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The *C. elegans* nuclear receptor NHR-6 functionally interacts with the JUN-1 transcription factor during spermatheca development

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Abstract

The NR4A nuclear receptor NHR-6 is an essential regulator of spermatheca organogenesis in *C. elegans*. In this study, we perform a focused, RNAi-based screen to identify modifiers of partial *nhr-6* loss of function. Ninety-eight genes that encode signaling proteins expressed in the spermatheca were screened for enhancement of the *nhr-6* RNAi phenotype. We identify the *C. elegans* gene *jun-1*, which encodes the homolog of the Jun transcription factor, as a strong enhancer of *nhr-6* partial loss of function. We show that *nhr-6* and *jun-1* function together to regulate development of the spermatheca and are necessary for generating an organ with the normal number of cells. *jun-1* is expressed in all cells of the developing spermatheca. We also provide evidence that NHR-6 and JUN-1 can physically interact in a yeast two-hybrid assay. Our results provide *in vivo* evidence that NR4A nuclear receptor and Jun transcription factor interactions are essential in regulating developmental processes in metazoans.

Keywords

RNAi interaction screen; NR4A; modifier

Nuclear receptors (NRs) belonging to the orphan NR4A subgroup in vertebrates (Nur77/NGFI-B/NR4A1; Nurr1/NR4A2, and Nor1/NR4A3) are essential mediators of numerous developmental and physiological processes (Maxwell and Muscat, 2006). At the cellular level, NR4A NRs have important roles in regulating cell proliferation, cell differentiation, apoptosis, cell survival, cell migration, inflammation, and carbohydrate metabolism depending on the particular cellular context (McMorrow and Murphy, 2011; Mohan *et al.*, 2012; Pearen and Muscat, 2010; van Tiel and de Vries, 2012). Thus, an important question regarding NR4A NR activity is the mechanism(s) by which NR4A NRs are able to regulate such diverse processes in a wide variety of cell and tissue types.

The cellular functions of NR4A NRs appear to have been conserved through evolution. Similar to vertebrates, the *Drosophila melanogaster* NR4A NR homolog, DHR38, regulates carbohydrate metabolism. It is also required for cuticle synthesis, a process associated with

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adult epidermal differentiation during metamorphosis (Kozlova *et al.*, 2009; Ruaud *et al.*, 2011). Nuclear hormone receptor-6 (NHR-6) is the sole NR4A NR homolog in *C. elegans* and exhibits both sequence and functional similarity to vertebrate NR4A NRs (Gissendanner *et al.*, 2008). NHR-6 has high sequence identity within the DNA-binding domain (74% identity with DHR38 and 76% with NR4A1/2/3) and can bind the canonical NR4A NR binding site *in vitro* and in mammalian cell transcription assays (Gissendanner *et al.*, 2004; Heard *et al.*, 2010). *nhr-6* is an essential gene and loss of *nhr-6* function causes severe reproductive defects associated with abnormal development of the spermatheca, a tubular somatic gonad organ that functions in ovulation, fertilization, and sperm storage (Gissendanner *et al.*, 2008; McCarter *et al.*, 1997). In spermatheca development, *nhr-6* is required for both cell proliferation and differentiation (Gissendanner *et al.*, 2008), indicating conserved functions between NHR-6 and vertebrate NR4A proteins in regulating these cellular processes. Given the experimental tractability of *C. elegans*, the spermatheca can be utilized as a unique organ development system to dissect NR4A NR signaling in an *in vivo* context.

