonstrates that RXR plays a dual role in nuclear hormone receptor signaling.

The ligands could play a role in dimerization and binding to DNA. For instance, thyroid hormone inhibits binding of homodimers but not heterodimers to DNA, thus promoting formation of heterodimeric complexes on the HRE (Ribeiro et al., 1992). In contrast 9-*cis*-retinoic acid in some instances can increase binding of RXR homodimers to a DR1 (Zhang, 1992), which can lead to unavailability for heterodimer formation with other receptors and to decreased levels of transcription for genes depending on heterodimers (Aranda and Pascaul, 2001).

3.2.2.1 Nuclear hormone receptor cofactors

TR and RAR repress basal transcription in the absence of ligands. The binding of a hormonal ligand to the receptor releases the transcriptional silencing and leads to gene activation (Baniahmad et al., 1992). The unliganded receptors are bound to the HRE and under these conditions are associated with corepressors responsible for the silencing activity. After ligand binding, the conformational changes cause the dissociation of corepressors and the recruitment of coactivator complexes responsible for transcriptional activation (Horwitz et al., 1996). This is applied to TR and RAR receptors, but not to the other receptors, such as the unliganded steroid receptors that do not bind corepressor proteins.

The coactivators and corepressors play a critical role in the regulation of transcription by the nuclear hormone receptor protein complex. The first identified transcriptional corepressors were a 270 kDa protein named Nuclear Corepressor (NcoR) (Horlein et al., 1995) and Silencing Mediator of Retionid and Thyroid Receptor (SMRT)

(Chen and Evans, 1995). NCoR and SMRT are related both structurally and functionally and are known to recruit a histone deacetylases (HDACs) through bridging molecules (Robyr et al., 2000). Other proteins different from NCoR and SMRT, that also act as corepressors of nuclear hormone receptors, have been identified (e.g. SUN-CoR, Alien) (Zamir et al., 1997).

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

3.2.3 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).

3.2.4 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 hormone receptor super-family, being found in nearly all animal species examined. Orphan nuclear hormone 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).

3.2.5 The nongenomic action of nuclear hormone receptors

The nongenomic actions of steroid and thyroid hormones were observed and reported for several decades (Hess and Martius, 1951; Hoch and Lipmann, 1954; Lardy et al., 1960) and preceded the discovery that thyroid and steroid hormones regulate RNA polymerase II dependent transcription (Tata, 1968; Tata and Widnell, 1966; Widnell and Tata, 1966). There are several lines of evidence indicating the nongenomic mechanism of actions of steroid and thyroid hormones including the rapid response of cells and tissues to the treatment by hormones (Hess and Martius, 1951; Hoch and Lipmann, 1954; Lardy et al., 1960) and a long list of documented “binding sites”, hormone localization in target cells and subcellular fractions specifically binding steroid and thyroid hormones (Davis et al., 1974; Samuels and Tsai, 1974; Sterling et al., 1977; Kostrouch et al., 1987).

Reported nongenomic actions of steroid and thyroid hormones are in recent years complementing the transcriptional regulatory potential of ecdysone (Srivastava et al., 2005), estrogen (Morley et al., 1992; Revankar et al., 2005), androgen (Benten et al., 1999), progesterone (Baldi et al., 1995), mineralocorticoid (Harvey and Higgins, 2000), glucocorticoid (Liu et al., 1995) and thyroid hormones (Davis and Davis, 1996), vitamin D (Inoue et al., 2008) and retinoic acid (Masia et al., 2007).

In some cases, the nongenomic actions are likely to be executed by cytoplasmic, mitochondrial and membrane “nuclear” receptors or their splice variants. It is expected that some functions of ligands of NHRs are dependent on receptors belonging to different protein families and acting through transduction cascades, G-protein family members, PI3K cascade (D'Arezzo et al., 2004; Kavok et al., 2001) and regulation of cytoskeleton functions

(Zamoner et al., 2008; D'Arezzo et al., 2004; Davis and Davis, 2002; Davis et al., 2008; Davis et al., 2002; Trainor et al., 2008).

3.2.6 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 hormone 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 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). The alignment of DBD is shown in Fig.4.

Fig. 4: Sequence analysis of NHRs from subgroup 8 in *C. elegans*

(A) Alignment of the DBD amino acid sequence derived from an NHR-40 cDNA and 17 other predicted *C. elegans* NHRs that are characterized by the P box sequence CNGCKT (Van Gilst et al., 2002). The P box of the DBD and the conserved cysteins are marked in bold. The alignment was generated using the MultAlin program (Corpet, 1988).

3.2.6.1 Nuclear hormone receptor-40 (NHR-40)

NHR-40 belongs to the class 1, subgroup 8 of *C. elegans* NHRs that have the P box sequence CNGCKT (Van Gilst et al., 2002) and is classified as an orphan receptor. *nhr-40* is expressed from at least two promoters and generates at least three transcripts (Brozova et at., 2006).

The down-regulation of *nhr-40* by RNAi, or a mutant with an intronic region deletion called *nhr-40 (ok667)* deletion in RB840 strain (840 bp long deletion within the first intron of all *nhr-40* isoforms) results in late embryonic and early larval arrest with defects in elongation and morphogenesis. The *nhr-40* loss of function phenotype includes irregular development of body wall muscle cells and impaired movement and coordination resembling neuromuscular affection (Fig. 5) (Brozova et al., 2006). The penetrance of these

phenotypes in larvae is only 20 % in both the mutant allele and following *nhr-40* RNAi, likely reflecting a reduction of function in NHR-40 and/or possible compensation by redundantly acting supnrs (Brozova et al., 2006).

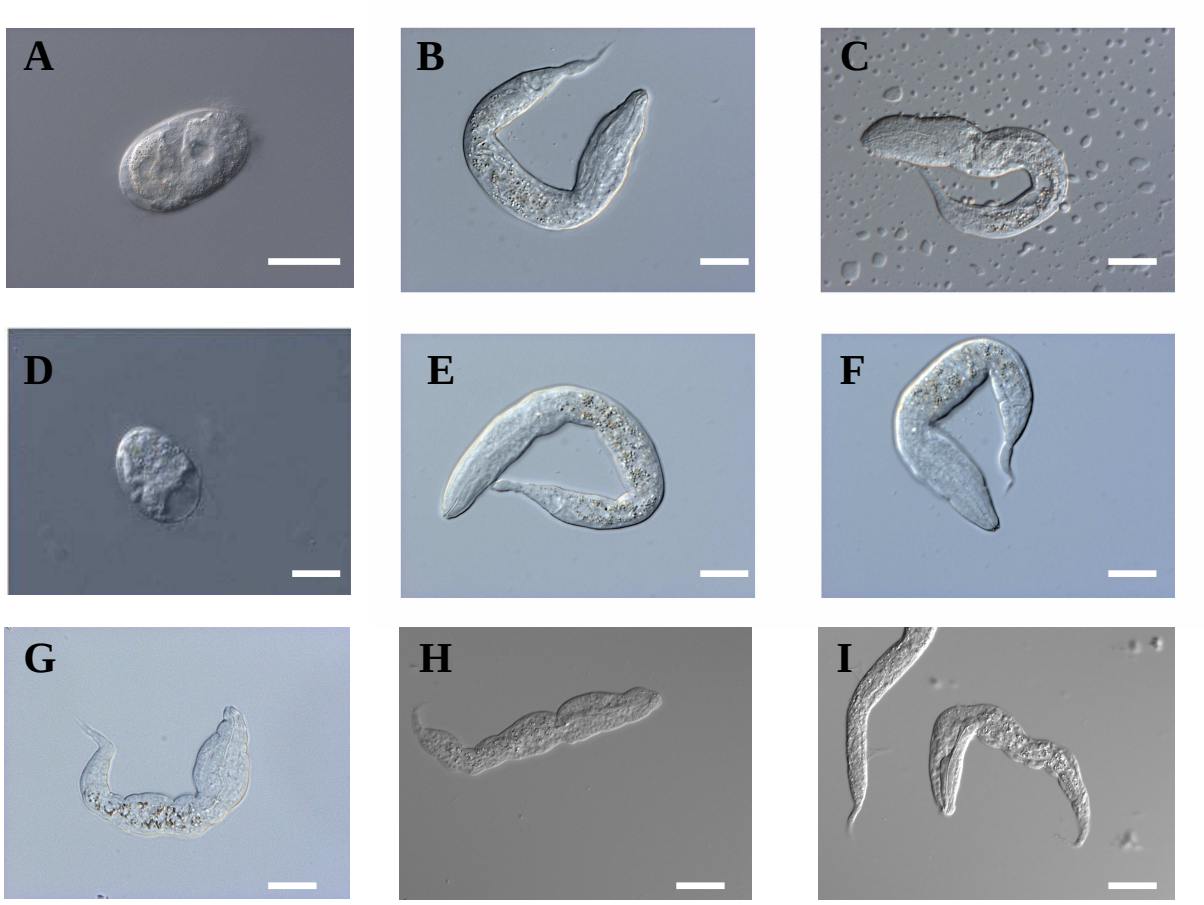

Fig. 5: **A comparison of the developmental defects induced by *nhr-40* RNAi and the phenotype of the deletion allele *nhr-40 (ok667)*.**

The pictures A, B, C, E and F show phenotypes induced by *nhr-40* RNAi. The picture A shows arrested embryo and the pictures B, C, E and F show larvae L1 with bulges and dpy phenotype. The pictures D, G, H and I show the phenotypic changes found in RB840 line. Embryonic arrest is shown in the picture D and in the pictures G - H are shown L1 larvae with severe defects of body shape, bulges, and strictures and with dpy phenotype (Brozova et al., 2006).

