Original Article

Supplementary Nuclear Receptor NHR-60 is Required for Normal Embryonic and Early Larval Development of *Caenorhabditis elegans*

(*Caenorhabditis elegans* / nuclear hormone receptor / development / NHR-23)

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Abstract. The *C. elegans* genome encodes an unexpectedly large number of NHRs, the majority of which are classified as supplementary nuclear receptors (supnrs) that are likely to have evolved from an ancestral protein related to vertebrate HNF-4. To understand the need for this large repertoire of potential ligand-activated transcription factors, we have begun to study an 18-member subgroup defined by DNA binding domain relatedness. Here we report on NHR-60, a supnr expressed ubiquitously throughout development with a distinct pattern of localization on the nuclear periphery. Both antibody staining and GFP reporter genes demonstrated high-level expression and accumulation of NHR-60 in seam cell nuclei that is dependent on NHR-23 activity. Interference with NHR-60 activity, by either RNAi or overexpression of a putative dominant negative isoform, results in embryonic and early larval lethality, including defects in seam cell development. This adds NHR-60 to the list of *C. elegans* NHRs playing important roles in development.

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Abbreviations: GFP – green fluorescence protein, HNF – hepatocyte nuclear factor, NHR – nuclear hormone receptor, qPCR – quantitative polymerase chain reaction, RNAi – RNA interference.

Introduction

Nuclear hormone receptors (NHRs) form a superfamily of transcription factors that are important for the regulation of cell metabolism and development in a variety of animal species (Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995; King-Jones and Thummel, 2005). 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 compounds (Chawla et al., 2001). However, most members of the NHR family do not have known ligands and are, therefore, called orphan receptors. The number of NHRs found in genomes of metazoan species varies from 48 in man, and 18 in *Drosophila melanogaster*, to more than 280 in the genome of *Caenorhabditis elegans* (Enmark and Gustafsson, 2001; Maglich et al., 2001; Van Gilst et al., 2002; Robinson-Rechavi et al., 2003; Ruau et al., 2004; Gissendanner et al., 2004; King-Jones and Thummel, 2005; Antebi, 2006). Among the predicted NHRs in *C. elegans*, 15 are orthologs of vertebrate and *Drosophila* genes. Most of these conserved receptors have regulatory functions in development (molting, dauer larva formation, epidermal cell development and differentiation, toxin resistance, neuronal development and sex determination); summarized in Sluder and Maina (2001), Gissendanner et al. (2004), Antebi (2006). The remaining 269 NHRs are now classified as supplementary nuclear receptors (supnrs). These receptors seem to be specific for nematode species and are distantly related to hepatocyte nuclear factor 4 (HNF-4) (Robinson-Rechavi et al., 2005). Vertebrate HNF-4 (NR2A) 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). Mouse HNF-4 is expressed in embryonic visceral endoderm and its disruption leads to arrest in gastrulation (Chen et al., 1994). HNF-4 is not required for specification of visceral endoderm, rather, it is needed for completion of differentiation (Duncan et al., 1997). HNF-4 has special ligand-binding properties for an NHR. Long-chain fatty acids in the form of acyl-coenzyme A thioesters bind to HNF-4α and modulate its transcriptional activity (Hertz et al., 1998). Nevertheless, the fatty acid thioesters are not classical ligands of HNF-4α (Bogan et al., 2000). HNF-4α differs from other NHRs also by the nature of its C-terminal F domain, which extends beyond the conserved activation domain AF2 that binds co-activators. The extended F domain can discriminate between functional interaction of HNF-4α with co-regulators and is able to bind both co-activators and co-repressors (Ruse et al., 2002; Petrescu et al., 2005). Surprisingly, crystallographic and functional studies indicate that homodimeric HNF-4α can adopt distinct conformations relative to the position of helix H10 and that fatty acids occupying the ligand-binding pocket are firmly bound to hydrophobic surfaces of adjacent helices. Such molecules serve as structural co-factors rather than ligands in the classically regulated NHRs (Dhe-Paganon et al., 2002; Aggelidou et al., 2004; Iordanidou et al., 2005). Moreover, it seems likely that the interaction between conformational forces induced by both ligand occupying the LBD and binding of co-factors to HNF-4α are necessary for locking the HNF-4 in its transcription activation state (Dhe-Paganon et al., 2002).

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., 2005a, b). 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).

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). We previously demonstrated that one member, NHR-40, of a subgroup of 18 genes with the P-box amino acids sequence CNGCKT (class I, subgroup 8 (Van Gilst et al., 2002)) was required for development. NHR-40 is expressed in muscle cells and neurons and its inhibition by RNA interference or mutation causes defective development of muscle cells (Brozova et al., 2006). Here we report on NHR-60, another member of this same subgroup of class I NHRs that also has a role in development. NHR-60 is expressed ubiquitously, with a higher level in the 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. Interestingly, the expression of NHR-60 in seam cells is dependent on NHR-23, another NHR known to regulate molting during development (Kostrouchova et al., 1998, 2001).

Material and Methods

Strains

The following *C. elegans* strains were used: wild-type animals, N2 (var. Bristol), were used when not specifically stated, and all strains were maintained as described (Brenner, 1974), JR667 - integrated strain of *unc-119*(e2498::Tc1)III; wIs51 expressing GFP in seam cell nuclei, Su93 – expressing *ajm-1::gfp* (Junction Associated Protein) (Mohler et al., 1998), *him-5* (e1490), PD7963 (*hlh-1::gfp)* integrated line (Krause et al., 1994), *chr3::gfp* (*nhr-23::gfp)* line #4991 and the *Caenorhabditis briggsae* strain. Standard NGM plates were used in all experiments, except the experiments in which mRNA and gDNA were isolated; 2% agarose capped plates were used in such case.

E. coli OP50 was fed to worms except for RNAi feeding experiments where transformed *E. coli* HT115 bacteria were used. All experiments except heat shock were performed at 16–22°C.

Total RNA isolation

Wild-type animals of *C. elegans* and *C. briggsae* (from mixed and individual stages) were grown on 2% agarose capped plates, washed with water and frozen at -80°C. The frozen pellet was re-suspended in 0.5 ml of re-suspension buffer (0.5% SDS; 5% 2-mercaptoethanol; 10 mM EDTA; 10 mM Tris/HCl (pH 7.5) with 12.5 µl of proteinase K (20 mg/ml)). After vortexing for 60 s, the mixture was incubated for 60 min at 55°C. Phenol-chloroform extraction and ethanol precipitation followed. The air-dried pellet was dissolved in water and treated with 1 unit of DNase (Promega, Madison, WI) per 1 µg of total RNA for 30 min at 37°C. Phenol-chloroform extraction and ethanol precipitation followed and RNA was re-suspended in DEPC water.