To identify other genes that function with *nhr-6* during spermatheca development, we performed an RNAi-based enhancer screen focused on 98 signaling genes expressed in the spermatheca (Supplemental Table 1). The screening strategy is shown in Supplemental Figure 1. The screen utilized *nhr-6* RNAi by feeding which causes a partial loss of *nhr-6* function (Gissendanner *et al.*, 2004; Gissendanner *et al.*, 2008). To identify signaling gene enhancers of *nhr-6*, we employed two RNAi approaches: a combinatorial double RNAi by feeding approach (*nhr-6* RNAi + signaling gene RNAi) and *nhr-6* RNAi by feeding performed on signaling gene mutants when viable homozygous mutants were available. In both cases we screened for an enhanced reduction in fecundity of the double loss of function animals. Enhancement was identified in a qualitative manner in the primary screen and then “hits” were confirmed in subsequent experiments that quantified brood sizes. An initial screen involved double or single (for mutant strains) RNAi analysis in a liquid media, 96-well plate format for a subset of the signaling genes. From this initial screen four genes were identified as candidate enhancers of *nhr-6* RNAi: *hmg-1.2*, *let-502*, *swsn-2.1*, and *jun-1*.

Further screening of genes by *nhr-6* RNAi in mutant backgrounds was performed on NGM agar plates (see Methods) and identified three additional candidate interacting genes: *cgef-1*, *pix-1*, and *nipi-3*. Thus, 7 genes out of 98 screened were found to be candidate enhancers of *nhr-6* RNAi (Supplemental Table 1).

RNAi experiments for the candidate interacting genes were repeated and reproductive fecundity was quantified by brood size analysis (Table 1). To compare the degree of interaction with *nhr-6* among the different genes and to establish a list of robust enhancers of *nhr-6*, we calculated a scaled epistasis value for each interacting gene. Scaled epistasis has been utilized for yeast gene interactions and the equation yields a value between -1 and 0 when scoring for phenotypic enhancement (Segre *et al.*, 2005). A value of -1 indicates a strong synergistic interaction causing lethality or sterility while a value of 0 indicates no enhancement. We utilized an arbitrary cutoff value of -0.5 for establishing our final list of *nhr-6* RNAi enhancers (Tables 1 and 2). This value represents a two-fold increase in the severity of the brood size phenotype over the expected brood sizes if the phenotypic effects were simply additive (compare W_{xy} to $W_x W_y$ in Table 2). The -0.5 value was therefore utilized as our criterion for identifying a strong synergistic interaction; indicating that *nhr-6* and the candidate gene function in the same biological process.

After this quantitative analysis, *swsn-2.1* (scaled epistasis = -0.24), *hmg-1.2* (scaled epistasis = -0.3), and *let-502* (scaled epistasis = -0.27) were excluded from a final list, leaving *jun-1*, *cgef-1*, *pix-1*, and *nipi-3* as the remaining strong interacting genes from our

screen (Table 1). *cgef-1* and *pix-1* encode guanine nucleotide exchange factors (GEFs) for the Rho- family GTPase CDC42 that are expressed in all somatic gonad cells throughout larval development (Kumfer *et al.*, 2010; Lucanic and Cheng, 2008; Ziel *et al.*, 2009). Interestingly, our screen consisted of 10 RhoGEF genes that are expressed in the spermatheca (Ziel *et al.*, 2009). A quantitative assessment of all RhoGEF genes on the screening list confirmed that only *cgef-1* and *pix-1* strongly interacted with *nhr-6* (Supplemental Fig. 2). Thus, these two genes may encode critical GEFs for Rho family signaling pathways regulating spermatheca development. *nipi-3* is expressed in the adult spermatheca and encodes a kinase with an innate immune system function in *C. elegans* (McKay *et al.*, 2003; Pujol *et al.*, 2008). It has similarity to the mammalian Tribbles homolog protein 1 which functions in a variety of cellular processes (Dobens and Bouyain, 2012). *jun-1* encodes the *C. elegans* homolog of the mammalian Jun (c-Jun, JunB, JunD) transcription factor (Hiatt *et al.*, 2009; Oommen and Newman, 2007). In addition to an enhanced average reduction in fecundity, double loss of function of *nhr-6* and the interacting genes also increased the percentages of sterile animals and the percentages of eggs laid that exhibit abnormal morphology (small, round or irregularly shaped eggs) caused by defective ovulation (Table 1) (Gissendanner *et al.*, 2008).