Scale bar: 20 µm.

To detect the changes in the affected muscles, Brozova et al. stained actin myofilaments with phalloidin in the RB840 line. The myofilaments were disorganized in regions corresponding to morphological defects (Fig. 6). Movement of RB840 L1 larvae was also affected. The larvae moved slowly, reversing directions several times, and very often moving in circles.

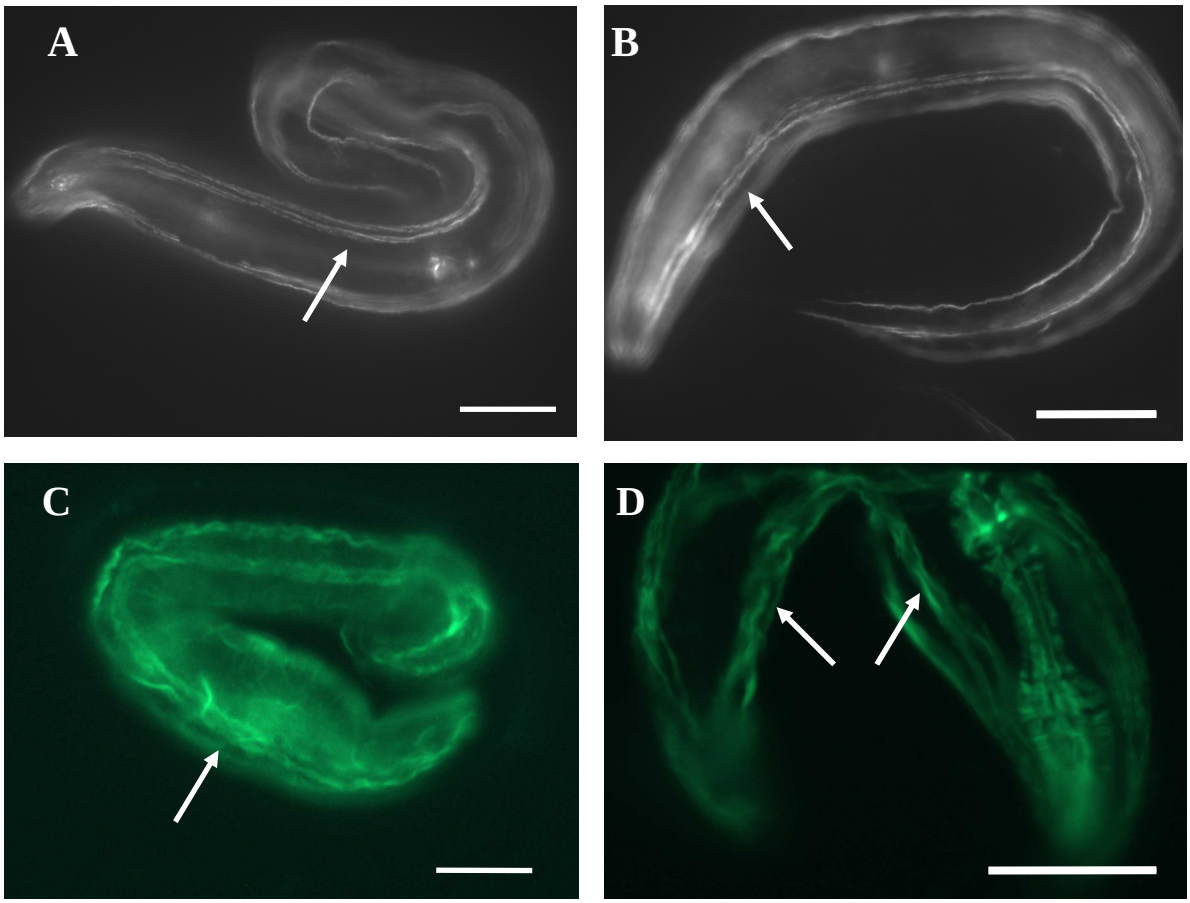

Fig. 6: Defective texture of actin myofilaments in RB840 line stained by phalloidin.
(A, B) Detection of actin myofilaments in wild-type larvae L1 by fluorescent microscopy.
(C, D) The defects in actin filaments in L1 of the RB840 line in two different focus planes.
The structure of the filaments is indicated by arrows.
Scale bar: 20 µm. (Brozova et al., 2006).

NHR-40 joins the list of *C. elegans* NHRs that regulate development and suggests that members of extensive nematode supnr family have acquired varied and novel functions during evolution (Brozova et al., 2006).

4 Materials and Methods

4.1 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 (NGM) (17 g of Bacto Agar, 2.5 g of Bacto Pepton, 3 g of NaCl, 1 ml of Cholesterol (5 mg/ml in ethanol), H_2O to 1 liter, autoclave, 25 ml of 1 M KH_2PO_4, 1 ml of 1 M $CaCl_2$, 1 ml of 1 M $MgSO_4$ add after autoclaving) plates and fed *Escherichia coli* OP50 as described (Brenner, 1974).

C. elegans are visualized using a dissecting microscope Olympus SD30 (Olympus, Tokyo, Japan). Worms could be transferred on a new plate using a chunk of agar from an old plate. Worms could be also picked individually using a platinum wire with a hook at the end, which could be easily sterilized in a flame.

4.2 Synchronization and cultures

Synchronized L1 larvae wild type and mutant animals were prepared by bleaching gravid adult hermaphrodites and hatching embryos overnight in liquid without food. These cultures were used for subsequent experiments or stored at -80 °C in aliquots that were later used for proteomic analyses. Synchronized and arrested L1 larvae were seeded on plates with a bacterial lawn grown overnight at room temperature after an initial seeding of 300 µl/plate of a bacterial culture containing approximately 10^8 *E. coli* (OP50)/ml. To segregate *nhr-40 (ok667)* mutant larvae with obvious movement defects (~20 % penetrance) 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.

4.3 Fractionation of the soluble proteomes by two-dimensional chromatography

4.3.1 Preparation of protein lysates for chromatography

Frozen pellets of worms were melted at 4 °C and the pellet was resuspended in 0.2 ml of 50 mM Tris-HCl (pH 8.0) and vortexed. The solution was mixed with 1.6 ml of lysis buffer (7.5 M urea, 2.5 M thiourea, 12.5 % glycerol, 50 mM Tris, 2.5 % n-octylglucoside, 6.25 mM Tris-(carboxyethyl) phosphine hydrochloride, 1x Protease Inhibitor Cocktail (Boehringer Mannheim, Mannheim, Germany)). The suspensions were incubated on ice for 10 minutes and sonicated in five cycles, each consisting of four times 10 s sonication /10 s interruption (20 kHz, amplitude 20 µm, 60 W) (Ultrasonic Processor (Cole-Parmers Instruments, Vernon Hills, IL)) using the internal sonication rod. Cell debris was removed by centrifugation at 20,000 x g for 60 minutes at 4 °C and the supernatant was harvested. The sample was supplemented with Start Buffer (Beckman Coulter, Inc., Fullerton, CA) to a final volume of 2.5 ml. The lysis buffer was then exchanged for the Start Buffer supplied by Beckman. The PD10 column (Amersham Pharmacia Biosciences, Uppsala, Sweden) was equilibrated with approximately 25 ml of Start Buffer for the first dimension separation (pH 8.5±0.1, pH adjusted with iminodiacetic acid and ammonium hydroxide). The sample was loaded onto the column and the first eluent was discarded. Start Buffer was used to elute the proteins that were collected in the first 3.5 ml fraction. The total protein concentration in the sample was measured using a BCA Assay Kit (Pierce, Rockford, IL). Two mg of total protein were used for a subsequent run.

4.3.2 First Dimension - Chromatofocusing HPLC (HPCF)

For the chromatofocusing separation, the first module of the Beckman Coulter ProteomeLab PF 2D system was used (Beckman Coulter, Inc.). The pH gradient is based on two buffers supplied by the manufacturer, the Start Buffer and the Elution Buffer (Beckman Coulter, Inc; pH adjusted with iminodiacetic acid and ammonium hydroxide). The upper limit of the pH gradient was determined by the Start Buffer (pH 8.5±0.1) and the lower

limit was determined by the Elution Buffer (pH 6.4±0.1). The proteins remaining in the column after elution by the pH gradient were eluted by ionic strength (1 M NaCl).

The HPCF column was equilibrated with 25 column volumes of Start Buffer. Two mg of total protein in 2 ml volume was applied to the injector compartment and separated by chromatofocusing at the flow rate 0.2 ml/min. UV detection was performed at 280 nm and the pH was monitored using a flow-through on-line probe. Fractions were collected in 96 well 1 ml plates either by time or by 0.3 interval pH difference.

4.3.3 Second Dimension – Reversed Phase HPLC (HPRP)

The second module of Beckman Coulter ProteomeLab PF 2D system was used for the separation of proteins according to the surface hydrophobicity with two solvents. Solvent A was 0.1 % trifluoroacetic acid (TFA) in water and solvent B was 0.08 % TFA in acetonitrile. The first dimension fractions were separated on HPRP columns packed with nonporous silica beads at 50 °C, at a flow rate 0.75 ml/min. The gradient was run from 0 to 100 % of solvent B in 35 minutes, followed by an elution with solvent B for 5 minutes to elute the remaining proteins from the column. The fractions were collected at 1 minute intervals into 96 well plates. The module was finally equilibrated with solvent A (by a 10 minute run) and prepared for another second dimension separation.

4.3.4 Chromatographic analysis

The chromatograms were integrated by Karat 32 software and exported automatically to storage files (.asc). The chromatograms of N2 and homozygous *nhr-40 (ok667)* worm lysates were compared by the Karat 32 software to identify peaks that clearly differed in the compared materials. The stored files were analyzed using computer programs ProteoVue and DeltaVue provided with the system and differential protein maps generated for corresponding fractions and for complete proteomes.

