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A functional NR4A nuclear receptor DNA-binding domain is required for organ development in *C. elegans*

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Abstract

NR4A nuclear receptors are a diverse group of orphan nuclear receptors with critical roles in regulating cell proliferation and cell differentiation. The ortholog of the NR4A nuclear receptor in *C. elegans*, NHR-6, also has a role in cell proliferation and cell differentiation during organogenesis of the spermatheca. Here we show that NHR-6 is able to bind the canonical NR4A monomer response element and can transactivate from this site in mammalian HEK293 cells. Using a functional GFP-tagged NHR-6 fusion, we also demonstrate that NHR-6 is nuclear localized during development of the spermatheca. Mutation of the DNA-binding domain of NHR-6 abolishes its activity in genetic rescue assays, demonstrating a requirement for the DNA-binding domain. This study represents the first genetic demonstration of an *in vivo* requirement for an NR4A nuclear receptor DNA-binding domain in a whole organism.

Keywords

NHR-6; NR4A; *C. elegans*; DNA-binding domain; spermatheca; organogenesis

The NR4A orphan nuclear receptors (NRs) are rapidly emerging as key regulators of a diverse array of physiological and developmental processes (Maxwell and Muscat, 2006). One of the critical biological functions of the NR4A NRs is the regulation of cell proliferation and cell differentiation. All three vertebrate NR4A paralogs (NR4A1–3) have described cell proliferation and cell differentiation functions in a variety of cellular contexts, including atherosclerosis, endothelial cell growth, myeloid homeostasis, and differentiation of dopaminergic neurons (Bonta *et al.*, 2007; Castro *et al.*, 2001; Kolluri *et al.*, 2003; Martorell *et al.*, 2008; Mullican *et al.*, 2007). How NR4A NRs can mediate opposing cell proliferation and cell differentiation processes is unknown. Structural studies demonstrate that NR4A NRs lack a ligand-binding pocket and thus are not regulated by the same canonical mechanism as other NRs (Baker *et al.*, 2003; Wang *et al.*, 2003). It is also apparent that NR4A NR activity is regulated through diverse interactions with signal transduction pathways (Wansa *et al.*, 2003) and these differential interactions are likely the key to understanding the diverse cellular functions of NR4A NRs. NR4A NRs are also known to have both nuclear and cytoplasmic functions in regulating cellular processes, such

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as apoptosis (Chintharlapalli *et al.*, 2005). It is not known whether NR4A NRs regulate cell proliferation and cell differentiation through a DNA-binding mechanism *in vivo*. In this study, we assessed the activity and *in vivo* function of the DNA-binding domain from the *C. elegans* ortholog of the NR4A NR, NHR-6. Previous analyses of *nhr-6* null mutants demonstrated a requirement for NHR-6 in the organogenesis of the spermatheca, a somatic gonad organ that regulates oocyte ovulation and fertilization (Gissendanner *et al.*, 2008). *nhr-6* mutants have low brood sizes and sterility due to defective spermatheca development. NHR-6 is necessary for both cell proliferation and cell differentiation during development of this organ, indicating a striking conservation of biological function between NHR-6 and its mammalian homologs.