The identification of a Jun homolog was of interest as nuclear receptors are known to interact with AP-1 proteins consisting of heterodimers of Jun, a basic leucine zipper (bZIP) protein, and a member of the Fos subfamily (c-Fos, FosB, Fra-1 and Fra-2) of bZIP proteins, or other bZIP protein subfamilies (ATF and JDP) (Hess *et al.*, 2004; Martin and Tremblay, 2009; Pascual and Glass, 2006). This interaction can occur as part of a protein complex or as cooperatively-acting transcription factors on target gene promoters. JUN-1 has been proposed to function with the *C. elegans* Fos homolog, FOS-1, in the regulation of uterine cell specification and in the transcriptional regulation of the phospholipase C gene, *plc-1*, in the spermatheca (Hiatt *et al.*, 2009; Oommen and Newman, 2007). *plc-1* functions in the calcium-mediated pathway that regulates ovulation in adults (Kariya *et al.*, 2004). We did not detect an interaction with *fos-1* in the double RNAi screen but were not able to further test this using a mutant strain as *fos-1* mutants are sterile. The sterility of *fos-1* mutants is not due to defective spermatheca morphology (Hiatt *et al.*, 2009).

To determine if the *nhr-6/jun-1* interaction is due to a shared function in the regulation of spermatheca development, we assessed spermatheca morphology in both single and double loss of function animals (Fig. 1). We utilized the spermathecal cell marker AJM-1::GFP which is localized to the adherens junctions of spermathecal cells (Aono *et al.*, 2004; Mohler *et al.*, 1998). Both *nhr-6* (hypomorphic) and *jun-1* single loss of function cause moderate developmental defects in the spermatheca (Fig. 1a–f). In *jun-1(gk551); nhr-6(RNAi)* animals, however, there is a dramatic effect on spermatheca morphology (Fig. 1g–i). The spermatheca is reduced in size and lacks defined proximal and distal regions, including absence of the spermatheca-uterine valve. This phenotype is very similar to the spermathecae of *nhr-6* null mutants (Gissendanner *et al.*, 2008). Also similar to *nhr-6* null mutants, the spermathecal cell number is reduced by half in the double loss of function animals (Fig. 2) and large, undivided cells are evident in these spermathecae (Fig. 1i). Thus, *nhr-6* and *jun-1* functionally interact during development of the spermatheca and the defective spermatheca development in these animals likely contributes to the severe decrease in brood size.

In previous studies, *jun-1* was shown to be expressed in uterine cells and in the adult spermatheca (Hiatt *et al.*, 2009; Oommen and Newman, 2007). *jun-1* encodes six isoforms (A–F) of JUN-1 that all contain the bZIP region (Hiatt *et al.*, 2009). Large expression surveys demonstrate broad somatic expression of *jun-1* (Feng *et al.*, 2012; Reece-Hoyes *et al.*, 2007). Given the spermatheca phenotypes observed in *jun-1(-); nhr-6(RNAi)* animals

we assessed the expression of *jun-1* in the developing spermatheca. We obtained the strain OP234 generated by the modEncode project (Gerstein *et al.*, 2010). This strain contains an integrated transgene (*wgIs234*) encoding JUN-1 fused at the C-terminus to GFP. The expression pattern should represent all JUN-1 isoforms described in Hiatt *et al.*, 2009 since all isoforms share the last exons that encode the bZIP region. JUN-1::GFP was nuclear-localized and expressed in all cells of the developing and young adult spermatheca (Fig. 3a–d) as well as the spermatheca-uterine junction core cells (sujc) in L4, which forms part of the spermatheca-uterine valve (Fig. 3e, f). Expression in the spermatheca-uterine valve was not observed in young adults (Fig. 3c). The expression of *jun-1::GFP* in the developing spermatheca is identical to *nhr-6* (Gissendanner *et al.*, 2008; Heard *et al.*, 2010).