4.4 Mass spectrometric analysis and data processing

Fractions were dried down using a Speedvac concentrator, 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 µl of tryptic peptide mixture was applied on the Magic-C18 column, 0.180x150 mm, 200 Å, 5 µm (Michrom Bioresources, Auburn, CA) and separated using water/acetonitrile gradient elution. The column was connected to a LCQDECA ion trap mass spectrometer (ThermoQuest, San Jose, CA) equipped with a nanoelectrospray ion source.

Spray voltage was held at 1.8 kV, tube lens voltage was 10 V. The heated capillary was kept at 150 °C with a voltage of 30 V. The LCQDECA was set to acquire a full MS scan between 350 and 1600 m/z followed by full MS/MS scans of the top two ions from the preceeding full MS scan. Activation time for collision- induced dissociation was 30 ms and the relative collision energy was set to 42 %. Dynamic exclusion was enabled with two repeat counts, repeat duration of 30 s and 3 min of exclusion duration. Spectra were searched with the SEQUEST^{TM} software against the SwissProt database. For spectra from a multiply charged peptide, an independent search was performed on both the 2+ and 3+ mass of the parent ion. The search parameters were as follows: trypsin specificity; mass errors 2.0 Da for precursor and 1.0 Da for fragment ions; possible modification: +16 Da for Met. SEQUEST results were automatically processed with the BioWorks Browser (Tabb et al., 2002) using the following criteria: XCorr values were 1.7 for singly charged, 2.2 and 3.0 for doubly and triply charged peptides, respectively.

4.5 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) as described and the bulk downloads were performed using WormMart access.

4.6 Western blot

Western blots were performed using a standard protocol: samples in water were mixed with 10x protease cocktail (Complete Mini, Boehringer Mannheim) to reach 1x final concentration, sonicated for a total time of 1 min (in six cycles consisting of 10 s sonication /10 s interruption times, Ultrasonic Processor (Cole-Parmers Instruments). 2x Laemmli sample buffer was added (Laemmli, 1970) and samples were boiled for 7 min. The mixture was centrifuged for 10 min using 12,000 x g and the supernatant was collected. Protein concentration was estimated using BCA Protein analysis kit (Pierce) as recommended. After the estimation of protein concentration, 2-mercaptoethanol was added (1 µl per 30 µl of final sample) shortly heated and mixed. 80 µg of proteins were loaded for each sample, separated by electrophoresis and blotted onto a nitrocellulose membrane using a Mini Protean II apparatus (Bio-Rad, Hercules, CA). Gels were stained by Coomassie blue (ICN Biomedicals Inc., Aurora, OH). The membranes were then incubated in 1 % Tween 20 (Serva, Heidelberg, Germany) in phosphate buffered saline, pH 7.4 (T-PBS) containing 5 % (w/v) dried non- fat milk overnight at 4 °C. 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 diluted as necessary in T-PBS containing 5% (w/v) dried milk. The membranes were incubated for 1 h with primary antibodies at room temperature, washed six times in T-PBS for a total duration of 1 h and incubated with the secondary antibodies labeled by horseradish peroxidase (Sigma Aldrich) diluted 1:10,000 in T-PBS for 1 h at room temperature and washed six times for a total of 1 h. Goat anti-rabbit IgG and goat-anti mouse IgG antibodies labeled by horseradish peroxidase were used. After the wash in T-PBS, the secondary antibody was visualized using ECL Plus chemiluminescent system (Amersham, Pharmacia) or Supersignal (Pierce). Films were

exposed from 10 s to 1 h and developed using an automated developer. The membranes were stripped from primary and secondary antibodies by three one hour incubations in 0.2 M glycine pH 2.5, 0.05 % (v/v) Tween 20, 100 mM 2-mercaptoethanol at 60 °C. The membrane was then washed four times in 20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1 % (v/v) Tween 20 at 4 °C and finally incubated overnight at 4 °C in T-PBS containing 5 % (w/v) non- fat milk. For densitometric analysis, the films were scanned and analyzed by Image J program available on http://rsb.info.nih.gov/ij/.

4.7 Immunocytochemistry and microscopy

L1 larvae were washed several times with water and placed on poly-L-lysine-coated slides. Five μl of the pellet containing animals were placed on the glass together with an equal volume of 5 % paraformaldehyde diluted in PBS, covered with a 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 has been chilled on dry ice. While frozen, the cover slide was flipped off to freeze crack animals, the samples were placed in cold methanol (-20 °C) for 10 min and rehydrated in a series of rehydration buffers in methanol: TTBS (Tween Tris-buffered saline – 4.5 g NaCl, 50 ml of 1 M Tris (pH 7.5), 500 ul of Tween, 500 ml of dH_2O)) in ratios 9:1, 7:3, 1:1 and 1:4. Each rehydration step lasted 10 minutes. The specific antibodies were used at a dilution of 1:200 and reactions incubated in a wet chamber at 4 °C overnight. Slides were washed three times in fresh TTBS and the secondary antibodies conjugated to fluorescent dye Alexa Fluor 568 (Molecular Probes, Eugene, OR)) were added in a dilution of 1:400. Slides were incubated at room temperature for 2 h, washed three times in TTBS and mounted in 10 μl of mounting medium (Shandon, Pittsburgh, PA).

The fluorescent and Nomarski pictures of worms were taken using Olympus SZX12 Stereomicroscope System or Olympus BX60 System Microscope both equipped with a light fluorescence attachment. BX60 Microscope is equipped also with Olympus DP30BW camera allowing taking pictures of observed objects.

4.8 Mutant phenotypic analysis

The penetrance of the homozygous *nhr-40 (ok667)* movement phenotype was determined using animals synchronized (described above) at 20 °C with larvae seeded outside the bacterial lawn at 20 °C and transferred immediately to the particular experimental temperatures (3 incubators set for 16 °C, 20 °C and 24 °C). All experiments were done at a minimum in duplicate and scored by two researchers independently. To test the effects of temperature on the homozygous *nhr-40 (ok667)* phenotypic penetrance, 4,291 mutant larvae and 1,567 control larvae were scored, broken down as follows: for 16 °C 1,691 mutants and 372 N2, for 20 °C 1,668 mutants and 195 N2, for 24 °C 932 mutants and 1,000 N2. To test the effects of food restriction, bacterial suspensions were transferred on plates with the increasing volume so that plates with the following bacterial amounts were prepared: none, 3.3×10^5, 3.3×10^6, 3.3×10^7, and 1.65×10^9. Plates were irradiated on the UV transluminator for 2 times 10 min each prior to inoculation of synchronized L1 larvae. Two independent experiments yielded counted larvae as follows: no food condition (n=328), 3.3×10^5 bacteria (n=551), 3.3×10^6 bacteria (n=661), 3.3×10^7 bacteria (n=741), 1.65×10^9 bacteria (n=763). The number of bacteria was calculated from diluted aliquots plated on separate LB plates and number of resulting colonies after 24 h of incubation.

4.9 Molecular biology methods

4.9.1 Total RNA isolation

Animals were washed with water and frozen at -80 °C. The frozen pellet was resuspended in 0.5 ml of RNA lysis buffer (10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 5 % (v/v) 2-mercaptoethanol, 0.5 % (w/v) SDS with 12.5 µl of Proteinase K (20 mg/ml). After 60 s vortexing, the mixture was incubated for 60 min at 55 °C. Phenol-chloroform extraction followed. For 250 µl of the sample, 1 µl of glycogen (20 mg/ml), 50 µl of 10 % (v/v) NaOAc and 0.75 ml of cold ethanol were added. The mixture was incubated for 30 min at -80 °C and centrifugated for 10 min using 12,000 x g at 4 °C. The air-dried pellet was dissolved in water and treated with 1 unit of DNase I (Promega) per 1 µg of total RNA

for 30 min at 37 °C. Phenol-chloroform extraction and ethanol precipitation followed and RNA was resuspended in water.

4.9.2 RT-PCR

cDNA was prepared by reverse transcription reaction with SuperScriptII Reverse Transcriptase (Invitrogen, Carlsbad, CA) of previously isolated DNA-free RNA. 1 µl of total RNA was mixed with 100 ng of random hexamers and heated for 5 min at 65 °C. Then 4 µl of first strand buffer, 1 µl of RNAse Inhibitor (Bioline USA Inc., Randolph, MA), 1 µl of 100 mM DTT, 1 µl of dNTP`s and 1 µl os SuperScript II were added. The mixture was incubated 10 min at 25 °C, 50 min at 42 °C and finally for 15 min at 70 °C. First strand cDNA served as a template of subsequent PCRs with specific primers.

4.9.3 PCR

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

4.9.4 Quantitative PCR

cDNA was prepared from 5 µg of total RNA in a 20 µl reaction using reverse transcription reaction with SuperScriptII Reverse Transcriptase (Invitrogen) and random hexamers. Quantitative PCR was performed as described (Kostrouchova et al., 2001) with modifications. The amplicons of selected regions of *myo-1*, *myo-2*, *myo-3*, *act-1*, *unc-52*, *unc-54*, *unc-60*, *unc-89*, *unc-95*, *unc-97*, *unc-98* and *nhr-60* were amplified using PCR, eluted from agarose gel using electrophoresis and semipermeable membrane, and the amount of DNA was determined spectrophotometrically. The purified DNA was used for

determination of the standard curve. The values were normalized relative to *ama-1* (encoding the large subunit of RNA Pol II (Johnstone and Barry, 1996)), using the LightCycler 480 and the LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, Basel, Switzerland). The primers were designed for an annealing temperature of 59 °C for all examined genes. The analysis was done in two independent experiments each in triplicate.