RT-PCR

C. elegans and *C. briggsae* cDNA were prepared in a similar way by reverse transcription (Superscript II kit, Invitrogen, Carlsbad, CA) of previously prepared RNA. Five µg of RNA were mixed with 100 ng of random hexamers and heated for 5 min at 65°C, and then 4 µl of 1st strand buffer, 1 µl of RNasin, 1 µl of 100 mM DTT, 1 µl of dNTPs and 1 µl of Superscript II were added. The mixture was incubated for 50 min at 42°C and 15 min at 70°C.

Quantitative PCR

Quantitative PCR (qPCR) was performed as described (Sun et al., 2004) with modifications. Five μg of total RNA were reverse transcribed using SuperScript II RNA polymerase and random hexamers. Selected amplicons of *nhr-60*, *ama-1* and *act-1* were chosen, amplified, separated by agarose gel electrophoresis and electro-eluted using a semi-permeable membrane. The DNA was extracted and precipitated in phenol-chloroform and ethanol. The amount of DNA was estimated spectrophotometrically and the DNA directly sequenced in both directions. This DNA was used for determination of standard curves. The real-time qPCR was done using a PTC200DNA EngineR thermal cycler equipped with ALS0296 96-well sample block (Bio-Rad, Hercules, CA) and the DyNAmoTM HS SYBR Green qPCR kit. The reaction mixture of RT was diluted 10 times and 1 μl used for PCR. The reactions were analysed using Opticon Monitor TM Version 3.0 computer program. The results were normalized against RNA polymerase II large subunit *ama-1* or actin with almost identical results. The primers used for *nhr-60* quantification were #6165 (taggtccgccaagactaccgaaac) and #4566 (gatccgtcaggctcaatgataacc), the primers for *ama-1* quantification were #4684 (ttccaagcgccgctgcgcattgtctc) and #4685 (cagaatttccagcactcgaggagcgga). Primers used for amplification of actin gene *act-1* were #5293 (atgtgtgacgacgaggttgccgc) and # 5294 (gctcattgtagaaggtgtgatgcc).

Cloning of nhr-60 and CBG22907

The *nhr-60* cDNA: all cDNA was amplified by PCR from yk470e6 with the sense primer #6014 (cggatccgtccatatgattcaatccagttc), where the ATG was included from our data and the antisense primer #6015 (cccatggtatgtacatttcccatttattcac).

C. briggsae cDNA of CBG22907 was prepared by PCR amplification of *C. briggsae* cDNA with primers #5156 (atgcttcaaccaacagaatcaccc) and #5157 (tgtccatgatatcttcaataacccg).

GFP (green fluorescence protein) reporter genes

All *nhr-60::gfp*-expressing constructs (transcriptional fusions) contain various sequences from the *nhr-60* upstream region. Putative promoter region fragments of *nhr-60::gfp* a, b and c (-1950 bp, -580 bp and -340 bp from ATG) were amplified by PCR from N2 gDNA with different sense primers #4535 (acgcgtcgactggccgaggcatactgtccacg), #6046 (acgcgtcgacgctcacatttctgatacccg), #6063 (acgcgtcgacccatcttttcaagctcaggcgtgtc) and the same antisense primer #4536 (cgggatcccaaatcaggcattcagttgggcg). Fragments were cloned into the promoter-less GFP vector pPD95.67, which contains the nuclear localization sequence, using restriction sites *Sal*I and *Bam*HI. Two transgenic lines were prepared for each construct.

The line #4991, which contains *chr3::gfp* (*nhr-23::gfp*), was prepared by PCR amplification of 1,600 bp long fragment upstream of ATG with primers: #4955 (cccaagcttcacggtcactctgccattgcc) and #4956 (cgcggatccgtatgcggatgaaacatgc) and contained the first and second exons. The fragment was cloned into vector pPD95.67, confirmed by sequencing, and two transgenic lines were prepared.

Transgenic lines

All transgenic lines were made by co-injection of a marker, pRF4 (50 ng/μl), and experimental plasmid (50 ng/μl) into the gonad of young adult N2 hermaphrodites (Mello and Fire, 1995). Progeny of microinjected animals was screened for a rolling phenotype. Positive transgenic animals in the third generation were checked by PCR to determine whether they also carried the experimental plasmid.

Overexpression of nhr-60

The clone #6056, which corresponds to the full-length *nhr-60* cDNA, was amplified by PCR with primers #6014 and #6015 and cloned into the pPD49.83 vector (which contains a heat-shock-regulated promoter). The clone #6057, which corresponds to *nhr-60* /AF2-, was made from PCR-amplified fragment from yk470e6 with primers #6014 and #6016 (cccatggtttcggagccacgagaagtagatg). This construct lacks the putative activation function 2 (AF2) domain, the amino acids VAKLPKDLVMRVIEDIMDS. This fragment was also cloned into pPD49.83 vector. The expression of transgenic animals harbouring # 6056 and #6057 was induced by heating of gravid hermaphrodites at 31°C for 2 h or at 34°C for 30 min.