Since *nhr-6* RNAi is hypomorphic in the interaction studies, the enhancement could reflect *nhr-6* functioning in parallel to the interacting genes or in common or overlapping pathways. One mechanism by which NHR-6 could function with JUN-1 in spermatheca gene regulation is through protein-protein interactions. To test if NHR-6 and JUN-1 have the ability to physically interact, we performed a yeast two-hybrid analysis. NHR-6 bait was found to interact with JUN-1 prey by induction of *URA3* expression in the assay, allowing growth on Ura⁻ plates and growth inhibition by 5-fluoroorotic acid (5-FOA) (Fig. 4a). This interaction was confirmed in an additional yeast two-hybrid assay using a *lacZ* reporter under regulation of a different promoter (Fig. 4b). Sequence analysis of the *jun-1* ORFeome library clone (Rual *et al.*, 2004) used to generate the yeast two-hybrid expression plasmid revealed that it corresponded to the JUN-1A isoform (the largest of the six isoforms) but lacked the bZIP region. This indicates that the bZIP region is not necessary for the interaction with NHR-6. We generated a yeast two-hybrid expression clone for full-length JUN-1A and demonstrated that a JUN-1 protein containing a bZIP region can also interact with NHR-6 (Fig. 4c). To broadly assess regions of NHR-6 necessary for the interaction, we constructed an NHR-6 expression clone lacking the D and E regions of NHR-6 and an expression clone lacking the A/B and C regions. Both deleted forms of NHR-6 failed to interact with JUN-1, indicating that both regions are necessary for the JUN-1 yeast two-hybrid interaction (Fig. 4c).

The *jun-1(gk551)* mutation deletes the bZIP coding region (last 2 exons) (http://www.wormbase.org/species/c_elegans/gene/WBGene00012005?query=jun-1), indicating that it should be a strong loss of function allele. However, *jun-1(gk551)* homozygotes have a less severe phenotype compared to *nhr-6* null mutants. Thus, if JUN-1 and NHR-6 function in a common pathway or cooperatively regulate the expression of common target genes, the activity of JUN-1 is probably not as critical as NHR-6. There is also a possibility that a mutant JUN-1 protein expressed from the *gk551* allele may be able to interact with NHR-6 since the bZIP domain is not required for the interaction. In this case, JUN-1 function in spermatheca development does not completely require DNA binding but is instead dependent on the NHR-6 interaction. An important aspect of this interaction is in the regulation of cell proliferation; a known function for both NR4A NRs and Jun. NR4A NR/Jun interactions may also be a broadly conserved feature of NR4A NR activity as the NR4A NR Nur77 and c-Jun physically interact at the *Star* gene promoter in mouse Leydig cells (Martin and Tremblay, 2009). The results described here provide a foundation for further exploring this interaction in an organ development context.

Methods

RNAi screening

Spermatheca expressed genes were identified from a list of signaling genes generated by Lehner *et al.*, 2006 (Lehner *et al.*, 2006a), by searching [wormbase.org](http://www.wormbase.org) and published literature (Gissendanner and Kelley, 2013; Hiatt *et al.*, 2009). The list of genes screened can

be found in Supplementary Table 1. HT115 RNAi by feeding bacteria clones were obtained from two existing RNAi libraries. The ORFeome-based library (Reboul *et al.*, 2003) was utilized for most of the genes (library obtained from Open Biosystems). Clones not found in this library were obtained from the “Ahringer” library (Fraser *et al.*, 2000) (obtained from Source Bioscience). Mutant strains for the screening were obtained from the *Caenorhabditis* Genetics Center. The mutant strains utilized can also be found in Supplemental Table 1.