Table 1: The list of primers.

Gene	Sense primer	Antisense primer
myo-1	tcaatggaaggccagatacgaagg	ggtattacggctgtcacgagtagt
myo-2	cgtgacctcaccgaatcactttcc	gctctgacttggccttggtctcgg
myo-3	atgtctggaaatccagacgcattc	tcctcggtcttgtcgaattttggc
act-1	atgtgtgacgacgaggttgccgct	gctcattgtagaaggtgtgatgcc
unc-52	gagtgttgtgtcttacggacccca	cggttggcttgcagtcactgctgc
unc-54	cgcggaaatgaggtcaccctcaag	ggttctcgtggtcttggagcatgt
unc-60	cggagtcaaagttgatccatcctg	gcgttgtctgggcagtattgaacg
unc-89	gcataagtggctggaacgtcgcgt	gtggaatttgtggtggttgtctat
unc-95	caagaacagatgagaatggaacgt	ctctggtgccggtgtaagtctcaa
unc-97	ggaacgctggacgagctctctgtc	gtgctccacgtgccagtgcttgcc
unc-98	gttgaagcatatgtcggaagttca	cttccgcattcacacttgtacccg
ama-1	ttccaagcgccgcgtcgcattgtctc	cagaatttccagcactcgaggagcgga
nhr-60/L4440	acgcgtcgacgtcggaatgaacccccttgc	cgggatcccggatcaacggtgcaacaga
nhr-60/qPCR	taggtccgccaagactaccgaaac	gatccgtcaggctcaatgataacc

4.9.5 Cloning

PCR amplified DNA fragment and appropriate vector were digested with specific restriction enzymes. After purification, the DNA fragments were ligated into the vectors by T4 DNA ligase (Invitrogen) for 1 hour at room temperature.

nhr-60 in L4440 – the sense primer *nhr-60/*L4440 with Sal1 restriction site and anti-sense primer *nhr-60/*L4440 with BamH1 restriction site were used for PCR using N2 cDNA mixture template. 690 bp DNA fragment was cloned into L4440 vector containing two convergent T7 polymerase promoters in opposite orientation separated by a multicloning site.

4.9.6 Transformation

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

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

4.9.7 In vitro transcription

Prior to in vitro transcription, the specific construct *nhr-60/*L4440 was linearised at the ends of inserted DNA using unique restriction sites. 500 ng of each linearised construct was mixed with 20 µl of rNTP´s, 10 µl of 5x transcription reaction buffer, 1 µl of 100 mM DTT, 1 µl of RNase Inhibitor, 4 µl of T7 polymerase (Promega) (2.5 µl for the first hour, next 1.5 µl for the second hour) and water was added up to final volume 50 µl. Both mixtures (for sense and antisense ssRNAs) were incubated at 37 °C for 2 hours. Equal

amounts of both ssRNAs (confirmed by electrophoresis) were mixed and incubated for 10 min at 70 °C and for 30 min at room temperature to anneal. dsRNA was purified by phenol-chloroform extraction and ethanol precipitation.

4.10 RNA interference – soaking and microinjections

Soaking

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

Microinjections

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

5 Results

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

5.1.1 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.

5.1.2 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 (Fig. 7 A).

The same result was obtained using the computer programs ProteoVue and DeltaVue (Fig. 7 B and C) 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. 8). Two complete analyses and one analysis with only selected second dimension separations were done.

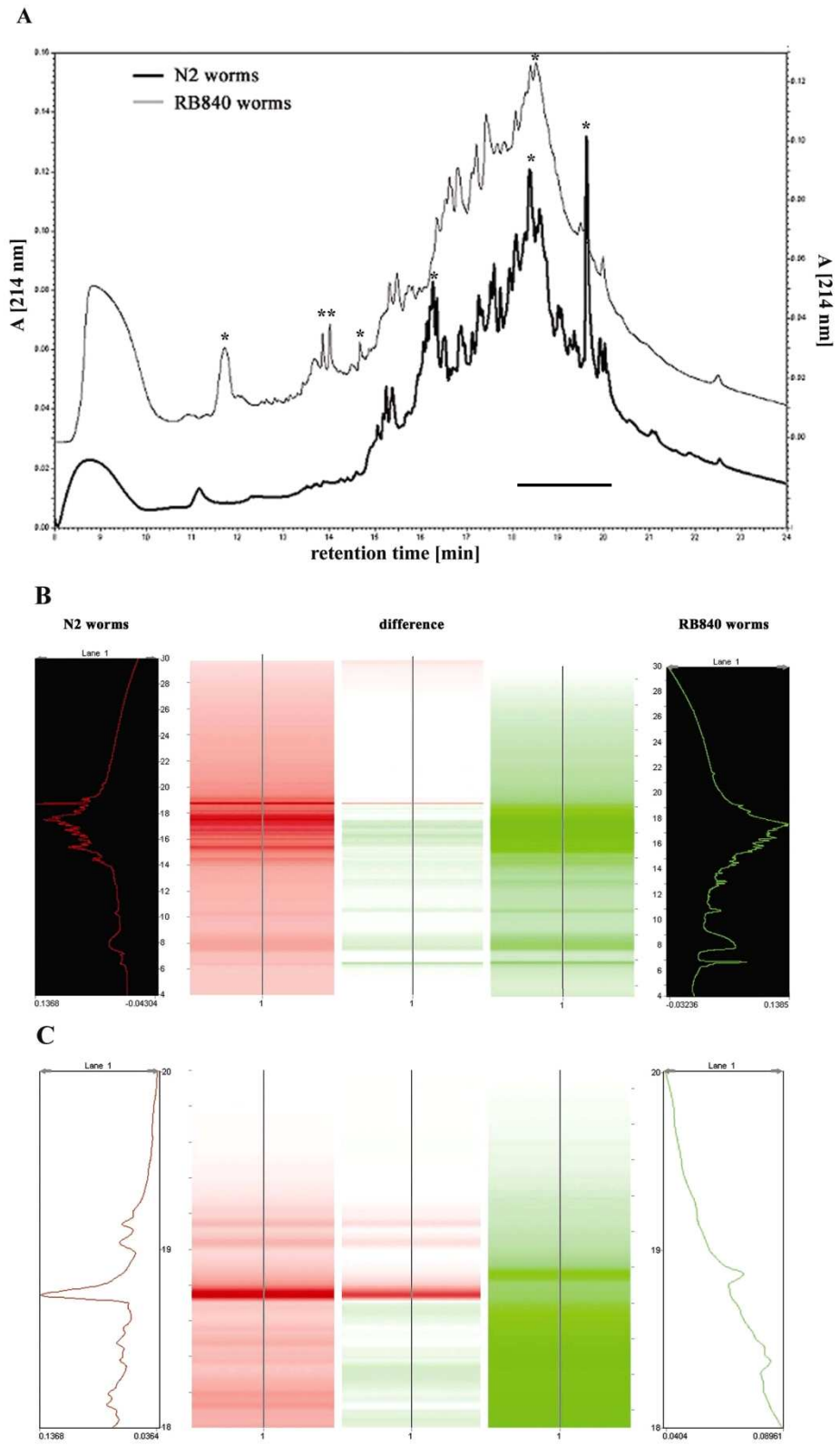
A
N2 worms
RB840 worms
A [214 nm]
A [214 nm]
retention time [min]
B
N2 worms
difference
RB840 worms
Lane 1
Lane 1
C
Lane 1
Lane 1

Fig. 7: Chromatograms of the second dimension – reversed phase HPLC

Representative comparison of the third fractions (A3) in which the *nhr-40 (ok667)* data was overlaid with an offset on the Y-axis (absorbance), for better visualization (wild type values left, *nhr-40 (ok667)* right). The peaks that significantly differed in both chromatograms (asterisks) were used for further analysis. (B) The comparison of chromatograms (shown in panel A) by the ProteoVue program. Panel C shows a detail of the region indicated in panel A by the black line and corresponds to a fraction that was further analyzed by mass spectroscopy.

