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

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ABSTRACT

Caenorhabditis elegans has an unexpectedly large number (284) of genes encoding nuclear hormone receptors, most of which are nematode-specific and are of unknown function. We have exploited comparative two-dimensional chromatography of synchronized cultures of wild type *C. elegans* larvae and a mutant in *nhr-40* to determine if proteomic approaches will provide additional insight into gene function. Chromatofocusing, followed by reversed-phase chromatography and mass spectrometry, identified altered chromatographic patterns for a set of proteins, many of which function in muscle and metabolism. Prompted by the proteomic analysis, we find that the penetrance of the developmental phenotypes in the mutant is enhanced at low temperatures and by food restriction. The combination of our phenotypic and proteomic analysis strongly suggests that NHR-40 provides a link between metabolism and muscle development. Our results highlight the utility of comparative two-dimensional chromatography to provide a relatively rapid method to gain insight into gene function.

Nuclear hormone receptors (NHRs) are transcription factors that regulate metabolism and development in all animal species studied to date [1]. The number of NHRs in genomes of species that have been sequenced varies from tens to several hundreds. The genome of *Caenorhabditis elegans* contains 284 sequences that share homology to vertebrate and insect NHRs [2] and the sequence analysis identifies 268 NHRs in the genome of *Caenorhabditis briggsae* [3,4]. The large number of genes coding for nematode NHRs contrasts with the 48 genes coding for NHRs in the human genome and 18 such genes in *Drosophila mellanogaster* [5].

Most NHRs found in *C. elegans* are related to human Hepatocyte Nuclear Factor 4 (HNF4; NR2A according to Nuclear Receptors Nomenclature Committee 1999 and NUREBASE [6]) and are called supplementary nuclear receptors (supnrs) [2]. The supnrs (NR2G [6]) are nematode-specific genes that have arisen by repeated and successive duplications in a relatively short period of time after the split of insects and nematodes [2]. The evolutionary pressure that led to the multiplication of NR2A group of NHRs in nematodes is not known. However, several supnrs have been shown to regulate development or metabolism. For example, ODR-7 regulates olfactory gene expression [7,8] whereas NHR-49 regulates genes important for nutritional response and fatty acid beta oxidation [9,10]. As the functions of more supnrs are determined, our understanding of development and metabolism will be enhanced and the driving force behind NHR gene expansion in nematodes may become clarified.

To this end, we have studied several *C. elegans* NHRs, including NHR-40 that is a supnr belonging to a subgroup of 18 NHRs characterized by the DNA-binding sequence CNGCKT [11]. Previously, we 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. The penetrance of these phenotypes in larvae is only 20% in both the mutant allele and following *nhr-40* RNAi [11], likely reflecting a reduction of function in NHR-40 and/or possible compensation by redundantly acting supnrs.

NHR-40 exemplifies the challenges 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 additional insight into supnr function. In this report we use comparative two-dimensional chromatogra-

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phy and mass spectrometry to further characterize the *nhr-40*(*ok667*) mutant phenotype at the proteome level. Using synchronized larval cultures of wild type 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 our 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.

Materials and methods

Wild type *C. elegans* N2, and the RB840 strain harboring the *nhr-40*(*ok667*) deletion generously supplied by the *Caenorhabditis* Genetic Center were cultured as described [12].

Chromatography. Two-dimensional chromatographic protein separation was performed using a commercially available system ProteomeLab™ PF 2D Protein Fractionation System (Beckman Coulter, Inc. Fullerton, CA) as recommended by manufacturer with the exception that Elution Buffer was adjusted to pH 6.4 ± 0.1 and proteins with lower isoelectric point were eluted by ionic strength with 1 M NaCl. The chromatograms were analyzed using computer software provided by the manufacturer.

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

Five microliters of tryptic peptide mixture was applied on the Magic-C18 column, 0.180 × 150 mm, 200 Å, 5 μm (Michrom Bioresources, Auburn, CA) and separated using gradient elution. The column was connected to a LCQ^{DECA} ion trap mass spectrometer (ThermoQuest, San Jose, CA) equipped with a nanoelectrospray ion source. Spectra were searched with the SEQUEST™ software against the SwissProt database. SEQUEST results were automatically processed with the BioWorks Browser software [13] using the following criteria: XCorr values were 1.7 for singly charged, 2.2 and 3.0 for doubly and triply charged peptides, respectively.

Molecular analysis. Westerns were performed using a standard protocol. Protein concentration was estimated using BCA Protein analysis kit (Pierce, Rockford, IL) as recommended and 80 μg of proteins were loaded for each sample. The primary rabbit polyclonal IgG anti-NHR-60 [14] and mouse monoclonal IgG anti-MYO-3 [15,16] antibodies were used as described [17]. For densitometric analysis, the films were scanned and analyzed by Image J program available at http://rsb.info.nih.gov/ij/.