Combinatorial RNAi screening was performed as previously described using the same bacterial growth and induction procedures (Lehner *et al.*, 2006b). The screening protocol we utilized is described in Supplemental Figure 1. Synchronized RNAi-sensitive *eri-1 (mg366)* (Kennedy *et al.*, 2004) L1 larvae were prepared by alkaline hypochlorite treatment of gravid adults to obtain eggs and the subsequent hatching of eggs in the absence of food. Ten to fifteen L1s in 10µl of M9 were added to wells of a 96-well plate containing 40 µl of induced HT115 bacteria. The media in the wells also contained 100 µg/ml ampicillin and 1 mM IPTG to maintain induction of dsRNA production. Each RNAi treatment was performed in triplicate on the plates. To identify enhancement, the wells containing 20µl *nhr-6* dsRNA producing bacteria and 20µl of signaling gene dsRNA producing bacteria were compared to wells containing 20µl *nhr-6* dsRNA producing bacteria plus 20 µl of control dsRNA producing bacteria and to wells containing 20µl of signaling gene dsRNA producing bacteria plus 20 µl of control dsRNA producing bacteria. We used one of two control dsRNA producing bacteria: 1) a bacterial strain producing *her-1* dsRNA, obtained from ORFeome library, was utilized as control as this gene is not expressed in hermaphrodites (Trent *et al.*, 1991) and would possess the same plasmid backbone sequences as other ORFeome clones; or 2) *GFP* dsRNA producing bacteria obtained by transforming HT115 bacteria with plasmid pPD128.110 (Addgene). No differences were observed for the different control dsRNAs (data not shown). The *GFP* dsRNA was used for the RNAi experiments that generated brood size data. *her-1* dsRNA was predominantly used for the initial 96-well plate screening and for RNAi experiments involving *ajm-1::GFP* (strain SU93). Wells were screened 3 and 4 days after set-up and incubation at 20°C. Although adult hermaphrodites in the liquid media screen were often egg-laying defective, enough F1 offspring were produced to identify large decreases in fecundity when compared to control wells. Liquid media RNAi screening with mutant strains involved feeding of Bristol N2 strain L1 larvae with 40 µl *nhr-6* RNAi bacteria and the selected mutant strain L1 larvae with *nhr-6* RNAi bacteria or control RNAi bacteria.

An initial liquid media screen was performed on 79/98 signaling genes by double RNAi. Of these 79 genes, 36 were also represented by mutant strains and screened by single *nhr-6* RNAi in liquid media. All of the interacting genes identified in the initial double RNAi screen were confirmed by sequence analysis of the RNAi plasmid. We found that it was problematic to perform an accurate quantitative brood size assessment on animals subjected to RNAi in liquid media. Even when transferred to an NGM agar plate as young adults, both control and experimental animals were routinely egg-laying defective (data not shown). This did not occur in animals subjected to double or single RNAi on NGM agar plates; therefore, the remaining 19 genes were represented by mutant strains and these genes were only screened by single *nhr-6* RNAi on NGM agar plates (Supplemental Table 1). All RNAi experiments for brood size quantification and microscopic analysis were performed on agar plates. For RNAi experiments on agar plates, RNAi bacteria was grown from a freshly streaked plate in a 15ml conical tube for 8 hrs in 2 ml LB broth containing 100 µg/ml ampicillin at 37°C with shaking. Bacteria was added to NGM Lite agar plates containing 200 µg/ml ampicillin and 1 mM IPTG and induced overnight at room temperature. For combinatorial RNAi on plates, equal volumes of RNAi bacteria were mixed prior to adding to plates. Developmentally arrested L1 were added after bacteria induction and screened 3 and 4 days after plating or removed as young adults for brood size analysis.