RNA interference

The *nhr-60* cDNA in L4440 (#6086) was prepared by PCR amplification of the ligand-binding domain (LBD) of *nhr-60* cDNA from the EST clone yk470e6 with primers #4520 (acgcgtcgacgtcggaatgaacccccttgc) and #4521 (cgggatcccggatcaacggtgcaacagac) and cloned into L4440. The construct was linearized with *Sal*I and *Bam*HI. ssRNAs were prepared by *in vitro* transcription (Promega, Madison, WI). Equal amounts of both ssRNAs (estimated by agarose gel electrophoresis) were mixed and incubated for 10 min at 68°C and for 30 min at room temperature. dsRNA was purified by phenol-chloroform extraction and ethanol precipitation and used at a concentration around 2 μg/μl. *nhr-60* RNAi was performed on N2 worms and on the following transgenic lines: *hlh-1::gfp, chr3::gfp (nhr-23::gfp),* Su93*,* JR667 and *nhr-60::gfp.*

dsRNA from the promoter region of *nhr-60* was made in order to assay a possible non-specific effect of prepared dsRNA. The construct was made by PCR amplification of *nhr-60* (non-coding) promoter sequence from

gDNA with primers #6060 (cgggatccgcacacgcctgaccttgaaaagatgg) and #6045 (acgcgtcgacccctgatcaacaccagtccatttcc) and cloned into the L4440 vector. dsRNA for *nhr-116* was prepared by cloning of a part of coding sequence of LBD of *nhr-116* into the L4440 vector as described above. The primers #6051 (catgccatggcttcaacttgttcaccaccgtag) and #6052 (acgcgtcgacgacactttggagcatgagccaaag) were used for PCR amplification.

chr3 (*nhr-23*) dsRNA for feeding experiments was prepared using plasmid #4666 (*nhr-23* in L4440) (Kostrouchova et al., 2001). All dsRNAs for RNAi were microinjected into the ovarial syncytium of young adult hermaphrodites (Mello and Fire, 1995). Embryos and larvae of microinjected animals were collected in 10 to 12 hour intervals, incubated for 12 h and screened for phenotypes and behavioral changes. For soaking experiments, 5 to 15 animals were incubated in a dsRNA solution for 12 h.

NHR-60 antibodies

To generate the anti-NHR-60 antibody, a 15 aa long polypeptide was designed from the C-terminus of the receptor: PKDLVMRVIEDIMDS. The polypeptide was used to immunize two rabbits. Bleeds at 4, 8, 10 weeks were tested by Western blots and by staining of fixed *C. elegans* embryos for reactivity. Both polyclonal antibodies (#4529 and #4530) worked well on Western blots (not shown) and #4529 was used in all shown experiments.

Western blot analysis

Worms from mixed and individual stages were washed twice in water and spun at 200 x g for 4 min at 4°C. The pellets were transferred to eppendorf tubes, the equal amount of Tris-Glycine SDS Sample Buffer (2x) (Invitrogen) was added. The samples were boiled for 5 min and then chilled. The protein concentrations in supernatants were estimated using a BCA kit (Pierce, Rockford, IL). Samples were boiled again after the addition of 1 µl of 2-mercaptoethanol. For Western blot analysis, 40 to 60 µg of protein samples were separated by 4%/15% SDS PAGE and proteins were blotted onto nitrocellulose membrane. The membrane was incubated overnight at 4°C in T-PBS-M (phosphate-buffered saline (PBS) pH 7.4, 0.1% (v/v) Tween 20, 5% low-fat milk powder (w/v)). Incubation in T-PBS-M with primary antibody (dil. 1 : 1000) was done for 1 h at room temperature. The membrane was washed in T-PBS for 10 min at room temperature six times, rocking. Incubation with secondary antibody coupled to horseradish peroxidase (Sigma, St. Louis, MO) diluted 1 : 5000 in T-PBS followed for 40 min at room temperature. The membrane was washed in T-PBS for 10 min at room temperature four times with rocking. SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used for detection of peroxidase activity.

Immunocytochemistry

A. Detection of NHR-60 protein

Embryos, larvae and adult animals were washed several times with water and placed on poly-L-lysine-coated slides. Ten µl of pellet containing animals were placed on the glass together with 5% paraformaldehyde diluted with PBS, covered with cover glass and incubated in a wet chamber for 10 min at room temperature and then frozen for 5 min on an aluminum block that had been chilled on dry ice. After freeze cracking, the samples were placed in cold methanol (-20°C) for 10 min and re-hydrated for 10 min each in a series of rehydration buffers in methanol: TTBS (Tris Tween-buffered saline) in ratios 9 : 1, 7 : 3, 1 : 1 and 1 : 4 and then NHR-60 antibody #4529 was used in dilution 1 : 200. Slides were kept in a wet chamber at 4°C overnight. On the next day, the slides were washed three times in TTBS and secondary goat anti-rabbit IgG antibody conjugated to Alexa Fluor 568 (Molecular Probes, Eugene, OR) was added in dilution 1 : 400. Slides were incubated at room temperature for 2 h, washed three times in TTBS, and 1 mg/ml DAPI (diamidinophenylindole) was added and mounted in 10 µl of mounting medium (Shandon, Pittsburgh, PA).

B. Staining of transgenic line overexpressing nhr-60

Young adult hermaphrodites carrying the complete *nhr-60* cDNA in a transgene regulated by the heat-shock promoter were heated at 31°C for 2 h or at 34°C for 30 min, incubated at room temperature for 2 h and for 12 h at 16°C. Staining with the antibody was performed as described above.

Fluorescence microscopy was done using an Olympus BX60 microscope and a Nikon Eclipse E800 microscope equipped with C1 confocal head and 543 nm laser line (Spectra-Physics Lasers, Mountain View, CA).

Results

Characterization of the nhr-60 gene

Comparison of the predicted 443 amino acid sequence of NHR-60 with other *C. elegans* NHRs shows that NHR-60 belongs to Class I *C. elegans* supnrs that have the P-box sequence CNGCKT (Van Gilst et al., 2002). NHR-60 falls more specifically into subgroup 8, which consists of 18 genes in *C. elegans*. This subgroup contains sequences that have 9, 11, 12 or 13 amino acids in the internal part of the second zinc finger (C-X2-C–X (9, 11, 12 or 13)-C-X2–C) within the DNA-binding domain (DBD). We have previously described the details of another member of this group, NHR-40 (Brozova et al., 2006).

The *nhr-60* gene is localized on chromosome V (a.k.a. F57A10.5) and consists of seven exons spanning about

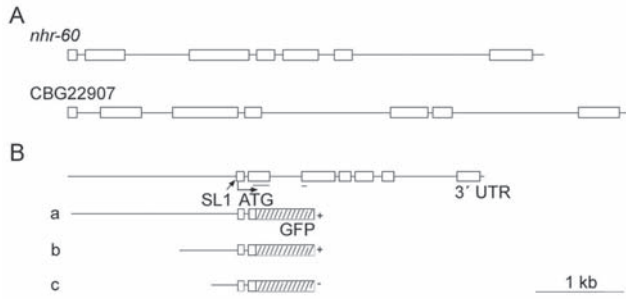

Fig. 1. Schematic representation of the genomic organization of *C. elegans nhr-60* and *C. briggsae* CBG22907. A. Comparison of genomic organization of *nhr-60* and CBG 22907. Exons are marked as open rectangles. Note the conserved number and length of exons of *nhr-60* and CBG 22907. B. Schematic representation of the genomic organization of *C. elegans nhr-60* and schematic representation of GFP fusion constructs used for analysis of *nhr-60* promoter activity. Three different regions of the sequence preceding the first exon of *nhr-60* were used for preparation of transgenic animals. Open rectangles represent the sequence of *nhr-60* and hatched rectangles the sequence of *gfp*.