Immunocytochemistry and light microscopy were done as described [11,14,17].

Quantitative PCR was done as described [17] 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*, and *unc-98* were amplified using PCR and the purified DNA was used for determination of the standard curve. The values were normalized relative to *ama-1* using the LightCycler 480 and the LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, Basel, Switzerland).

Meta-data analysis. Gene ontology analysis was done as described [18]. 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).

Detailed Materials and methods can be found in Supplementary information.

Results and discussion

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 separated proteins using chromatofocusing. First dimension chromatography separated all proteins into 37 fractions based on isoelectric point. Each fraction was then automatically separated into an additional 35 fractions by 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 (not shown).

Chromatographic profiles of nhr-40(ok667) reveal muscle and metabolic abnormalities

Chromatograms from samples to be compared can be overlaid using one of several computer programs (e.g. 32Karat). We used ProteoVue and DeltaVue that graphically represent the chromatographic peaks as artificial electrophoretic bands of different colors (green and red) for each compared sample. The colored overlays highlight the chromatographic peaks containing more protein in one sample than the other, as estimated by absorbance at 214 nm. In the majority of cases, the baseline shape was almost identical in paired wild type and mutant chromatograms. However, in some cases one of the chromatograms had a higher relative baseline causing a green or red coloration throughout the fraction profile. Nevertheless, grossly different peaks were easily recognized by the ProteoVue and DeltaVue computer programs and were always controlled on classical chromatogram curves in 32Karat software.

Comparison of paired wild type and *nhr-40*(*ok667*) mutant chromatograms showed similarities in most major peaks and in the trend of the curves. However, several major peaks were clearly different between the mutant and control animals (Fig. 1). The comparison of complete chromatograms identified approximately 50 major peaks recognized by the DeltaVue program as more abundant in control fractions and 10 peaks as more abundant in *nhr-40*(*ok667*) worms (Fig. 2). Chromatographic peaks represent O.D. readings from the collected fractions, not individual protein profiles, and novel peaks simply reflect changes in the proteome elution profile between compared samples.

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 pro-

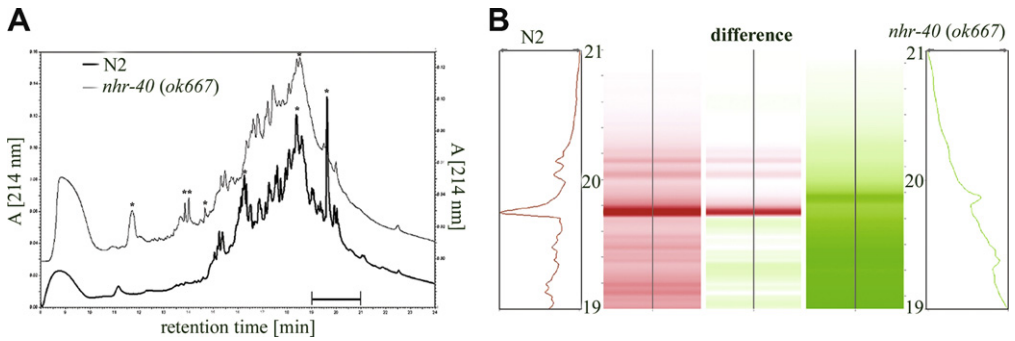

Fig. 1. (A) Chromatograms of second dimension—reverse phase HPLC. Representative comparison of the third fractions (A3) in which the *nhr-40*(*ok667*) data was overlaid with an offset on the *Y*-axis (absorbance), for better visualization (wild type values left, *nhr-40*(*ok667*) right). The peaks that significantly differed in both chromatograms (asterisks) were used for further analysis. (B) The comparison of chromatograms [region indicated by line segment in the bottom of (A)] using the ProteoVue program.