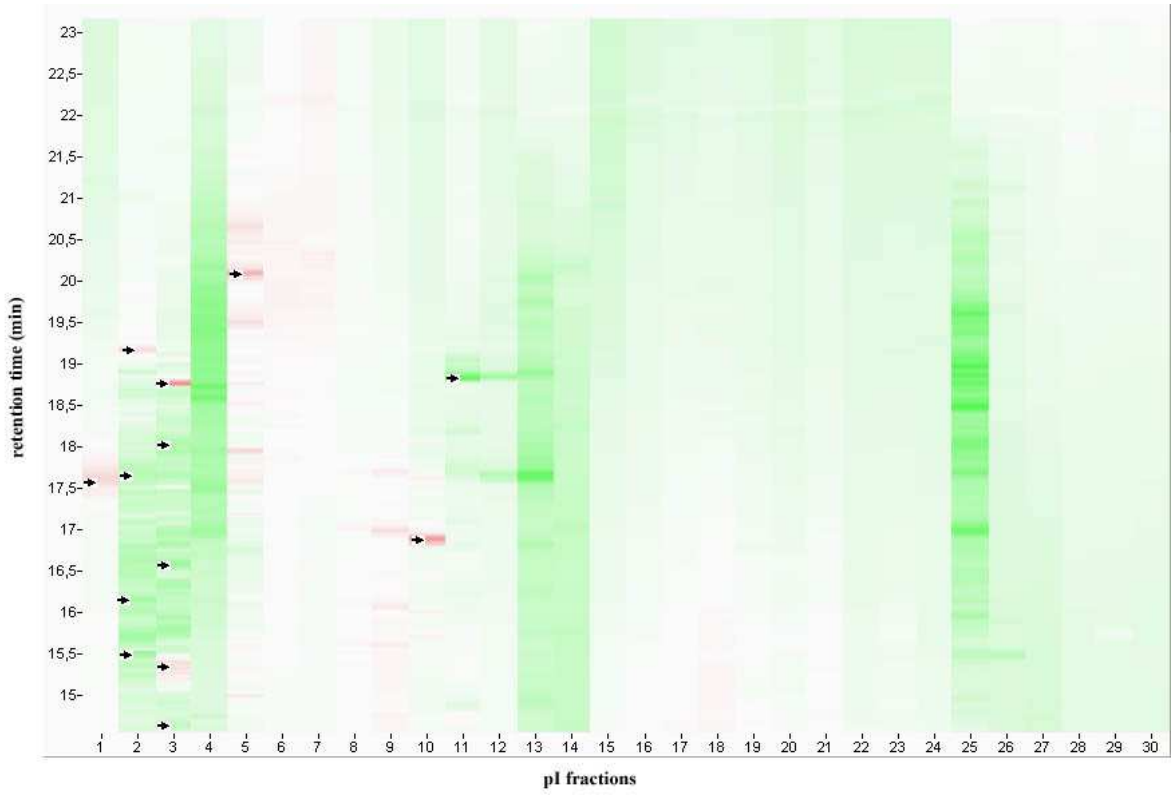

Fig. 8: Representative part of the whole proteome differential display of second dimension chromatograms prepared using the program DeltaVue

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 (Table 2, Appendix). 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 (Table 3, Appendix).

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. 9). 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).

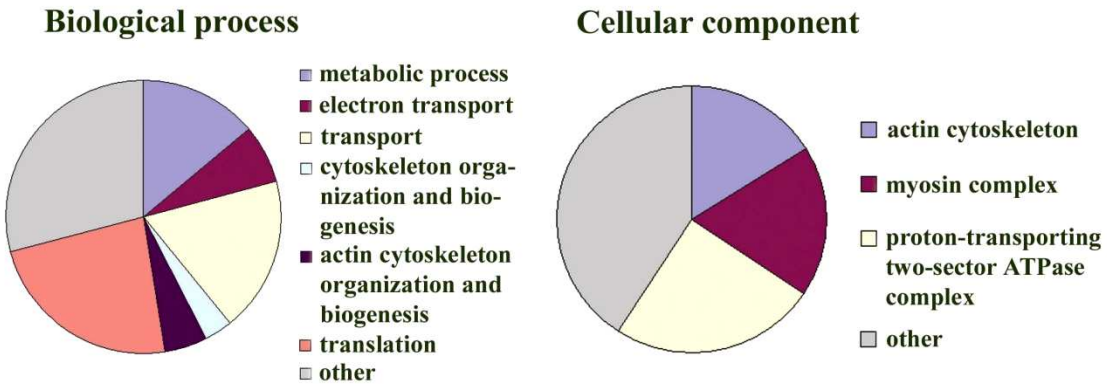

Fig. 9: Gene Ontology analysis of differentially eluted proteins

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

5.1.3 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), immunohistochemistry 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. 10).

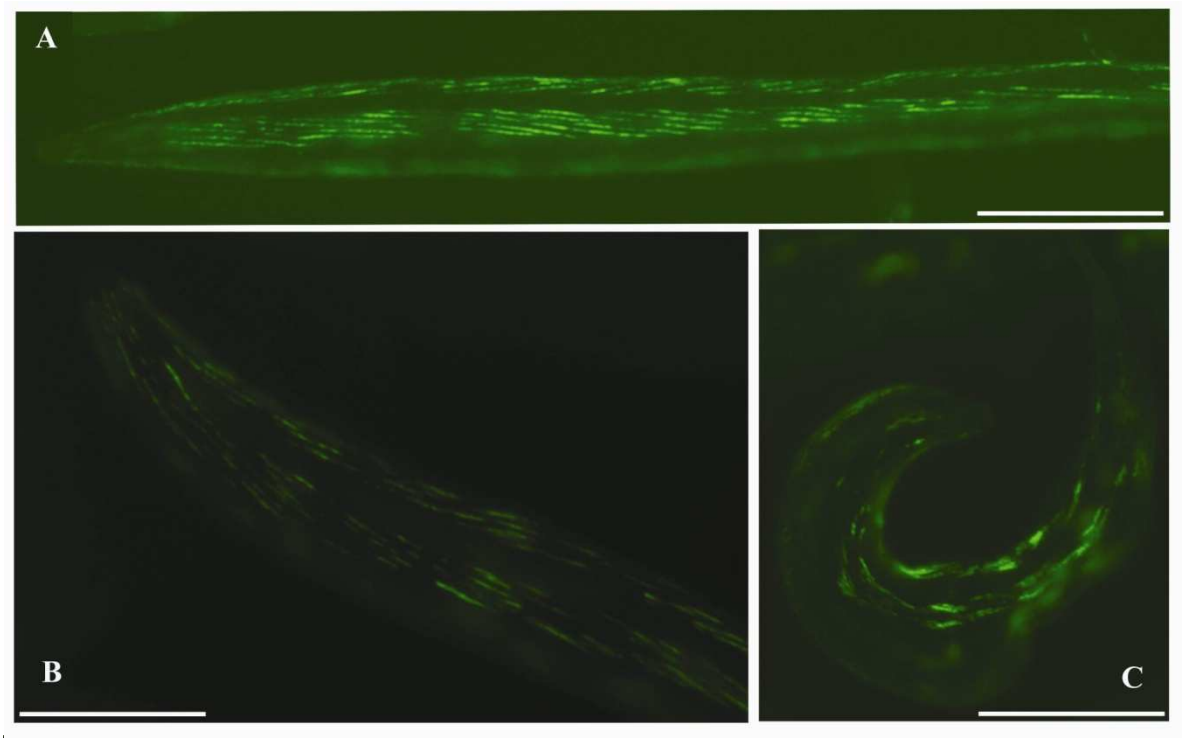

Fig. 10: Detection of MYO-3 by immunocytochemistry

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 et al., 1989, Moerman and Williams, 2006). Body wall muscle comprises 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 (qRT-PCR) 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. 11).

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.

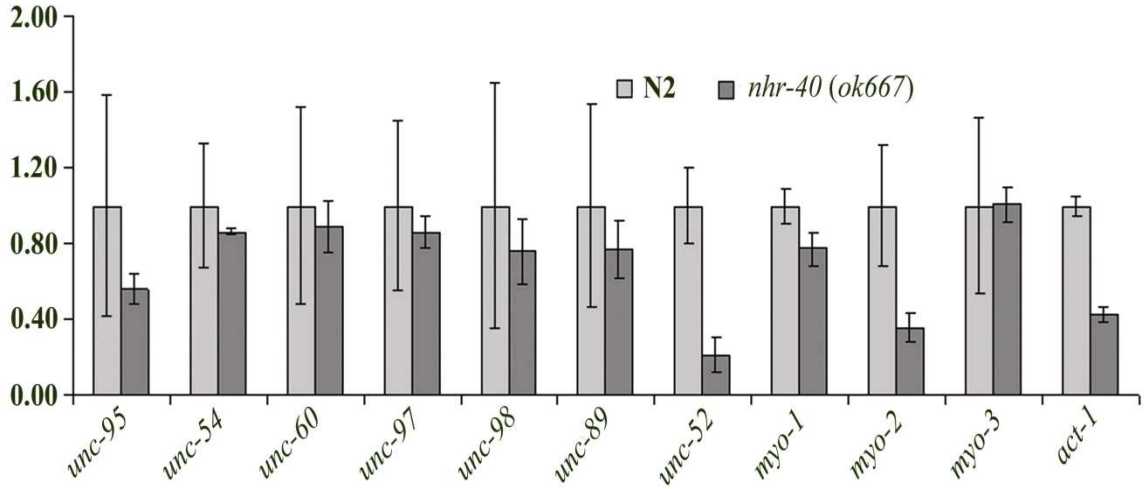

Fig. 11: The expression of selected muscle genes using qPCR

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. 12). 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.

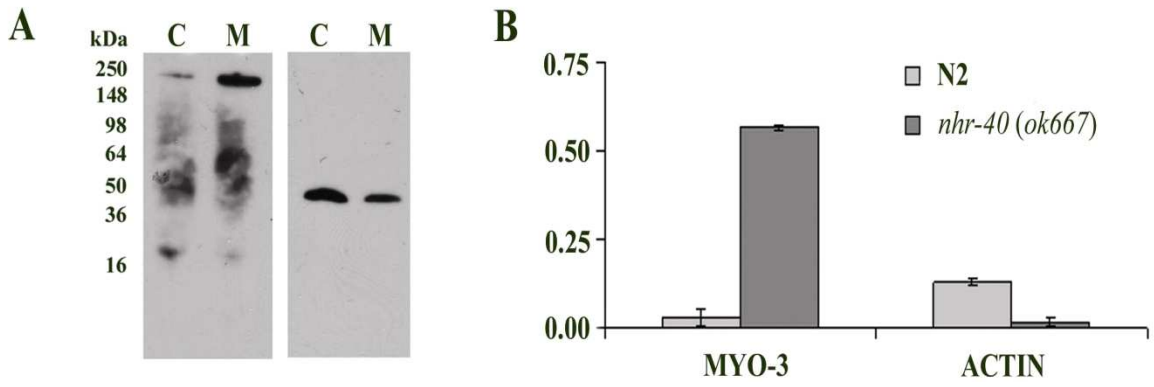

Fig. 12: Detection of MYO-3 and actin in wild type and *nhr-40 (ok667)* larvae by Western blot

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).