Vertebrate NR4A NRs bind DNA as a monomer to the canonical sequence: 5'-AAAGGTCA-3' (also known as the NGFI-B response element, NBRE) (Wilson *et al.*, 1992). We investigated if NHR-6 can bind the NBRE *in vitro* and in a cellular context. A GST-NHR-6 fusion protein containing the DNA-binding domain (DBD) and ligand-binding domain (LBD) binds the NBRE in a dose-dependent manner in electrophoretic mobility shift assays (Fig. 1a). The binding to a radiolabeled NBRE probe is competed using molar excess of cold NBRE but not by NBRE with two nucleotide substitutions (5'-CCAGGTCA-3'), indicating specificity of binding to the NBRE sequence. To determine if the NHR-6 DBD is necessary for the *in vitro* binding, we generated a cysteine to serine substitution (C288S; see Fig. 4a) in the first zinc finger of the DBD. The C288S mutation is located within the P-box, the region of the DBD important for NR binding to specific DNA sequences. Mutation of this cysteine to the similar sized serine is known to eliminate DNA binding activity in other NRs (Severne *et al.*, 1988). Binding to the NBRE was abolished by the C288S mutation, demonstrating a requirement for a functional NHR-6 DBD in the gel-shift experiments. We next asked if NHR-6 can activate transcription from the NBRE in a cellular context. NR4A NRs can activate transcription in a variety of mammalian cell lines when co-transfected with an NBRE-*luc* reporter that contains three copies of the NBRE sequence (Forman *et al.*, 1995; Perlmann and Jansson, 1995). Co-transfection of *nhr-6* cDNA and the 3XNBRE-*luc* reporter into mammalian HEK 293 cells resulted in 3-5-fold activation of luciferase expression over empty vector controls in multiple independent experiments (Fig. 1b, c). As a comparison, we also performed co-transfection of mammalian NR4A3 and 3XNBRE-*luc* in parallel. In our experiments, the level of activation by NR4A3 from a 3XNBRE-*luc* reporter in HEK293 was similar to previously published observations (Castro *et al.*, 1999). Consistent with previous studies (Wilson *et al.*, 1991), the level of activation over empty vector controls was dependent on the number of NBRE sites as reporters with 2XNBRE and 4XNBRE exhibited different levels of activation by both NHR-6 and NR4A3 (Supplemental Fig. 1). NHR-6 exhibited less activity compared to NR4A3, a result that is not surprising considering the evolutionary divergence of nematodes and mammals. Nonetheless, NHR-6 is able to recognize the NR4A response element and recruit the mammalian transcriptional machinery, indicating that there is structural conservation between NHR-6 and mammalian NR4A NRs. A functional DBD of NHR-6 was required for the activation as NHR-6 with the C288S mutation in the DBD failed to activate transcription of the reporter (Fig. 1b, c).

Previous studies of NHR-6 expression utilized either transcriptional reporter fusions to green fluorescent protein (GFP) or translational fusions to GFP that did not encode the complete NHR-6 protein (Gissendanner *et al.*, 2008). Thus, cellular localization could not be addressed in those studies. To assess the localization of NHR-6 protein during spermatheca development, we constructed an *nhr-6::GFP* reporter transgene (pLG0097) to express full-length NHR-6 with a C-terminal GFP fusion (Fig. 2a). Transgenic nematodes bearing this transgene were generated in an *nhr-6(lg6001)* homozygous null mutant background. Six transgenic nematode lines that expressed NHR-6::GFP exhibited near wild-type brood sizes and did not exhibit any *nhr-6* mutant phenotypes of low brood size/sterility, abnormal egg

morphology, or defective spermatheca morphogenesis (Fig. 2b and Fig. 5). Thus, the GFP-tagged NHR-6 is fully functional for spermatheca development. A transgenic *nhr-6* mutant line, *sgEx19*, that did not express GFP (see Methods) was not rescued, indicating that rescue requires expression of NHR-6::GFP (Fig. 2b and Fig. 3k, l). The expression pattern of NHR-6::GFP was nearly identical to the previously reported expression of transcriptional and partial translational fusions of *nhr-6::GFP* (Fig. 3). However, we did notice a few differences in the current study, including expression of NHR-6::GFP in precursor cells of the spermatheca-sheath lineages (SS cells) and in the precursors and descendants of the dorsal-uterine lineage (DU cells) (Figure 3a–j). Another difference was sheath cell expression in late L4 animals (data not shown). The GFP expression in both dorsal uterine and sheath cells was less intense than spermathecal cell expression. The slightly dissimilar expression pattern could reflect differences in the regulatory regions between the two reporter transgenes (see Methods) and/or differences in expression intensity as the GFP expression intensities of the reporter transgenes in the previous study were comparatively weaker.