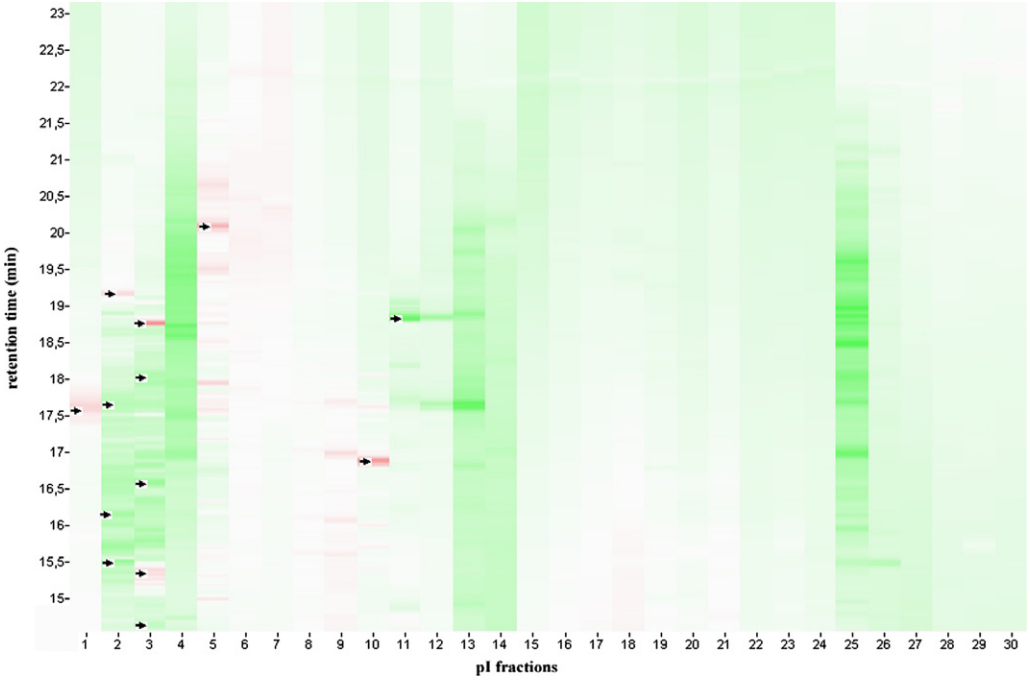

Fig. 2. Representative part of the whole proteome differential display of second dimension chromatograms prepared using the program DeltaVue. Green bands represent fractions with greater absorbance at 214 nm in *nhr-40*(*ok667*) worms whereas red bands were higher in control animals. The tendency of a single dominant color in individual lines reflects higher baseline in the particular chromatogram. Fractions corresponding to these bands were analyzed by mass spectrometry as marked by arrows.

teins that were reduced or missing in a given fraction from one strain were actually eluted in a neighboring fraction in either the first or 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 (Supplementary Table 1). 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 (Supplementary Table 2).

Gene Ontology (GO) analysis for the terms "biological process" and "cellular component" classified most proteins that were missing in *nhr-40*(*ok667*) fractions as muscle and metabolism related (Fig. 3). 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).

Our observation that proteins related to muscle function have abnormal chromatographic patterns in *nhr-40*(*ok667*) mutants was consistent with our previous phenotypic characterizations that demonstrated defects in myogenesis and locomotion in these mutants. 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 bodywall muscle that forms the central component of the muscle thick filament and nucleates filament assembly [15,16,19,20]. Bodywall muscle comprises more than 10% of the body mass in *C. elegans* [21] 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 transcriptase polymerase chain reaction (qRT-PCR) analysis, we found that *myo-3* is expressed at similar levels in wild

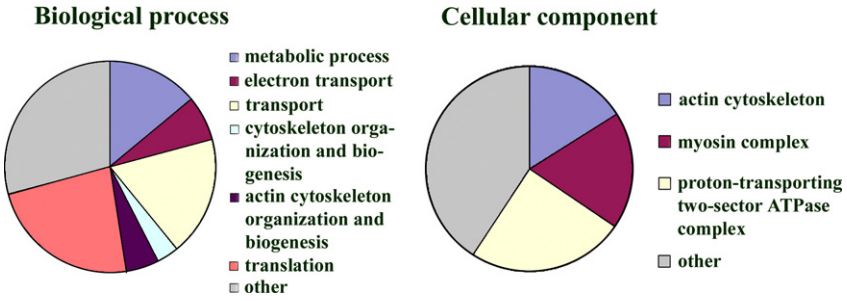

Fig. 3. Gene Ontology analysis of differentially eluted proteins. Proteins identified as decreased or absent in *nhr-40*(*ok667*) worms were linked to GO categories [18] of biological process or cellular component. An algorithm that is provided in Supplementary data was used.

type and homozygous *nhr-40*(*ok667*) mutants, as were several other muscle structural genes we assayed (Fig. 4C). 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.