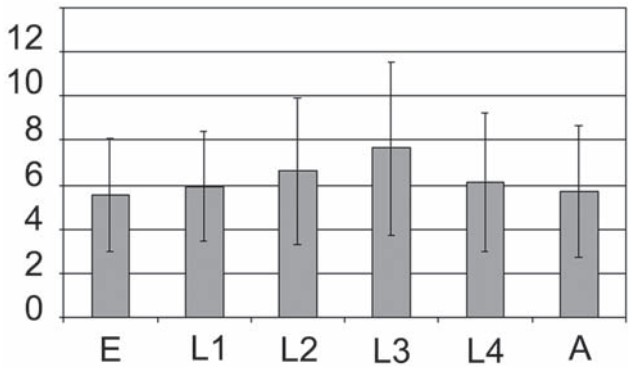

Fig 2. Analysis of *nhr-60* mRNA expression during *C. elegans* development.
Results show average and SD from three independent experiments and are expressed as log of number of copies per 250 ng of total RNA that were normalized with expression of *ama-1*. Normalization to *act-1* showed a similar pattern. Total RNA was prepared from synchronized *C. elegans* cultures harvested as embryos obtained by lysis of adult hermaphrodite cultures (E), or grown as synchronized larval stages L1 to L4 and young adult animals (A). Five µg of total RNA were reverse transcribed and used for quantitative PCR as described in Material and Methods. The results show high expression of *nhr-60* in all stages with the trend of increased expression in larval stages L1, L2 and L3.

3 kb. We cloned the 5' end of *nhr-60* cDNAs, revealing it is trans-spliced to SL1 (Krause and Hirsh, 1987; Blumenthal and Spieth, 1996) five nucleotides upstream of the predicted translational start codon. The full length of *nhr-60* cDNA was obtained using gene-specific primers and either the Kohara EST clone yk470e6 or total RNA from mixed populations as a template. Cloning and sequencing of products confirmed the gene structure predictions shown in Wormbase (www.wormbase.org release WS150, Nov.30, 2005) and were consistent with only a single prominent spliced message at steady state.

A search in the *Caenorhabditis briggsae* database detected a single *nhr-60* orthologue (CBG22907). The predicted protein product (CBP05478) shows 70% similarity and 62% identity at the amino acid level to CeNHR-60. We prepared cDNA from *C. briggsae*, amplified CBG22907 cDNA with gene-specific primers and sequenced the PCR product. The CBG22907 cDNA differs from the predicted transcript, likely reflecting a mis-prediction of the *C. briggsae* gene structure (Fig. 1A). Based on our results, both *C. elegans* and *C. briggsae nhr-60* genes contain the same number of exons with sizes conserved. We suggest naming CBG22907 as *Cbnhr-60*.

To determine the developmental pattern of *nhr-60* expression in *C. elegans*, we prepared cDNA from a mixed population as well as from embryos, larval stages, and adults. Gene-specific primers for *nhr-60* were used for RT-PCR and quantitative RT-PCR (qRT-PCR) (Libý et al., 2006) and expression levels were normalized to *ama-1*, encoding the large subunit of RNA Pol II (Johnstone and Barry, 1996), or to *act-1*, encoding actin. We found that *nhr-60* expression is high in all developmental stages and is increased in larval stages with a peak at L3 and a decrease in L4 and adult animals (Fig. 2).

NHR-60 distribution

Polyclonal antibodies were prepared in rabbits using a polypeptide derived from the C-terminal part of NHR-60 and their specificity was confirmed by ELISA using the synthetic peptide and by Western blots. Western blot analysis of protein extracts from a mixed population of N2 animals revealed the presence of two *C. elegans*-specific bands. The prominent band, had an approximate size of 64 kDa and a minor band representing in some preparations up to approximately 10% of the intensity observed for the prominent band, had about 50.3 kDa (Fig. 3). One additional non-specific protein migrating at approximately 36 kDa was seen in Western blots that contained material from *E. coli* OP50 used for feeding. Western blot analysis of developmental stages showed that the 64 kDa NHR-60 protein was detected in embryos, during all larval stages, and in adult animals, consistent with our expression data. The 50 kD NHR-60 protein was detected in all stages in Western blots when large quantities of lysate were used for electrophoresis (60 µg per lane). Inhibition of *nhr-60* by RNAi decreased the intensity of the 64 kDa protein and eliminated the 50 kDa protein, suggesting that both proteins are indeed forms of NHR-60.

Immunocytochemistry using fixed embryos, larvae, and adults detected NHR-60 in all cell nuclei beginning