Expression of NHR-6::GFP was predominantly localized to the nuclei of spermathecal cells throughout somatic gonad development (Fig. 3). Diffuse expression in non-dividing cells, which would be indicative of both nuclear and cytoplasmic localization, was never observed at any stage. As observed in previous studies, adult animals did not express NHR-6::GFP in the spermatheca. The nuclear localization of NHR-6::GFP suggests that NHR-6 has a nuclear function in the regulation of spermatheca development.

Since NHR-6::GFP is localized to the nucleus an important question is whether NHR-6 functions through a DNA-binding mechanism. The functional NHR-6::GFP allowed us to use this reporter in a genetic rescue assay to determine if a functional DBD is necessary for NHR-6 activity. We generated the same C288S mutation in NHR-6::GFP that abolished activity of NHR-6 in the gel shift and transcriptional reporter assays. We then generated transgenic *nhr-6 (lg6001)* homozygous mutant nematodes under the same conditions as with the wild-type transgene. We generated four transgenic lines, three of which expressed GFP. None of the GFP expressing lines exhibited any level of rescue, as brood sizes were not statistically different from those for *nhr-6(lg6001)* homozygous animals (Fig. 4b). Additionally, all transgenic lines exhibited a highly penetrant (>99%) abnormal egg morphology phenotype. As was the case with the wild-type fusion protein, GFP was expressed throughout spermatheca development despite the disruption of the DNA binding activity. The expression intensity of NHR-6(C288S)::GFP in the transgenic lines was qualitatively equivalent to the range of expression intensities in transgenics expressing wild-type NHR-6::GFP. Nuclear localization was also not affected by the DBD mutation (Fig. 4c, d). The C288S mutation did not have any dominant effects as *nhr-6(lg6001)/+* heterozygous animals bearing the transgene were phenotypically the same as non-transgene bearing heterozygotes (Fig. 4b). Morphological analysis of these animals also revealed a lack of spermatheca-uterine valve and distal constriction development (Fig. 5). Therefore, we conclude that a wild-type DBD is necessary for spermathecal development, with the C288S mutation likely disrupting the ability of NHR-6 to bind NR4A response elements *in vivo*.

An important question with respect to nuclear receptor biology is the extent by which NRs function through a DNA-binding mechanism. Few studies have been performed in whole animal systems demonstrating an *in vivo* requirement for NR DNA binding. Mice bearing mutations in the glucocorticoid receptor (GR) that renders it unable to bind DNA are largely phenotypically normal, demonstrating that GR DNA binding is not essential for viability (Reichardt *et al.*, 1998). Conversely, deletion of the germ cell nuclear factor (GCNf) DBD in mice does demonstrate a requirement for DNA binding *in vivo* for this NR during embryonic development (Lan *et al.*, 2002). NR4A NRs have been documented to have both

genomic and non-genomic functions, the latter being important in T-cell apoptosis (Li *et al.*, 2000). While NR4A2 has been shown by chromatin immunoprecipitation to bind DNA in the ventral mid-brains of mouse embryos (Jacobs *et al.*, 2009), no genetic studies have been performed in a whole animal system that demonstrates a requirement for the DNA-binding domain of NR4A NRs. The null-like phenotype of *nhr-6 (lg6001)* animals expressing NHR-6(C288S) suggests that DNA-binding activities of NHR-6 are required for spermatheca development. Therefore, based on these results, a likely mechanism of NHR-6 activity during spermatheca development is direct binding to promoter regions to regulate target gene transcription. More importantly, the genetic rescue assay developed in this study will provide a powerful animal-based system to further investigate the molecular mechanisms of NHR-6 activity, allowing an opportunity to identify, with *in vivo* relevance, downstream effectors of NHR-6 as well as the use of mutational studies to identify other regions of NHR-6 necessary for its activity.