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 [15,16]. We were surprised to see that MYO-3 levels, as detected by this antibody, were much more abundant in the mutant compared to wild type animals (Fig. 4A and B). 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 results to suggest that MYO-3 undergoes a different modification in the mutant, perhaps due to mis-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, ubiquination, etc.) by

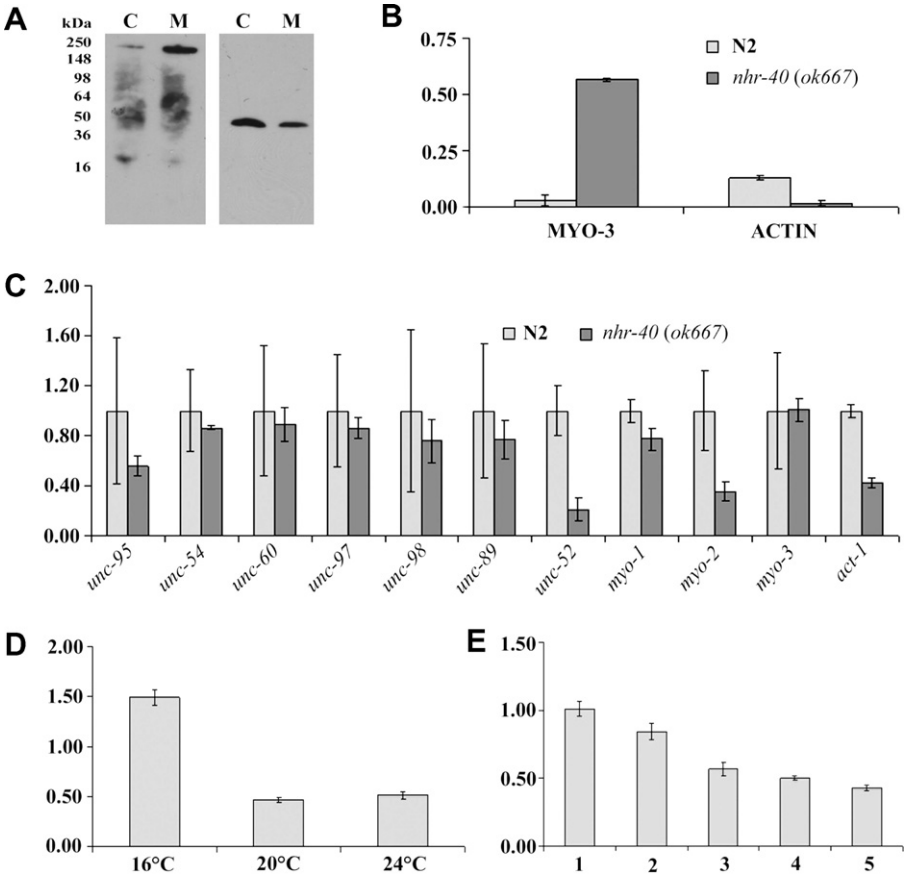

Fig. 4. *nhr-40*(*ok667*) mutant muscle analysis. (A) Levels of myosin heavy chain MYO-3 (left panel) and actin (right panel) in wild type (C) and *nhr-40*(*ok667*) (M) larvae detected by Western blot. MYO-3 levels are elevated in mutant larvae relative to an actin on the same, reprobed blot. (B) Densitometric analysis of MYO-3 and actin Western blot signals. (C) Relative expression of selected muscle genes in wild type and mutant L1 larvae under normal conditions from two independent experiments done in triplicate (in arbitrary units). (D) 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 ($n = 4291$ for *nhr-40*(*ok667*). (E) The effect of food restriction on the *nhr-40*(*ok667*) mutant phenotypes. *nhr-40*(*ok667*) larvae cultured on plates with no food, 3.33×10^5, 3.33×10^6, 3.33×10^7, and 1.65×10^9 bacteria (numbered 1–5, 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 ($n = 3044$; $P < 0.05$ for all food restricted cultures 1, 2, and 3). *Y*-axis of (D) and (E) shows ratio of severely affected non-moving larvae/larvae with milder phenotype able to reach food.

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.

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). 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, 20, and 24 °C). Larvae were placed outside the normal bacterial lawn on plates and incubated at one of these temperatures for 24 h 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. 4D).

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 on the animals to be assayed. Decreased availability of bacterial food led to a significantly higher penetrance of the locomotion defect compared to their well-fed siblings (Fig. 4 E). No similar effect was observed on N2 control larvae.

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 are more normal at lower temperatures [22–24] that favor correct protein folding. Food restriction causes an extension of lifespan in *C. elegans* [25,26] and can decrease the penetrance of the mutant phenotype of *unc-103* (ERG like K^+ channel) [27].

Our data suggest that NHR-40 provide a link that mediates connections between metabolism and orchestrated events necessary for normal muscle development. It seems likely that metabolic perturbances, especially those connected to food shortage and decreased temperatures, are revealing a requirement for synchronization between nutrient status and developmental programs. Genes related to nuclear hormone receptors from the NR2A group [6], constituting the nematode supnrs, may have been coopted during evolution to provide the necessary fine tuning of development in nematodes as they cope with environmental fluctuations. Employing stress culture conditions and whole proteome unbiased analyses may be particularly attractive for probing the functional repertoire of *C. elegans* NHRs that have mostly unknown functions [28].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.06.115.

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