5.1.4 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).

5.4.4.1 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. 13).

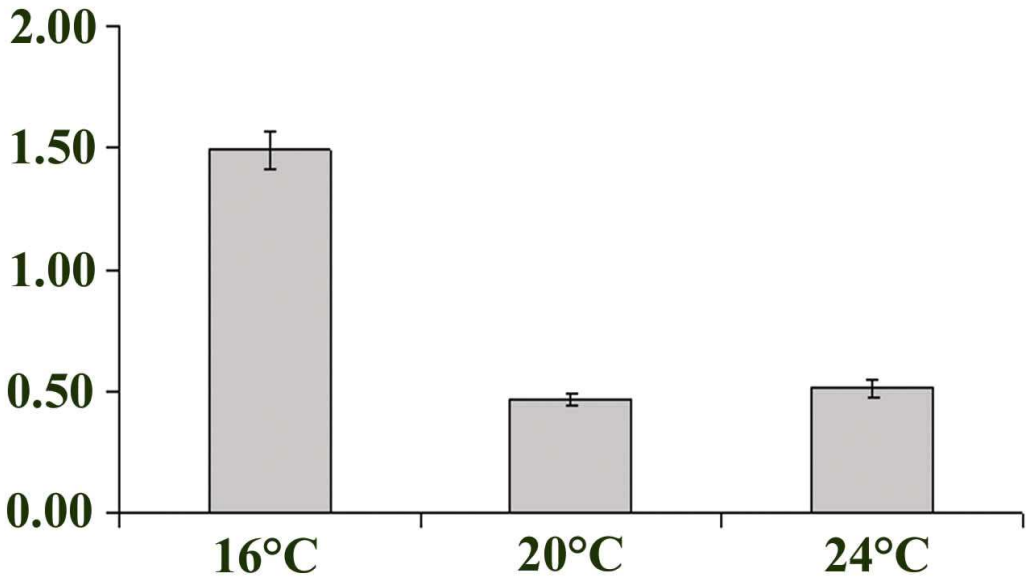

Fig. 13: The influence of culture temperature on the penetrance of *nhr-40 (ok667)* locomotion phenotype

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. 14). No similar effect was observed on N2 control larvae.

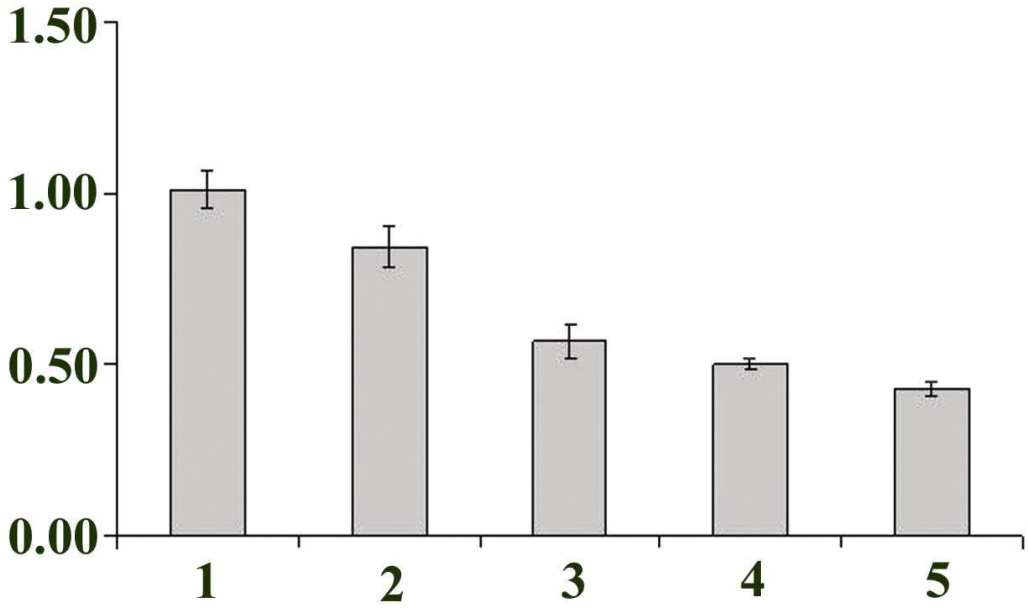

Fig. 14: The effect of food restriction on the *nhr-40 (ok667)* mutant phenotypes

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).

5.2 Proteomic characterization of NHR-60

5.2.1 *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 as well. 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. 15).

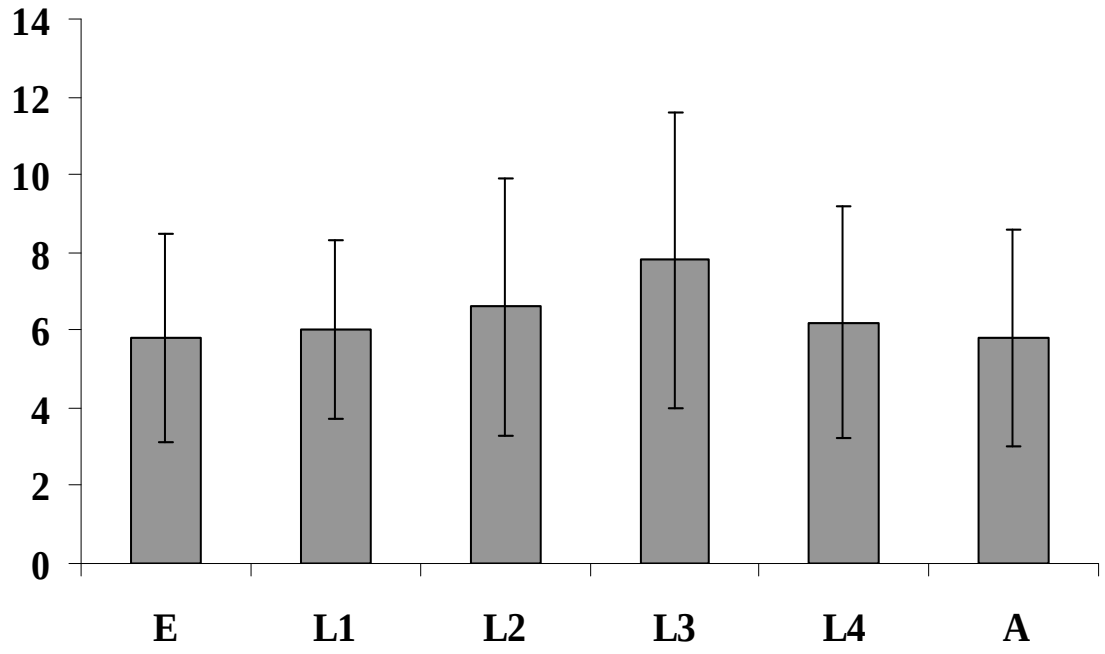

Fig. 15: The analysis of *nhr-60* mRNA expression during *C. elegans* developmental stages.

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).

5.2.2 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. 16). Because the expected size of NHR-60 protein is approximately 50 kD we suppose NHR-60 to be posttranslationally modified.

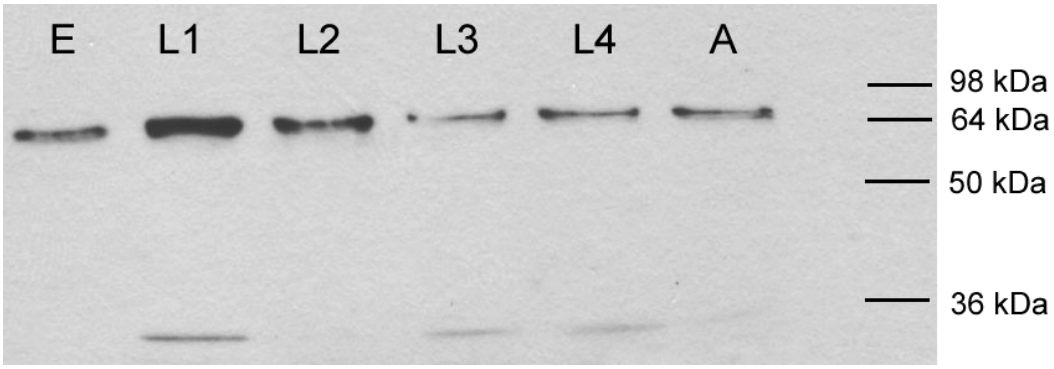

Fig. 16: Detection of NHR-60 in *C. elegans* developmental stages using Western blot
60 µg of protein lysates from developmental stages of *C. elegans* were used for Western blot detection of NHR-60 using rabbit polyclonal antibody against NHR-60. NHR-60 is expressed in all stages. The detection of 36 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. 17). 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.

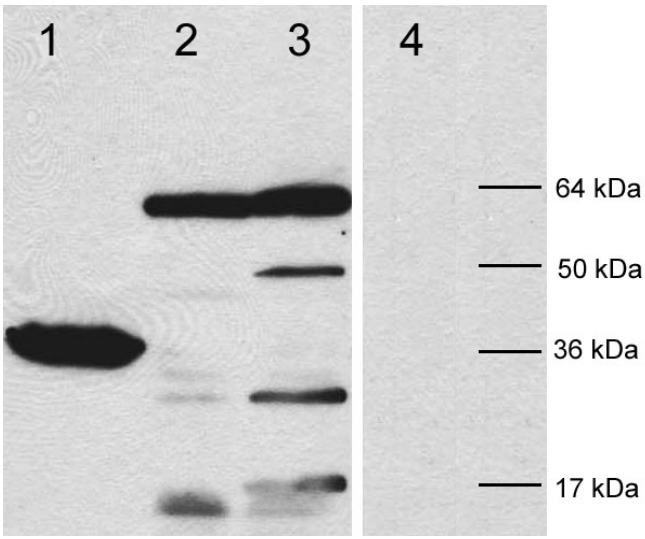

Fig. 17: Analysis of wild type NHR-60 by Western blot

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. 18).