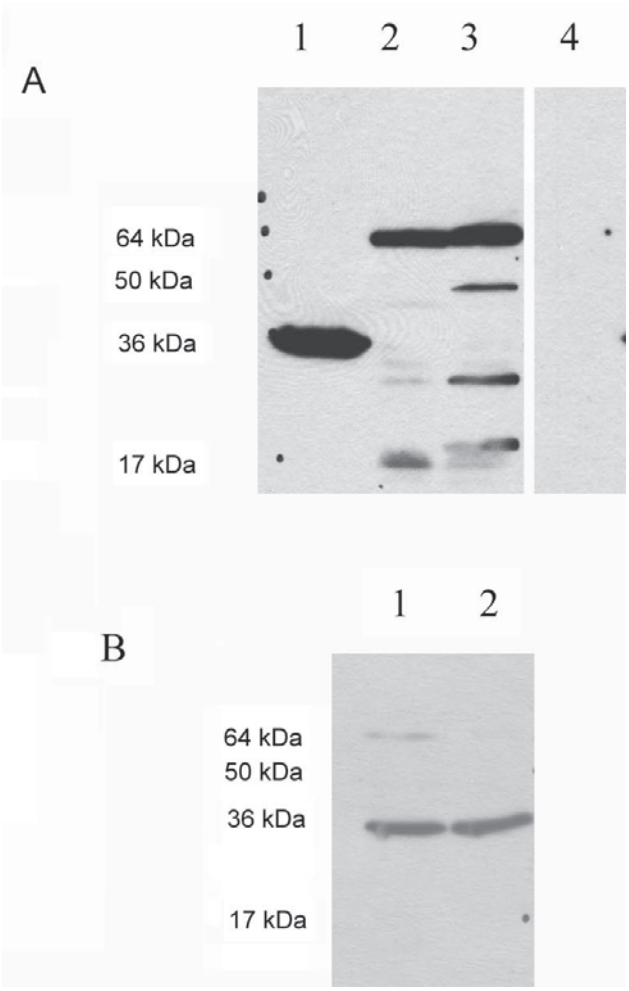

Fig. 3. Analysis of NHR-60 antibody specificity. A. Protein lysates from *E. coli* (lane 1) or *C. elegans* embryos (lanes 2 to 4) were analysed by Western blot using rabbit polyclonal antibodies (# 4529) (lanes 1 to 3) or pre-immune serum (lane 4). Forty micrograms of protein were loaded in lanes 1, 2, and 4 and 60 micrograms in the lane 3. Antibody recognizes protein migrating at 64 kDa. A protein with a size corresponding to that predicted for NHR-60 is detected by the antibody in larger protein loadings (lane 3). The 36 kDa protein that is detected by the antibody in bacterial lysate is a non-specific antibody interaction. B. Analysis of antibody specificity in embryos of N2 *C. elegans* that were subjected to *nhr-60* RNAi (lane 2) or controls. RNAi of *nhr-60* results in decreased detection of the 64 kDa protein by the antibody. The 36 kDa protein detected by the antibody is a contamination from the bacterial food source.

as early as the 1-cell stage of development, strongly suggesting maternal contribution (not shown). Interestingly, the antibody staining revealed prominent staining at the periphery of nuclei in all cells (Fig. 4). Although Western blots showed that our NHR-60 antibodies recognized NHR-60 in *C. elegans* extracts, we thought it was important to demonstrate that the *in vivo* staining pattern faithfully reflected NHR-60 distribution. As additional tests of specificity, we depleted NHR-60 using RNAi or over-produced NHR-60 in transgenic strains. The NHR-60 antibody signal was reduced to near background levels following *nhr-60* RNAi (Fig. 4N). Conversely, the NHR-60 antibody signal was increased in transgenic animals overexpressing NHR-60 from a full-length cDNA driven by the heat-shock promoter and it was localized primarily at the periphery of nuclei (Fig. 4O). Although ubiquitous throughout development, NHR-60 was specifically up-regulated in larval seam cells (see below) as well as in the cells of the germ line (Fig. 4M).

nhr-60 reporter genes reflect high seam cell expression

In order to determine whether the high levels of NHR-60 detected in seam cells reflected *nhr-60* expression, we prepared various transgenic lines expressing a *nhr-60::gfp* reporter from extra-chromosomal arrays containing the putative promoter region and limited coding sequences of the gene. The constructs tested (a, b and c) included either 1,950 bp, 580 bp or 340 bp upstream of the predicted start of translation as well as *nhr-60* genomic sequences up to, and including part of the second exon (Fig. 1B). Expression of both constructs (a) and (b) was documented in many different cell types, beginning in embryogenesis. Construct (c) did not show any expression although the presence of the transgene was confirmed by PCR, suggesting that the minimal *nhr-60* promoter is not fully contained within the 340 bp immediately upstream of the translational start. The strongest expression of *nhr-60::gfp* was in seam cells, consistent with our antibody localization of the NHR-60 protein.

Seam cells are epidermal cells forming two parallel rows of 10 cells each. They divide at each larval stage and generate an anterior daughter cell, which fuses with the main epidermal syncytium, and a posterior daughter, which functions as a seam cell. These posterior stem-like seam cells continue to divide until the L4 stage. At the L4/adult molt, seam cells fuse to each other to form a syncytium (Sulston and Horvitz, 1977). The seam cells are important for the formation of stage-specific cuticle structures called alae in L1, dauer larvae and adults.

Seam cell expression of *nhr-60::gfp* started in embryonic precursors of these cells at about 260 minutes after fertilization and continued during all larval stages (Fig. 5). It was present in the proliferating seam cells and expression levels were constant through larval development, disappearing in adult animals after seam cells undergo homotypic cell fusion. Additionally, we saw strong GFP signals in four pharyngeal gland cells, VC4, VC5 neurons, and the hemaphrodite uterine vulval uv1 cells (Fig. 5B, C, D). In males, the two expressing *nhr-60::gfp* transgenes were expressed in all ray cells of the mature tail (data not shown) (Sulston and Horvitz, 1977).