Quantification of brood sizes

Single young adults from the RNAi plates were added to brood count plates that contained a narrow thin lawn of OP50 bacteria. Worms were moved to new brood count plates every 8–12 hours until eggs were no longer being laid. After worms were moved, eggs and larvae on the brood count plates were counted. Statistical analyses were performed using SigmaPlot 11.0.

jun-1 strains and quantification of spermatheca nuclei number

Strain VC1184 (*jun-1(gk551)*) was generated by the *C. elegans* Reverse Genetics Core Facility at the University of British Columbia, which is part of the international *C. elegans* Gene Knockout Consortium (Consortium, 2012). The strain was obtained from the *Caenorhabditis* Genetics Center. VC1184 was outcrossed 4× to Bristol N2 to generate strain DN73. This outcrossed strain was used for the data shown in Table 1. The 832 bp *gk551* deletion was tracked during the crosses by PCR. PCR primer sequences were provided by the *C. elegans* Gene Knockout Consortium (<http://www.celeganskoconsortium.omrf.org>). The *ajm-1::gfp* strain, SU93, contains the integrated transgene *jclIs1* (Mohler *et al.*, 1998) and was obtained from the *Caenorhabditis* Genetics Center. This transgene was crossed to *jun-1(gk551)* mutants to generate strain DN74 (*jun-1(gk551); jclIs1*). Strain OP234 (*unc-119(ed3); wglIs234*) was utilized for the *jun-1::GFP* reporter analysis. This strain was generated by the modEncode project and obtained from the *Caenorhabditis* Genetics Center.

Spermatheca nuclei number data were generated using strains SU93 and DN74 subjected to control or *nhr-6* RNAi. Multiple image planes were taken for each spermatheca. Individual nuclei were then counted in each image plane and confirming that no individual nucleus was counted more than once. Expression of *ajm-1::GFP* was utilized to mark spermathecal tissue. Given the severe morphological defects of *jun-1(gk551); nhr-6 (RNAi)* spermathecae, it is possible that some nuclei counted in these animals belonged to valve or uterine cells as it was sometimes difficult to distinguish proximal spermatheca tissue from valve or uterine tissue in these animals. Statistical analysis was performed using SigmaPlot 11.0.

Yeast two-hybrid analysis

Yeast two-hybrid analysis was performed using the ProQuest™ System (Invitrogen). GAL4 DNA binding domain (using plasmid pDEST™32) and activation domain (using pDEST™22 plasmid) fusion constructs were generated from the ORFeome library RNAi clones using Gateway cloning, according to manufacturers' instructions (Rual *et al.*, 2004). The *jun-1* ORFeome clone corresponds to *jun-1A* but lacks the last two exons of coding sequence which encode the bZIP region. Details of this clone can be found here: <http://wormfdb.dfci.harvard.edu/searchallwormorfs.pl?by=name&sid=T24H10.7+>. To test full-length JUN-1A expression in the yeast two-hybrid experiments we generated a full-length *jun-1A* cDNA by RT-PCR from mixed-stage *C. elegans* total RNA and cloned this cDNA into pDEST22 using the Gateway protocol. The *nhr-6* ORFeome clone encodes an NHR-6 protein that lacks the first 156 amino acids of the N-terminal A/B domain. cDNAs encoding NHR-6 containing only the A/B/C domains (amino acids 157 – 355) and NHR-6 containing only the D/E domains (amino acids 356 – 619) were amplified by PCR from the ORFeome library and cloned into pDEST32 using the Gateway protocol. For all clones sequence analysis confirmed in-frame fusions to the N-terminal GAL4 sequences and to a stop codon at the 3' end. DNA binding domain and activation domain plasmids were co-transformed into yeast strain MaV203, according to manufacturers' instructions and confirmed by Leu-Trp- selection. The data shown in Figure 4 were generated using GAL4DB-NHR-6 (bait) and GAL4AD-JUN-1 (prey). DB-JUN-1 (bait) exhibited strong autoactivation (data not shown); therefore, only NHR-6 (bait)/JUN-1(pre) experiments could be performed.

lacZ reporter expression in the yeast-two hybrid studies was quantified using the chlorophenol red- β -D-galactopyranoside (CPRG) assay, according to instructions provided by the ProQuest™ System.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. *nhr-6* and *jun-1* interaction during spermatheca development