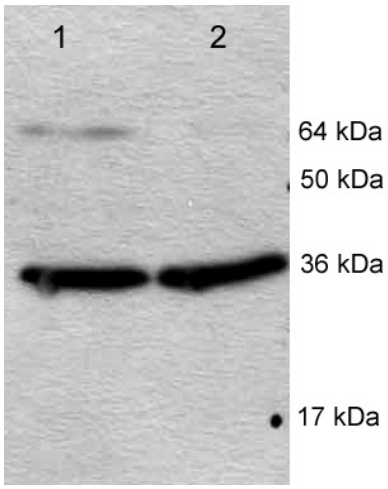

Fig. 18: Western blot analysis of NHR-60 in wild type embryos and in embryos following *nhr-60* RNAi

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.

We were interested in the posttranslational modification of NHR-60. In this sense, we designed a sequence of experiments to uncover it. Firstly, we cultured a mixed population of *C. elegans* and we prepared protein lysate for chromatographic separations. In the first dimension, we separated proteins according to their isoelectric point by HPLC (Beckman Coulter, Inc.) (Fig. 19A) and identified a fraction that contained the modified NHR-60 by Western blot analysis. The fraction was then used for the next HPLC separation (Fig. 19B) according to protein hydrophobic properties. Again, we identified a fraction that contained modified NHR-60 protein by Western blot analysis. The separated fraction contained less different proteins than the protein lysate and therefore was suitable for mass spectrometric analysis. Tandem MS surely uncovered around 10 proteins with a high score of probability. Unfortunately, NHR-60 and its modification were not detected. On the other hand, the two dimensional separation of protein with tandem MS detection could be used for identification of posttranslational modification of higher abundant proteins.

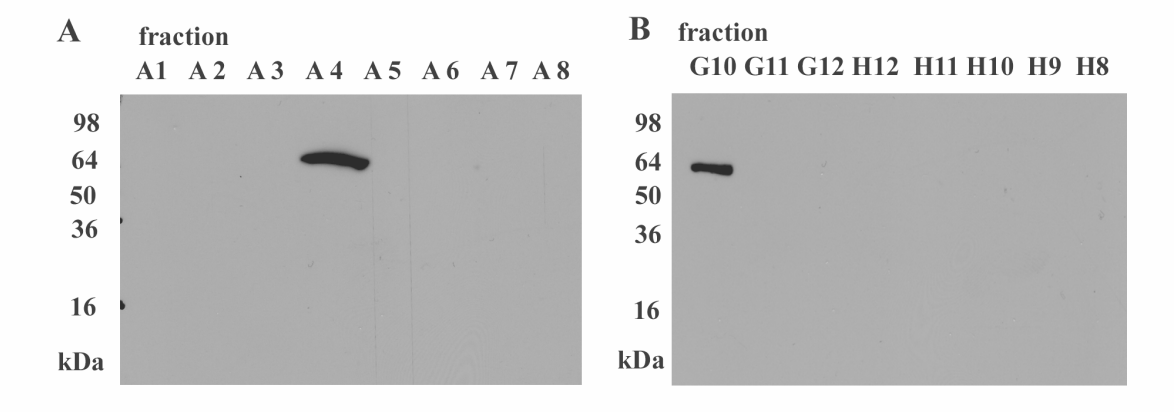

Fig. 19: Detection of NHR-60 in the fractions by Western blot

A. 40 µl of the fractions were loaded into lanes. The rabbit polyclonal antibody anti-NHR-60 recognized a protein migrating at 64 kDa in the fraction A4. The fraction was then used for the second dimension separation on reversed phase HPLC.

B. 40 µl of the fractions were loaded into lanes. The rabbit polyclonal antibody anti-NHR-60 recognized a protein migrating at 64 kDa in the fraction G10. The positive fraction was overdried by SpeedVac and prepared for MS analysis.

6 Discussion

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

Unbiased proteomic techniques are powerful complements to genome wide expression studies. Increased sensitivity of mass spectroscopy in recent years allows unprecedented precision in the determination of proteins and peptides in protein mixtures. Complete protein maps of procaryotic and eucaryotic organisms are likely to add a new dimension to biology (Renzone et al., 2005). Decades long literature track of proteomic studies documents the enormous plasticity and complexity of proteomes of organisms and tissues. The major obstacle of proteomic studies is the inherent reciprocal influence of proteins in mixtures affecting the sensitivity and validity of both separation techniques and mass spectrometry. This brings the necessity to separate the complex mixtures to samples deprived of abundant proteins that mask and prevent detection of other proteins that are less abundant but often biologically more important. In most methods, the experimental material has to be processed for removal of predominant protein species or to restrict the complex materials to less complex samples. In comparative studies, this is an unwanted step which may affect the proportionality of compared materials and prevent detection of some proteins. A recently developed fully automatic chromatographic system (ProteomeLab™ PF 2D Protein Fractionation System, Beckman Coulter) for two dimensional separation of complex protein mixtures allows comparison of relatively unrestricted materials with high reproducibility.

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.

6.2 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.

7 Conclusion

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.

8 References

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9 Appendix

9.1 The list of figures

9.2 Table 2: MS/MS analysis of proteins detected differentially in wild type N2 larvae and *nhr-40 (ok667)* larvae

Part A. Proteins found only in N2 larvae. Part B. Proteins found only in *nhr-40 (ok667)* larvae.

Part A. Proteins found only in N2 larvae.

	Peptide	MH+	Score XC	RSp	Ions (Hits)
Hypothetical protein C06A8.3 [Caenorhabditis elegans]					
	K.VAADWADFQK.F	1151.25293	1.72	1	11/18
	K.VQAWVNQQGGK.V	1215.34338	2.52	1	16/20
	K.IQAYLNSLPANVK.A	1431.66174	2.21	1	11/24
	K.KADADLTAISNDSSLSVQAK.G	2035.19910	5.23	1	24/38
	K.GQQGQAEAAHQAAVSNFSPAAK.K	2169.30005	4.36	1	31/84
Hypothetical protein T02H6.11 [Caenorhabditis elegans]					
	R.AETSGLIPPYQMK.Q	1435.67065	1.71	1	13/24
	R.LNLQEPHVFDQR.K	1496.65234	2.76	1	13/22
	K.AVTFAYNGTIASTLR.K	1585.78577	2.25	1	12/28
	R.EYGLQFHDTYFEPAPEVTEALR.R	2613.81836	3.69	1	17/42
	K.AEWTQWDQESWYLKPYLDEIEAEK.K	3059.28638	3.55	1	25/92
Hypothetical protein C06H2.1 [Caenorhabditis elegans]					
	K.VADGLQEAK.K	931.02551	2.46	1	14/16
	R.VATSSVNWSK.L	1079.18823	2.04	1	10/18
	K.IPYGEVPAEYLK.E	1379.58203	2.60	1	17/22
	K.ALPAHSAVLDSLQK.Q	1450.66504	2.22	1	15/26
	K.GVSGTFQSAVSQLPADLPK.I	1903.12549	3.64	1	21/36
Hypothetical protein Y105E8A.16 [Caenorhabditis					

elegans]				
R.LINLHAPAEVLR.Q	1346.60315	3.38	1	15/22
R.LTLTSQNVKPLEK.V	1471.72412	2.95	1	16/24
R.QITSISIEPGVDIEVTR.A	1858.08301	2.87	1	18/32
Hypothetical protein F40A3.3b [Caenorhabditis elegans]				
K.WDAEPGALYTLIK.T	1477.68567	2.32	1	17/24
K.FNSGVEANLGNVLTPTQVK.D	1989.21838	2.01	13	10/36
K.LGAPVFGNLFQAEYDDYVPILNK.Q	2584.90649	2.72	1	18/44
Lipid binding protein protein 9 [Caenorhabditis elegans]				
K.WNFVSSENFDEYLK.E	1778.89819	3.22	1	17/26
R.DVSSVFSIENDHLVQIETGK.G	2218.40601	3.92	1	19/38
Lipid binding protein protein 6 [Caenorhabditis elegans]				
K.SVVNFENGK.F	994.08331	2.10	1	12/16
K.LGQEFDETTPDGR.T	1465.50391	3.54	1	17/24
Hypothetical protein Y44A6D.2 [Caenorhabditis elegans]				
K.DVLSTAADATK.G	1092.18201	2.72	1	16/20
K.GYASQAQQAIGK.I	1222.33264	3.53	1	19/22
heat shock protein 16-1 [Caenorhabditis elegans]				
K.FAINLNVSQFKPEDLK.I	1864.13403	2.41	1	13/30
R.VILLPEDVDVGAVASNLSEDGK.L	2241.48120	3.43	1	27/84
S41018 hypothetical protein T07C4.5 – [Caenorhabditis elegans]				
R.VSEYLIPK.E	949.12561	2.32	1	11/14
K.IGGTYDMTYVTLDILSAK.D	1962.25305	3.15	1	16/34
Hypothetical protein H28O16.1a [Caenorhabditis elegans]				
R.VVDALGNPIDGK.G	1198.35095	3.36	1	18/22
K.HALIIFDDLSK.Q	1272.47473	2.53	1	13/20
K.TAIAIDTIINQK.R	1301.51440	2.76	1	17/22
R.TGAIVDVPVGDGLLGR.V	1539.75854	3.33	1	22/30
R.ILGTETGINLEETGK.V	1575.74292	3.45	1	18/28
B Chain B, Structural Genomics Of Caenorhabditis				

Elegans: Triose Phosphate Iso					
	R.HVFGESDALIAEK.T	1416.56079	2.70	1	17/24
	K.TASGEQAQEVHEWIR.A	1741.84277	3.01	1	14/28
	K.KPDIDGFLVGGASLKPDFVK.I	2104.43408	5.31	1	31/76
Calponin protein 3 [Caenorhabditis elegans]					
	R.DNFHNLLK.D	1001.12067	2.23	5	11/14
	R.TTAGGIGFAVR.Q	1050.19324	2.63	1	17/20
Hypothetical protein Y18D10A.20 [Caenorhabditis elegans]					
	K.GADIEGVHYVVPR.T	1412.57532	3.16	1	17/24
gpd-2 gene product [Caenorhabditis elegans]					
	K.GTVAHEGDYLLVAK.E	1473.65552	2.50	1	15/26
Aconitase protein 2, isoform a [Caenorhabditis elegans]					
	R.LDFNPLTDELTAADGSK.F	1807.93616	2.85	1	18/32
Hypothetical protein K12F2.1 [Caenorhabditis elegans]					
	K.DLTDQLGEGGR.S	1161.20447	2.61	1	16/20

Part B. Proteins found only in *nhr-40 (ok667)* larvae.