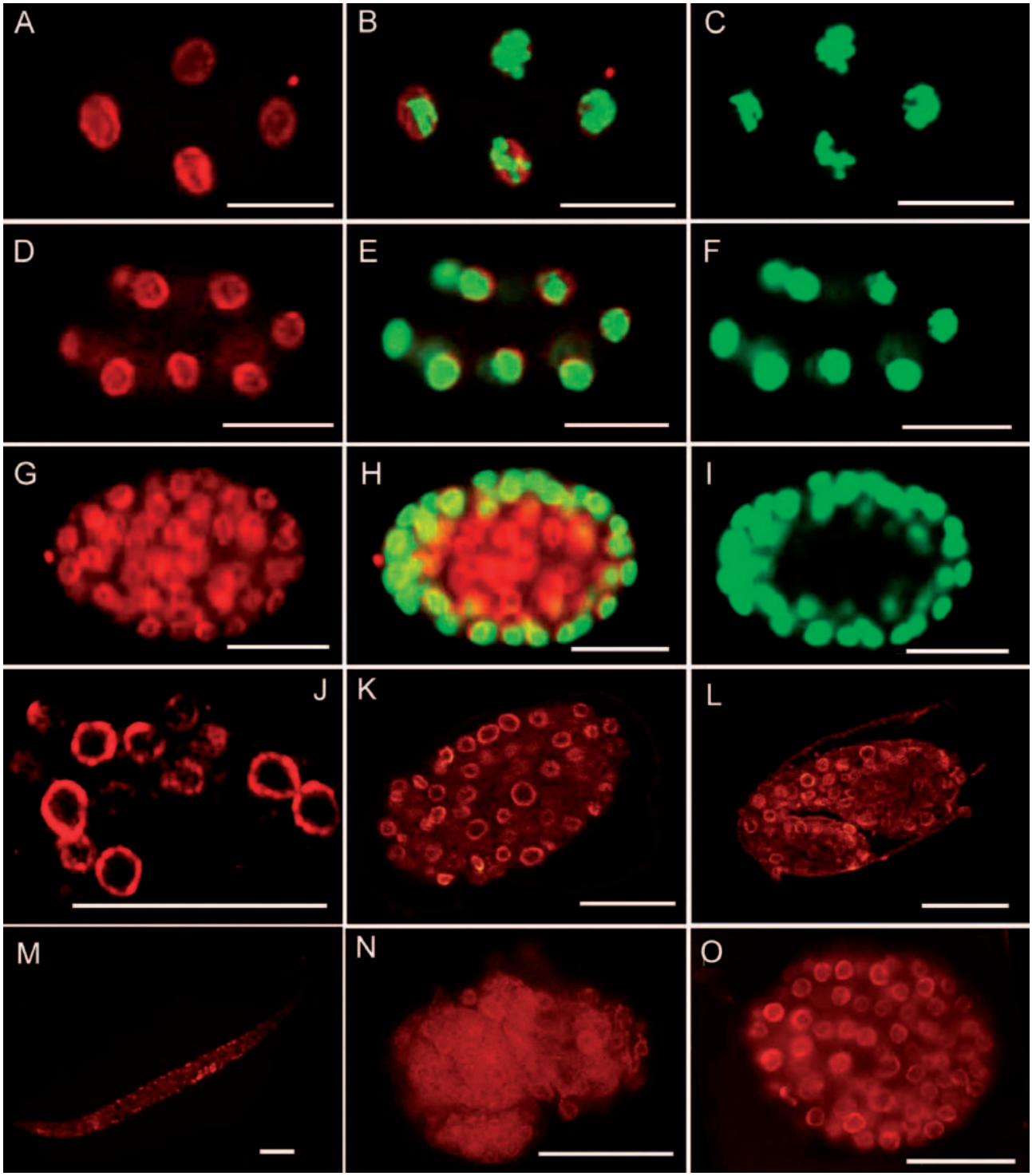

Fig. 4. Detection of NHR-60 in embryos and larvae. Rabbit polyclonal antibody was used to detect the distribution of NHR-60 in embryos and larvae. NHR-60 is detected in the 4-cell stage (A) and continues to be present in 8-cell embryo (D) and approximately 100-cell embryo (G) (C, F and I show the same embryos stained with DAPI). NHR-60 staining is always associated with the nucleus as shown in the merged DAPI-stained images (B, E and H). Note the prominent localization of NHR-60 at the nuclear periphery that becomes evident in confocal microscopy (J, K and L) of a 50-cell embryo, comma stage embryo and one and half-fold stage embryo, respectively. Panel M shows an L1 larva with expression of NHR-60 in most cells and prominent expression in the germ line. The NHR-60 antibody staining pattern is decreased following *nhr-60* RNAi (N) and is increased with *nhr-60* overexpression (O). Bars represent 20 μm.

Since the expression of *nhr-60::gfp* in seam cells overlaps with the expression of *nhr-23* (Kostrouchova et al., 1998), we tested the possibility that the regulation of these two genes was linked. We examined the expression of *nhr-60::gfp* construct (a) following *nhr-23* RNAi. *nhr-23* RNAi markedly decreased *nhr-60::gfp*

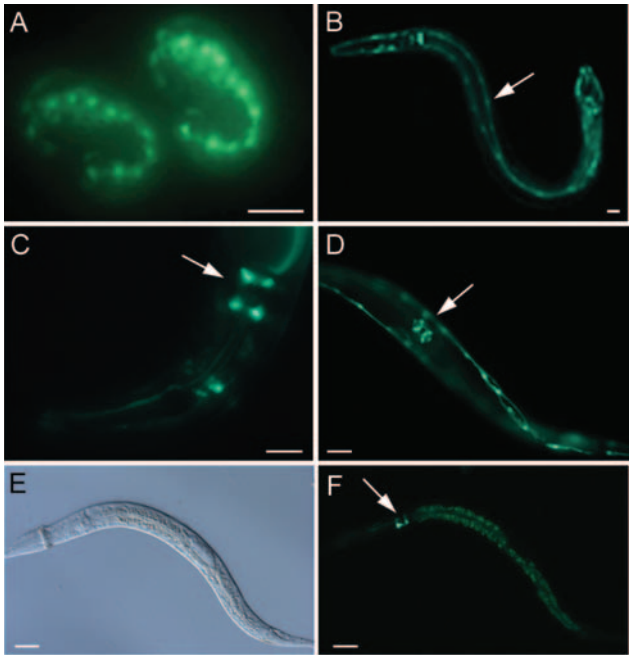

Fig. 5. Expression of *nhr-60::gfp* in transgenic strains. High-level expression of *nhr-60::gfp* in two-fold embryos is in the seam cell precursors (A). In larval stages (B-D) *nhr-60::gfp* is expressed in seam cells (B, arrow), in pharyngeal gland cells (C, arrow), and in the uterine vulval cells uv1 (D, arrow). The expression of *nhr-60::gfp* is lost in seam cells following *nhr-23* RNAi (F), while expression of the reporter in the pharyngeal gland cells is unaffected (F, arrow). The panel E shows the same animal as in the panel F in Nomarski optics. Note the molting defect induced by *nhr-23* inhibition. Bars represent 20 μm.