DIC (a, c, e, g, i) and AJM-1::GFP epifluorescence (b, d, f, h) micrographs of spermathecae from control, *nhr-6*(RNAi), *jun-1*(gk551), and *jun-1*(gk551), *nhr-6*(RNAi) animals. “P” indicates the proximal and “D” indicates the distal regions of the spermatheca. Some of spermatheca (Sp) nuclei in the plane of focus are indicated with arrows. When observed, sheath cell pair 5 nuclei (S5) are also indicated. Vu, vulva; Va, spermatheca-uterine valve; G, germline. In (i), two large, undivided spermathecal cells are indicated. Scale bar = 10µm.

Figure 2. Spermatheca nuclei number in *jun-1(-); nhr-6(RNAi)* animals

Spermatheca nuclei were counted in young adult animals expressing *ajm-1::GFP*. N=7, 31, 27, and 30 spermathecae were counted for control, *nhr-6(RNAi)*, *jun-1(gk551)*, and *jun-1(gk551); nhr-6 (RNAi)* animals, respectively. Error bars, $\pm$ standard deviation. Due to the invariant nature of the spermatheca cell lineages, all control animals have 24 spermatheca nuclei. Spermathecal nuclei number was decreased in 100% of *nhr-6* RNAi animals and in 85.2% of *jun-1(-)* animals. Spermatheca nuclei number was significantly decreased in the double versus single loss of function animals ($p < .0001$; One Way ANOVA).

Figure 3. Expression of *jun-1::GFP* in the developing spermatheca

Epifluorescence (a, c, e) and DIC (b, d, f) micrographs of JUN-1::GFP expression (strain OP234) in L4 and young adult animals. Nuclear-localized expression is seen in two rows of spermatheca (Sp) cells at mid-L4 (a, b). Expression in the uterus (Ut), sheath cell pairs 4 and 5 (S4 and S5), and in an intestinal nucleus (In) are indicated. Spermatheca expression is also observed in young adults. No expression is evident in adult valve (Va) cells but *jun-1::GFP* is expressed in the spermatheca-uterine junction core (sujc) cell in L4 (e, f). The binucleate sujc cell eventually forms the core of the spermatheca-uterine valve. Contrast in (e) has been digitally enhanced to better define sujc expression.

Figure 4. NHR-6 and JUN-1 interact in a yeast two-hybrid assay

(a) Serial dilutions of transformed yeast cultures on selection plates. Positive control plasmids were provided with the ProQuest™ System. The JUN-1 protein encoded by the yeast two-hybrid expression plasmid corresponds to the JUN-1A isoform and lacks the bZIP region. The NHR-6 protein encoded by the yeast two-hybrid expression plasmid lacks the first 156 amino acids of the A/B domain. Negative control: pDEST™32 (empty) + pDEST™22 (empty) plasmids; NHR-6 (bait): pDEST™32 (GAL4DB-NHR-6) + pDEST™22 (empty) plasmids; JUN-1 (prey): pDEST™32 (empty) + pDEST™22 (GAL4AD-JUN-1) plasmids; NHR-6/JUN-1: pDEST™32 (GAL4DB-NHR-6) + pDEST™22 (GAL4AD-JUN-1) plasmids. Positive control and NHR-6/JUN-1 transformants

express the *URA3* gene as shown by robust growth on Ura- plates and growth inhibition in the presence 5-FOA. (b) Expression of the *lacZ* reporter in the yeast two-hybrid assay. CPRG assays for the same transformants shown in (a), except that the positive control (provided by the ProQuest™ System) is for a weak interaction. NHR-6 (bait) shows some autoactivation in this assay but the NHR-6/JUN-1 experiment shows a significantly increased signal ($p < .01$ (*); Student's *t* test). Error bars, $\pm$ standard error. $N = 3$ independent assays for each experiment. (c) Both JUN-1A (FL) and JUN-1A Δ bZIP interacts with the NHR-6 (same library expression clone (Lib.) as shown in (a) and (b). The JUN-1A Δ bZIP is expressed from the library clone shown in (a) and (b). Both forms of JUN-1 fail to interact with NHR-6 A/B/C, which contains the same A/B/C domain sequences as the library clone but lacks the D and E domains, and NHR-6 D/E, which contains D/E domain sequence but lacks A/B/C domain sequences. Two independent transformants are shown for each test.