Peptide	MH+	Score XC	RSp	Ions (Hits)
Hypothetical protein K08H10.1 [Caenorhabditis elegans]				
ASDIADSFK.A	954.01593	2.32	1	13/16
K.AGDAISGAYDSVK.E	1254.32825	2.81	1	17/24
K.VGDSISGAYDTVK.E	1312.40771	2.71	1	18/24
K.IGDSISGAWESTK.D	1351.44397	3.09	1	17/24
S41018 hypothetical protein T07C4.5 – [Caenorhabditis elegans]				
R.VSEYLIPK.E	949.12561	2.12	3	10/14
K.IGGTYDMTYVTLDILSAK.D	1962.25305	3.73	1	20/34
Ribosomal protein, small subunit protein 28 [Caenorhabditis elegans]				
R.VEFINDQNNR.S	1249.31494	3.07	1	15/18
R.EGDILTLLESER.E	1375.50696	3.18	1	16/22
Sod (superoxide dismutase) protein 1, isoform a [Caenorhabditis elegans]				
K.SENDQAVIEGEIK.G	1432.51538	3.66	1	20/24
Sod (superoxide dismutase) protein 5 [Caenorhabditis elegans]				
R.HVGDLGNVEAGADGVAK	1609.72266	3.53	1	30/64
Histone protein 60 [Caenorhabditis elegans]				
R.DNIQGITK.P	888.98865	2.65	1	12/14
K.VFLENVIR.D	990.18091	2.76	1	12/14
R.ISGLIYEETR.G	1181.32043	1.82	5	10/18
R.DNIQGITKPAIR.R	1326.52710	2.17	1	14/22
Ribosomal protein, large subunit protein 7 [Caenorhabditis elegans]				
K.VPQVPETVLK.R	1110.32849	2.08	2	11/18

	K.KVPQVPETVLK.R	1238.50134	2.37	1	13/20
	R.VPITDNTIVEQSLGK.F	1614.82227	3.41	1	20/28
Hypothetical protein C50F4.13 [Caenorhabditis elegans]					
	R.HLQLAVR.N	837.00494	2.27	1	11/12
	K.LLAGVTIAQGGVLPNIQAVLLPK.K	2286.78564	3.21	1	16/44
Elongation factor protein 4, isoform d [Caenorhabditis elegans]					
	R.LPLQDVYK	976.15100	2.51	2	12/14

9.3 Table 3: Discrete set of proteins that had significantly altered chromatographic profiles

Part A. Proteins found only in N2 larvae. Part B. Proteins found only in *nhr-40 (ok667)* larvae.

Part A. Proteins found only in N2 larvae.

Proteins found in N2 worms			
	Protein name	**NCBI KOGs**	**Characterization**
1	C06A8.3	Unnamed protein	C06A8.3 gene
2	PHI-44	Ubiquinol cytochrome c reductase, subunit QCR7	T02H6.11 gene
3	ATP-5	Mitochondrial F1F0-ATP synthase, subunit d/ATP7	none available
4	RPS-20	40S ribosomal protein S20	rps-20 encodes a small ribosomal subunit S20 protein
5	F40A3.3B	Phosphatidylethanolamine binding protein	F40A3.3b gene
6	LBP-9	Fatty acid-binding protein FABP	none available
7	LBP-6	Fatty acid-binding protein FABP	none available

8	Y44A6D.2	none available	Y44A6D.2 gene
9	HSP-16.1	Alpha crystallins	*hsp-16.1* encodes a 16-kD heat shock protein (HSP) that is a member of the hsp16/hsp20/alphaB-crystallin (HSP16) family of heat shock proteins.
10	TTR-15	Uncharacterized protein with conserved cysteine	none available
11	PHI-37	F0F1-type ATP synthase, alpha subunit	H28O16.1 gene
12	TPI-1	Triosephosphate isomerase	*tpi-1* encodes a putative triosephosphate isomerase orthologous to human TPI1 and *Drosophila* WASTED AWAY.
13	CPN-3	Calponin	*cpn-3* encodes a calponin homolog, most closely related to its paralog CPN-4 in *C. elegans;* CPN-3 is more similar to the calponin paralogs transgelin (SM22 alpha) or neuronal protein NP25 than to calponin per se.
14	PFN-1	Profilin	none available
15	GPD-2	Glyceraldehyde 3-phosphate dehydrogenase	GPD-2 is predicted to reversibly catalyze the oxidation and phosphorylation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate during glycolysis.
16	ACO-2	Aconitase/homoaconitase (aconitase superfamily)	*aco-2* encodes an aconitase homolog that is required for embryonic viability, fertility, locomotion, and vulval morphogenesis, and perhaps for normal lifespan.
17	MYO-3	Myosin class II heavy chain	*myo-3* encodes MHC A, the minor isoform of MHC (myosin heavy chain) that is essential for thick filament formation, and for viability, movement, and embryonic elongation.

Part B. Proteins found only in *nhr-40 (ok667)* larvae.

Proteins found in *nhr-40* (*ok667*)			
	Protein name	**NCBI KOGs**	**Concise description**
1	LEA-1	Uncharacterized conserved protein	The *lea-1* gene encodes a protein that is predicted to be hydrophilic and heat-resistant, and that might participate in anhydrobiosis.
2	TTR-15	Uncharacterized protein with conserved cysteine	none available
3	RPS-28	40S ribosomal protein S28	*rps-28* encodes a small ribosomal subunit S28 protein.
4	SOD-1	Cu2+/Zn2+ superoxide dismutase SOD1	*sod-1* encodes the copper/zinc superoxide dismustase, an enzyme that is known to protect cells from oxidative damage.
5	SOD- 5	Cu2+/Zn2+ superoxide dismutase SOD1	none available
6	HIS-60	Histone H4	*his-60* encodes an H4 histone; by homology, HIS-60 is predicted to function as a nucleosome component required for packaging of DNA into chromatin.
7	RPL-7	60S ribosomal protein L7	*rpl-7* encodes a large ribosomal subunit L7 protein required in mass RNAi assays for embryonic viability and normally rapid growth.
8	HIS-35	Histone 2A	*his-35* encodes an H2A histone.
9	EFT-4	Translation elongation factor EF-1 alpha/Tu	*eft-4* encodes a translation elongation factor 1-alpha homolog that is required for embryonic viability, fertility, and a normal postembryonic growth rate.

9.4 List of author`s publications and presentations

9.4.1 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)

9.4.2 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.

M. Pohludka: ProteomLab PF2D/BioLogicLP navzdory určení poměrně versatilní systém. Oral presentation. *The proteomic workshop, Biotech*, Praha, Czech Republic, May 07, 2007.

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.

P. Liby, **M. Pohludka**, P. Hrabal, M. Kostrouchova, Z. Kostrouch: The expression of SKI-interacting protein (SKIP) is elevated in human astrocytic tumors. Oral presentation. *Kunců v memorial*, Neurochirurgicka klinika 1. LF UK a UVN, Praha, Czech Republic, March 15 – March 16, 2007.

M. Pohludka, J. Vohánka, P. Novák, M. Kostrouchová, P. Yilma, P. Libý, Z. Kostrouch: Použití microarrays a komparativní dvoudimensionální chromatografie pro analýzu exprese mRNA a proteinů v prvním larválním stádiu *Caenorhabditis elegans* v závislosti na potlačení exprese CHR-3 (*nhr-23*). Oral presentation. *Proteo 2006*, Liběchov, Czech Republic, Sep 26 – Sep 27, 2006.

J. Vohánka, **M. Pohludka**, E. Brožová, P. Novák, Z. Kostrouch, M. Kostrouchová: Studium fenotypu mutanty *C. elegans* RB840 metodou komparativní dvoudimensionální chromatografie. Oral presentation. *Proteo 2006,* Liběchov, Czech Republic, Sep 26 – Sep 27, 2006.

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. Oral presentation. *7th Student Scientifical Conference,* Prague, Czech Republic, May 22, 2006.

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.

P. Liby, **M. Pohludka**, P. Yilma, P. Hrabal, M. Kostrouchova, Z. Kostrouch: The expression of HDAC3 in human glial tum ors of astrocytic origin. Oral presentation. *Kuncův memorial*, Neurochirurgicka klinika 1. LF UK a UVN, Praha, Czech Republic, March 16 – March 17, 2006.