METHODS

Plasmid construction

To generate an *nhr-6::GST* fusion, *nhr-6* cDNA was PCR amplified and cloned into the *NotI/BamHI* site of the pGEX5-1 vector. The encoded protein has GST fused to amino acids 260-619 of NHR-6 which includes both the DBD and LBD. For expression of NHR-6 in HEK293, full-length *nhr-6* cDNA was PCR amplified and cloned into the *KpnI/XbaI* of pCMV6-XL5 vector (OriGene) to place *nhr-6* expression under control of the CMV promoter. There are two mRNA isoforms of *nhr-6*, alpha and beta (Gissendanner *et al.*, 2008). The shorter beta isoform encodes an NR4A protein with a shortened N-terminal A/B domain. We chose to use the full-length alpha isoform for the cell transfection assays described here.

pLG0097 is a genomic clone of *nhr-6* that contains the last ~2.4 kb of intron 1 and all coding sequences with sequence in the last exon fused in frame to EGFP (the last 11 amino acids of NHR-6 are not included in the fusion). Primers LG29.1 (5'-AGTCTGCAGTGCAGGTGTTTCTCTCAAAA-3') and LG29.2 (5'-AGTGGATCCGACGAGGAAGATGGTCCAAA-3') were used to amplify a 3108 bp PCR-fragment from *C. elegans* genomic DNA (containing the *nhr-6* promoter (intron 1) and exon 2 (which contains the start codon)) that was cloned into the *PstI/BamHI* site of plasmid pBY1153 (gift from Ralf Baumeister, containing EGFP) resulting in plasmid pLG0029. Primers LG97.3 (5'-AGTGGATCCTCGCAAAGGCTCC-3') and LG97.4 (5'-AGTCCATGGAACCTCCAGATGGGGCAAC-3') were then used to amplify a 4040 bp PCR-fragment (containing *nhr-6* genomic sequence downstream of exon 2) from *C. elegans* genomic DNA and cloned into the *BamHI/NcoI* site of plasmid pLG0029 resulting in plasmid pLG0097.

Site-directed mutagenesis to generate the C288S mutation was performed using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene). All three mutagenesis reactions utilized the same primer combinations (5'-CCAGAACTTGCGAAGGAAGCAAAGGATTCCTCAAGC-3' and 5'-GCTTGAAGAATCCTTTGCTTCCTTCGCAAGTTCTGG-3') and the same general reaction conditions according to manufacturer's protocols. All constructs that were generated were sequenced to verify the C288S mutation and that no other mutations were present. For pLG0097, only the coding regions (with splice junctions) of the construct were sequenced.

Electrophoretic Mobility Shift Assays (EMSA)

GST-NHR-6 protein expression was induced at 37°C with 100mM IPTG in NEB Express *E. coli* (New England Biolabs). The GST-NHR-6 was purified using GST SpinTrap columns (GE Healthcare) according to manufacturer's instructions.

For the EMSA assays primers 5'-GAGTTTTTAAAAGGTCATGCTCAATTT-3' and 5'-AAATTGAGCATGACCTTTTAAACTC-3', 2.5 pmoles each, were labeled with $\gamma^{33}\text{P}$ ATP in a 20 ml reaction using T4 Polynucleotide Kinase (New England Biolabs) according to manufacturer's instructions. Kinase was inactivated and primers were annealed by heating to 95°C for 5 minutes then allowing the reaction to cool to room temperature.

Purified GST-NHR6 protein, wild-type and mutant, was incubated in EMSA Buffer (10 mM Tris pH 7.5, 50 mM NaCl, 1 mM MgCl_2 , 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol) containing 50 mg/ml poly dI/dC, 50 ng/ml ssDNA, and 0.25 pmoles of labeled annealed primers in a 10 μl reaction at room temperature for 5 minutes. 12.5 pmoles of unlabeled annealed primers (above) or annealed mutant primers (5'-GAGTTTTACCAGGTCATGCTCAATTT-3' and 5'-AAATTGAGCATGACCTGGTAAACTC-3') were added as competitor in some reactions. Samples were then electrophoresed on a 6% polyacrylamide gel in 0.5X TBE Buffer at 100 volts for 75 minutes. Gels were subsequently fixed in 5% methanol, 5% acetic acid, dried and exposed to film.