Table 1

nhr-6 interactions with *pix-1*, *cgef-1*, *jun-1*, and *nipi-3*

Experiment ¹	N	Avg. Brood Size $\pm$ S.D.	Range	% Sterility	% Abnormal Egg Morphology	Scaled Epistasis
<i>GFP</i> (RNAi)	10	243 $\pm$ 19.7	209 – 265	0	0	
<i>nhr-6</i> (RNAi)	15	113 $\pm$ 16.8	92 – 148	0	10.1	
<i>pix-1(ok982); GFP</i> (RNAi)	13	138 $\pm$ 64.4	65 – 283	0	1.9	
<i>pix-1(ok982); nhr-6</i> (RNAi)	20	31.6 $\pm$ 20.1 ²	7 – 96	0	28.8	–0.50
<i>cgef-1(gk261); GFP</i> (RNAi)	13	111 $\pm$ 70.5	0 – 217	15.4	5.1	
<i>cgef-1(gk261); nhr-6</i> (RNAi)	20	26.7 $\pm$ 32.8 ²	0 – 90	30	30.3	–0.48
<i>GFP</i> (RNAi)	6	261 $\pm$ 34.9	206 – 292	0	0.5	
<i>nhr-6</i> (RNAi)	39	139 $\pm$ 61.5	0 – 299	2.6	13.4	
<i>jun-1(gk551); GFP</i> (RNAi)	40	55.5 $\pm$ 34.5	0 – 134	2.5	3.5	
<i>jun-1(gk551); nhr-6</i> (RNAi)	38	6.2 $\pm$ 7.8 ²	0 – 39	26.3	41.2	–0.79
<i>GFP</i> (RNAi)	5	219 $\pm$ 45.7	161–279	0	0	
<i>nhr-6</i> (RNAi)	10	109 $\pm$ 52.4	26 – 221	0	35.2	
<i>nipi-3(fr4); GFP</i> (RNAi)	10	184 $\pm$ 27	144 – 236	0	0.6	
<i>nipi-3(fr4); nhr-6</i> (RNAi)	10	34.4 $\pm$ 35.4 ²	0 – 114	10	57.3	–0.62

¹ *pix-1* and *cgef-1* mutants were analyzed together with wild-type controls. *jun-1* and *nipi-3* mutants were analyzed in separate experiments with a separate set of controls.

² Double loss of function is statistically significant ($p < 0.05$; One way ANOVA on Ranks with Dunn's test) compared to *nhr-6* single RNAi and mutant with *GFP* RNAi.

Table 2

Calculation of scaled epistasis values

Experiment	W_{x1}	W_{y2}	W_{xy3}	W_{xWy}	Scaled Epistasis ⁴
<i>nhr-6/pix-1</i>	0.465	0.568	0.130	0.264	-0.508
<i>nhr-6/cgef-1</i>	0.465	0.458	0.110	0.212	-0.481
<i>nhr-6/jun-1</i>	0.533	0.213	.0236	0.114	-0.793
<i>nhr-6/nipi-3</i>	0.5	0.842	0.16	0.42	-0.619

¹ Average brood size of *nhr-6* RNAi in wild-type background/Average brood size of *GFP* RNAi in wild-type background
² Average brood size of *GFP* RNAi in mutant background/ Average brood size of *GFP* RNAi in wild-type background
³ Average brood size of *nhr-6* RNAi in mutant background/ Average brood size of *GFP* RNAi in wild-type background
⁴ Scaled Epistasis = $(W_{xy} - W_x W_y)/(W_x W_y)$