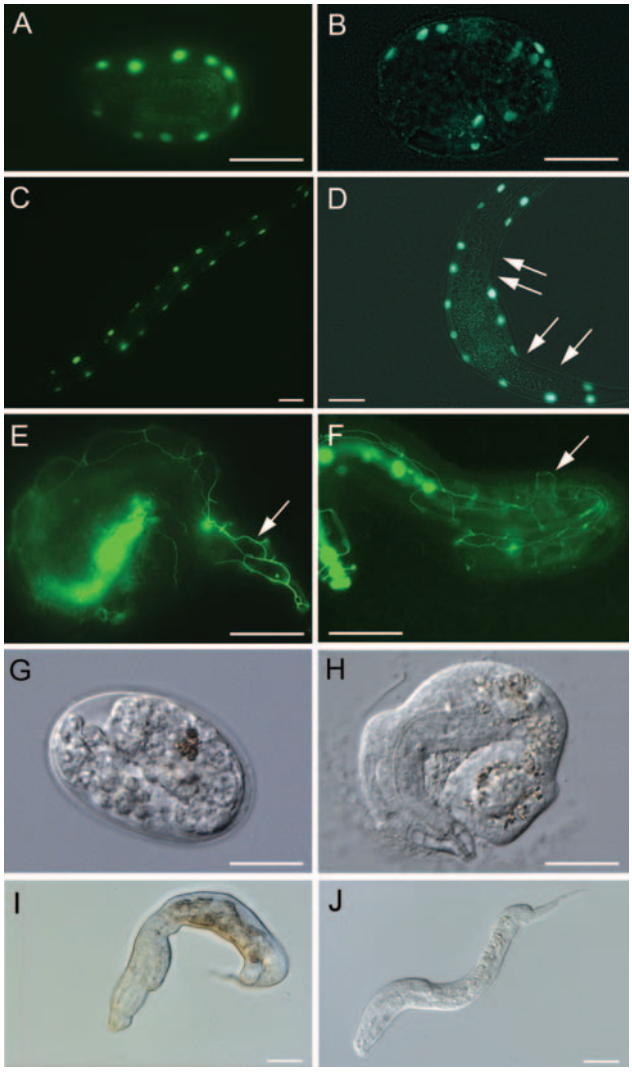

Fig. 6. Developmental defects induced by *nhr-60* RNAi (panels B, D, E and F) and by expression of *nhr-60* lacking the putative AF2 domain (panels G to J). Panels B and D show the effect of *nhr-60* RNAi on JR667 strain and panels E and F on Su93 strain (Mohler et al., 1998). Panels A and C are controls expressing the transgene in all seam cells (A is a lateral view on an embryo with the focus on one row of seam cells and C is a dorso-ventral view which allows to visualize both rows of seam cells in the L1 larva). Panel B shows a typical malformed embryo with a decreased number and mis-positioning of seam cells. Larvae with typical body shape defects are shown in panels D to F. The body shape defects are accompanied by mis-position or completely missing seam cells visualized by the expression of the transgene in the JR667 strain (D) or Su93 (E and F). Defects similar to NHR-60 inhibition are detected in embryos (panels G and H) and larvae L1 (panels I and J) overexpressing the NHR-60 lacking the putative AF2 region. Bars represent 20 μm.

expression in seam cells in embryos and larval stages. This effect was seam cell-specific because *nhr-60::gfp* expression in pharyngeal gland cells was unaltered by *nhr-23* RNAi (Fig. 5E, F). In contrast, *nhr-60* RNAi had no effect on the expression of *nhr-23::gfp* (data not shown). We concluded that *nhr-60* expression in seam cells was downstream of NHR-23 activity, either directly or indirectly.

nhr-60 is essential for embryonic development

RNA interference was used to study the consequence of NHR-60 reduction- or loss-of-function. Double-stranded RNA specific for *nhr-60* was prepared from cDNA covering exons 3 to 6 (690 bp) that encodes the putative ligand-binding domain (LBD). The dsRNA was delivered by either microinjection, incubation of larval stages in solutions containing dsRNA, or by feeding nematode cultures dsRNA-producing bacteria (Fire et al., 1991; Mello and Fire, 1995; Timmons and Fire, 1998); all methods gave similar results but with variable degrees of penetrance. The most severe effects were seen in the progeny of hermaphrodites receiving dsRNA injections. Following microinjection of *nhr-60* dsRNA, 21% (N = 5,858) of the progeny failed to hatch. The majority of the embryos were arrested at the two-fold stage of embryogenesis and appeared to have incomplete ventral closure, resulting in a protrusion of cells ventrally. In addition, arrested embryos had severe de-

fects in morphogenesis, including problems with elongation (Fig. 6). Defects were also observed in a small fraction of progeny larvae from *nhr-60*-injected hermaphrodites, presumably reflecting less effective knockdown of NHR-60 activity. These larvae often had morphogenic abnormalities along with vacuoles indicative of general cell death (Fig. 6). The effect of *nhr-60* inhibition on the number of progeny was studied on N2 L3 larvae soaked in a solution of *nhr-60* dsRNAi and controls. Inhibition of *nhr-60* by RNAi also led to a decreased number of progeny (182 SD = 30 in *nhr-60* inhibited worms (N = 10), compared to 222 SD = 44.3 in controls (N = 12), $P < 0.05$).

To identify the tissues most affected following *nhr-60* RNAi, we employed several GFP reporter genes that allowed us to assay defects in seam cells (strain JR667 or *nhr-60::gfp*), epidermal and seam cells (*nhr-23::gfp* or strain SU93), and body wall muscles (*hlh-1::gfp*). Most tissues were not overtly affected and, in most cases, the arrest occurred after all embryonic cell divisions were complete. However, a striking observation was that the seam cells in arrested embryos were often displaced. Frequently, *nhr-60* RNAi-arrested embryos had mis-positioned seam cells in the head and tail regions; arranged as side by side doublets rather than in a single row as in wild-type embryos; in some embryos the seam cells were completely missing or disorganized (Fig. 6B, D, E, F). The average number of observable seam cells in the JR667 line treated with *nhr-60* RNAi decreased to 9.83 seam cells per animal (N = 20, SD = 5.4) compared to 19.1 observed in controls (N = 20, SD = 1.01) indicating a statistically different result at > 99.9% probability. That seam cells show high-level expression of *nhr-60::gfp*, and the most obvious phenotypic defect following *nhr-60* RNAi suggests that NHR-60 activity is particularly important for development of this cell type.