Transient cell transfection assays

For the 3XNBRE-*luc* transfections, low passage HEK293 cells were seeded in 24 well plates and cultured in routine growth media for 24 hours or until cells reached approximately 60–70% confluency. Before transfection, old growth media was replaced with fresh routine growth media containing no antibiotics and 1% FBS. Transfection of plasmid constructs was performed using Lipofectamine LXT (Invitrogen). Each well received 100ng of experimental DNA, 100ng NBRE reporter (a gift from Thomas Perlmann), and 1 μl of Lipofectamine LXT (Invitrogen) diluted into 100ul of 1x DMEM. Each well also received 3ng of pRL-CMV vector (*Renilla* luciferase vector) (Promega) to serve as a control for transfection efficiency. Cells were incubated in normal conditions for 24 hours. As a positive control, cells were transfected with a pCMV-NR4A3 expression plasmid (OriGene). As a negative control, cells were transfected with the empty pCMV vector. Luciferase activity was determined using the Dual Glo Luciferase Assay System (Promega). All luciferase values were normalized to *Renilla* luciferase values.

For the 2XNBRE and 4XNBRE *Gaussia* luciferase reporter transfections, HEK293 cells at 80% confluency were transfected with 100ng experimental DNA, 100ng NBRE reporter, and 20ng pCMV-lacZ. Cells were harvested the next day and assayed for luciferase using Luciferase Assay Reagent (Promega). β -galactosidase was assayed using the β -galactosidase Assay Reagent (Tropix).

For Western analysis of transfected cells, cell pellets were lysed with 150 μl of cold RIPA buffer (Sigma) and incubated on ice for 20 minutes. Blots were incubated for 18 hours at 4°C with 10 μl of primary chicken polyclonal NHR-6 antibody or monoclonal α -tubulin antibody diluted in 10ml of blocking buffer. Membranes were washed three times in TBS-T (0.1% Tween 20) and incubated at room temperature in 10 μl of alkaline phosphate-linked secondary antibody diluted in 10ml of blocking buffer for three hours. Blots were washed 3 times with TBS-T buffer. The color reaction was observed by incubating the membrane with 5 ml of Western Blue Substrate (Promega).

Generation and analysis of nematode transgenics

C. elegans was cultured at 20°C under standard conditions (Brenner, 1974). Generation of transgenic nematodes was performed by microinjection as described (Mello and Fire, 1995). Constructs (either 5ng/μl or 1ng/μl) were co-injected with 100 ng/μl of pRF4 (*rol-6* (*su1006*)) dominant mutation) as a transformation marker. Injections were performed on young, gravid IP1001 (*nhr-6(lg6001)/qC1 dpy-19(e1259) glp-1(q339)* hermaphrodites. Stable transgenic lines were isolated in both *nhr-6(lg6001)/qC1 dpy-19(e1259) glp-1(q339)* heterozygous and *nhr-6(lg6001)* homozygous backgrounds (*qC1 dpy-19(e1259) glp-1(q339)* homozygotes are sterile). *nhr-6(lg6001)* homozygotes bearing transgenic arrays were recovered by segregation from heterozygous transgenic lines. Initial injections were performed with 5ng/μl of pLG0097. Four lines (two in heterozygous backgrounds and two in homozygous backgrounds) were recovered. The two *nhr-6(lg6001)* homozygous lines bearing the transgenic arrays *sgEx13* and *sgEx14* were rescued for brood size (236 ± 27 and 172 ± 63 , respectively, $n=6$). The other two lines failed to transmit the transgenic array in subsequent generations indicating dominant negative effects of the transgenic array in these lines. This has been previously observed for transgenics bearing other *nhr-6* coding sequences (Gissendanner *et al.*, 2008). Therefore, all subsequent injections were performed with 1ng/μl of wild-type and mutant constructs. From the wild-type construct injections, five lines were isolated (bearing arrays *sgEx15-19*). *sgEx15-18* bearing lines expressed GFP and rescued *nhr-6(lg6001)* homozygotes (Fig. 2). The line bearing *sgEx19* did not express GFP and did not rescue. Given the nature of transgenic array formation in *C. elegans* (Mello and Fire, 1995) the *sgEx19* array likely did not incorporate the pLG0097 plasmid due to the low concentration of DNA injected. For the pLG0097 (C288S) construct, four transgenic lines bearing the arrays *sgEx20-23* were recovered. Lines bearing the array *sgEx21* did not express GFP. None of the four arrays rescued *nhr-6(lg6001)* homozygotes (Fig. 4). Brood size and other phenotypic assessments (abnormal egg morphology and spermatheca morphogenesis) of individual animals were performed as previously described (Gissendanner *et al.*, 2008). Statistical analyses were performed using two-tailed *t*-tests assuming unequal variance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIG. 1. NHR-6 binds to the canonical NR4A response element and can transactivate in mammalian HEK293 cells

(a) Electrophoretic mobility assay of GST-NHR-6. GST-NHR-6 (lanes 1-5) and GST-NHR-6 (C288S) (lanes 6, 7) incubated with radiolabeled 26-mer oligonucleotide containing the NBRE (5'-AAAGGTCA-3'). An increase in intensity of the shifted oligonucleotide is seen with increasing amounts of protein (lanes 1-3, 140ng, 280ng, and 560ng of input protein, respectively). Two major shifted bands are observed (arrows). The two bands could be due to dimerization of the protein in the EMSA reaction. Both shifted bands are competed by 50X cold NBRE (lane 4, 280ng of input protein). The shifted bands were not competed by 50X cold mutant NBRE (5'-CCAGGTCA-3') (lane 5, 280ng of input protein). GST-NHR-6 (C288S) did not cause a shift (lanes 6 and 7, 280ng and 560ng of input protein, respectively). Coomassie stained gel of the GST-NHR-6 and GST-NHR-6 (C288S) protein preparations is shown below the EMSA, indicating equal amounts of protein in the preparations (arrow). Both proteins are ~68KDa in predicted size. 15 µl of protein preparation were loaded for each lane. (b) Representative graph showing transactivation by NHR-6 from the 3XNBRE in HEK293. Cells were co-transfected with pCMV-*nhr-6*, pCMV-*nhr-6*(C288S), pCMV-NR4A3, or empty pCMV vector (100ng each), along with 100ng of 3XNBRE-*luc*. Experiments were performed in triplicate and luciferase values (± SD) were determined 24 hours post-transfection. Luciferase values were normalized to *Renilla* luciferase (RLU). (c) Western blot analysis indicating expression of NHR-6 and NHR-6 (C288S) in HEK293 cells 24-hours post-transfection, as detected by anti-NHR-6 LBD polyclonal antibodies. Two specific bands are detected (arrows) as well as a non-specific band (*) that is also detected in cells transfected with empty vector. Detection of α-tubulin is used as a loading control.

FIG. 2. Rescue of *nhr-6* (*lg6001*) mutants by transgenes expressing NHR-6::GFP
(a) Diagram showing the fusion of GFP to NHR-6. Domains of NHR-6 are indicated. The numbers above the diagram indicate amino acid positions of NHR-6. EGFP is fused at the C-terminus at amino acid 608 of NHR-6. NHR-6 has 619 amino acids so the final 11 amino acids of the LBD are not included in the fusion. **(b)** Brood size analysis of transgenic and non-transgenic nematodes. “+/+” refers to animals homozygous for the wild-type *nhr-6* allele (*C. elegans* N2 strain). “-/-” refers to animals homozygous for the *nhr-6* (*lg6001*) allele, and “+/-” refers to *nhr-6*(*lg6001*)/*qC1 dpy-19*(*e1259*) *glp-1*(*q339*) heterozygotes (strain IP1001, see Methods). Animals bearing independent transgenic arrays containing the *nhr-6::GFP* transgene are indicated by the *sgEx* designation. Five or six animals were scored for +/+, +/-, and -/-; *sgEx13*; *15-18* genotypes. Ten animals were scored for the -/-; *sgEx19* genotype and fifteen animals were scored for the -/- genotype. Nematodes bearing arrays *sgEx15-18* exhibited rescue of the brood size phenotype with brood sizes that were not significantly different from heterozygotes ($p > 0.2$). -/-; *sgEx19* animals were not rescued and these animals do not express GFP presumably due to the lack of the *nhr-6::GFP* transgene in the array (*).