A dominant-negative NHR-60 isoform affects embryonic development and displaces endogenous NHR-60 from the nuclear periphery

NHRs lacking transcriptional activation domains can function as dominant negative factors (Pemrick et al., 1994; Johnson et al., 1999; Liu et al., 2002; Park et al., 2003). In order to identify the putative activation domain 2 (AF2), we performed multiple sequence analysis of NHR-60 and several vertebrate nuclear receptors. The alignments indicated that NHR-60 contains an AF2 domain located at the end of C-terminus, which is typical for most vertebrate nuclear receptors (Barettino et al., 1994) and is unlikely to posses the negative regulatory domain F that is present in vertebrate HNF-4α (Petrescu et al., 2005). We used a heat-inducible promoter to drive expression of *nhr-60* cDNA lacking the coding sequences homologous to the part of the putative AF2 (NHR-60/AF2-). Heat-shock induction of NHR-60/AF2- in adult hermaphrodites for 2 h at 31°C resulted in 21 % affected embryos (N = 252), while a 30 min pulse at 34°C resulted in 30% affected embryos (N = 313), suggesting that the terminal part of NHR-60 may indeed function as AF2 and that our construct was acting as dominant-negative. As a control, we similarly induced expression of a wild-type *nhr-60* cDNA from the heat-shock promoter; this treatment had no adverse effects on progeny. NHR-60/AF2- caused both early and late embryonic arrest. The early embryonic arrest occurred at the several hundred cell stage with embryos containing many vacuoles indicative of general cell death. The late embryonic arrest observed was at the 2- and 3-fold stage with embryos failing to complete elongation and having reduced and/or misplaced seam cells (Fig. 6). Because the NHR-60/AF2- protein lacks the C-terminal peptide used for antibody production, we were able to assay endogenous NHR-60 abundance and localization following heat-shock induction of the putative dominant-negative isoform. We observed an obvious decrease in the intensity of NHR-60 antibody labelling in animals expressing NHR-60/AF2- *in vivo* or by Western blot analysis (data not shown). These results demonstrate that NHR-60/AF2- is likely functioning as a dominant-negative factor, adversely affecting development and displacing endogenous NHR-60 from sites of nuclear localization.

Discussion

In this study, we show that NHR-60, a nuclear receptor from the large group of supplementary receptors, is required during *C. elegans* embryogenesis and early larval development. This is the second member of the group of supnrs that share the P-box sequence CNGCKT, along with NHR-40, that has been shown to have an important developmental role (Brozova et al., 2006). Contrary to NHR-40, which has the classical arrangement of the DNA-binding domain with 9 amino acids forming the inner part of the second zinc finger, NHR-60 has 11 amino acids in this region and can be linked with 10 more divergent NHRs in this subgroup (Brozova et al., 2006). Our findings indicate that many of the 269 supnrs will join the majority of the 13 canonical NHRs that play critical development roles.

The functions of NHR-60 are likely to be quite general based on ubiquitous expression and the character of developmental defects observed after knockdown of expression. These general functions likely underlie the arrested embryos with severe and widespread morphogenesis defects. These general functions would also be consistent with the detection of NHR-60 in the germ line, its apparent maternal contribution to the embryo, and its inhibition resulting in a decreased number of progeny.

It is also possible that NHR-60 has additional cell-type-specific functions. One set of cells that seem particularly dependent on NHR-60 function are the seam cells. These cells show high-level expression of *nhr-60*

reporter genes, they accumulate high levels of the NHR-60 protein, and they are affected in embryos and larvae after *nhr-60* RNAi. The role of NHR-60 in the seam cells appears to be after their specification, given the onset of the up-regulation in these cells and the persistence of clearly defined seam cells following *nhr-60* RNAi. Interestingly, we found that *nhr-60::gfp* expression in seam cells is downstream of NHR-23, an NHR required for development and molting (Kostrouchova et al., 1998, 2001). Future studies will be needed to explore these links in more detail and to determine whether NHR-23 is acting at the *nhr-60* promoter to regulate its expression.

As epidermis, the seam cells must participate in the coordination of larval molts among all hypodermal cells in the animal. Keeping with the critical role of seam cells in the regulation of larval transitions and integration of developmental regulatory events, this particular cell type is dependent on several NHRs. In addition to NHR-60 and NHR-23, NHR-25 is required for proper elongation of seam cells and formation of their processes and subsequent seam cell fusions (Silhankova et al., 2005). Additional receptors are likely to be involved in the regulation of transcription in seam cells. Among 28 ODR-7-related supnrs, eight genes were expressed in seam cells: *nhr-72*, *nhr-73*, *nhr-74*, *nhr-75*, *nhr-77*, *nhr-81*, *nhr-82* and *nhr-89*. Although RNA interference did not reveal any developmental phenotype, three genes, *nhr-77*, *nhr-81* and *nhr-82* showed severe developmental phenotype connected with an altered number of seam cells induced by the expression of GFP fusion transgenes in seam cells (Miyabayashi et al., 1999). However, the potential for redundancy of function among the supnrs is a real possibility, suggesting caution in interpretations. However it is clear that development of this dynamic and responsive tissue is heavily dependent on NHRs.

An unusual feature of NHR-60 biology is its localization at the nuclear periphery; this is not a typical cellular pattern for nuclear hormone receptors. Most NHRs are reported to be shuttling between the cytoplasm and the nucleus or constitutively nuclear, in both cases forming a patchy or diffuse pattern (Ktistaki et al., 1995; Zhu et al., 1998; Hager et al., 2000; Maruvada et al., 2003). A peripheral localization for several transcription factors has been reported and for these the pattern was shown to be related to interactions with the nuclear lamina and lamins. For some factors, this interaction was shown to be linked to transcriptional inhibition (Liu et al., 2000; Gruenbaum et al., 2003). Although we cannot exclude the possibility that NHR-60 localization at the nuclear periphery is also linked to transcription inhibition, we favour the hypothesis that it reflects interactions with metabolic or other regulatory proteins. This is consistent with our observations in animals with over-expressed NHR-60 having increased peripheral presence of NHR-60 without observable developmental phenotypes.

Unlike most NHRs, HNF-4α contains a negative regulatory region positioned at the C-terminus of the molecule, which if deleted leads to enhanced activation of HNF-4α-dependent gene expression (Petrescu et al., 2005). The sequence alignment of NHR-60 with NHRs of known crystal structures indicates that the NHR-60 C-terminal region has similarity to an AF2 region which interacts with co-activators. Consistent with this, expression of the C-terminally deleted isoform lacking the most terminal 19 amino acids of NHR-60 induces a phenotype similar to NHR-60 inhibition. Because overexpression of wild-type *nhr-60* had no obvious phenotypes, we believe the AF2- construct is functioning as a dominant-negative factor. This may provide a useful tool for further exploration of NHR-60 function and other supnrs that have similar domains.

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