FIG. 3. Expression and nuclear localization of NHR-6::GFP in cells of the developing spermatheca

Epifluorescence micrographs (a, c, e, g, i, k) and Nomarski micrographs (b, d, f, h, j, l) of nematodes bearing the *sgEx13* array. White arrow indicates spermatheca nuclei and the pink arrow indicates dorsal uterine nuclei. Only nuclei in the plane of focus are indicated. *C. elegans* nuclei have a button appearance and contain a prominent nucleolus. Expression of GFP is restricted to the nucleus at all stages. (a, b) Expression of NHR-6::GFP in the SS and DU precursor cells in early L3 larval stage. (c, d) Expression in SS and DU descendents at mid-L3. (e, f) Expression in developing spermatheca and dorsal uterine cells during late L3. (g, h) Expression in early L4 spermatheca and dorsal uterus. (i, j) Expression in the spermatheca and dorsal uterus at mid-L4. Dorsal uterine expression has become very faint. Brackets indicate the regions of the developing anterior and posterior spermathecae in L4. (k, l) Absence of GFP expression in the spermatheca of $-/-$; *sgEx19* animals (white bracket). The image in (k) was digitally enhanced and the diffuse fluorescence signal observed is due to autofluorescence of the gut.

FIG. 4. Genetic rescue by NHR-6::GFP requires a wild-type DNA-binding domain
(a) Diagram of the two zinc fingers of the NHR-6 DNA binding domain showing the C288S mutation (blue circles indicate the zinc ions). **(b)** Brood size analysis of transgenic nematodes expressing NHR-6::GFP (C288S). See legend to Fig. 2 for description of genotypes. Arrays *sgEx20-23* fail to rescue the brood size phenotype of *nhr-6(lg6001)* homozygotes (compare to *-/-*, $p > 0.8$). Array *sgEx21* does not express GFP (*). *sgEx22* (genotype *+/-; sgEx22*) does not exhibit dominant effects in a heterozygous background (compare to *+/-*, $p > 0.4$). $n = 15$ for *-/-*, *-/-; sgEx21-22*; $n = 8$ for *-/-; sgEx20*; $n = 12$ for *-/-; sgEx23*; $n = 5$ for *+/+*, *+/-*, and *+/-; sgEX22*. **(c, d)** Epifluorescence **(c)** and Nomarski **(d)** of NHR-6::GFP (C288S) expression during the mid-L4 stage from the *sgEx23* array in *nhr-6(lg6001)* homozygous animals. Arrow indicates nucleus in focal plane.

FIG. 5. Spermatheca morphogenesis phenotypes of rescued and non-rescued animals
(a, a') Wild-type (+/+) and *nhr-6(lg6001)/nhr-6(lg6001); sgEx18* young adult hermaphrodites. The spermatheca-uterine valve at the distal end of the spermatheca is formed properly (arrow) and the spermatheca has a normal size and cell number (bracket). In **(a')**, most of the distal constriction in the proximal end of the spermatheca is out of the plane of focus. **(b, b')** *nhr-6(lg6001)/nhr-6(lg6001); sgEx23* young adult hermaphrodites. A spermatheca-uterine valve is absent (arrow) and the spermatheca is smaller and abnormally formed (bracket). The animal in **(b)** is slightly older and sperm has entered the spermatheca through the defective distal constriction. sp=sperm in proximal gonad; ut=uterus.