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NHR-23 dependent collagen and hedgehog-related genes required for molting

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Abstract

NHR-23, a conserved member of the nuclear receptor family of transcription factors, is required for normal development in *C. elegans* where it plays a critical role in growth and molting. In a search for NHR-23 dependent genes, we performed whole genome comparative expression microarrays on both control and *nhr-23* inhibited synchronized larvae. Genes that decreased in response to *nhr-23* RNAi included several collagen genes. Unexpectedly, several hedgehog-related genes were also down-regulated after *nhr-23* RNAi. A homozygous *nhr-23* deletion allele was used to confirm the RNAi knockdown phenotypes and the changes in gene expression. Our results indicate that NHR-23 is a critical co-regulator of functionally linked genes involved in growth and molting and reveal evolutionary parallels among the ecdysozoa.

Keywords

Nuclear hormone receptor; *Caenorhabditis elegans*; NHR-23; transcription; gene expression; development; hedgehog; molting; ROR

1. Introduction

Nuclear hormone receptors (NHRs) are transcription factors regulating metabolism and development in all metazoan species studied to date. NHR-dependent pathways include those involved in developmental patterning and timing. In insects, transitions through developmental stages are initiated by pulses of the steroid hormone 20-hydroxyecdysone (Ecdysone, 20E) that regulates ecdysis, the periodic exchange of exoskeleton that occurs during larval development [1–7]. Ecdysis, or molting, is a common mechanism used by insects, nematodes and other bilateral species that allows for growth and progression from larval stages to adult forms and is a defining feature of the clade ecdysozoa [8]. In *Drosophila*, pulses of ecdysone result in increased expression of early hormone response

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genes, including those encoding the ecdysone receptor (ECR) itself and two other nuclear receptors, DHR3 and E75B [2, 9]. ECR, a conserved member of the NHR family with greatest similarity to farnesyl X receptors (FXR, NR1H4), forms a heterodimer with Ultraspiracle (USP), the insect homologue of Retinoid X receptors (RXRs) [10, 11]. This pathway serves as a paradigm of developmental regulation by NHRs and their sterol ligands.

In the nematode *C. elegans*, molting and larval development are regulated by NHR-23, a close homologue of *Drosophila* DHR3 [12, 13]. Expression of *nhr-23* oscillates, reaching peaks during intermolt periods and decreasing just prior to molting [13]. This timing roughly coincides with entry into lethargus, an approximately two-hour period during which movement and pharyngeal pumping decrease. The wave like pattern of *nhr-23* expression during the third and fourth larval stages (L3/L4) is, in part, regulated by the micro RNAs (miRNAs) *let-7* and *miR-84* [14]. Moreover, the regulatory RNA *let-7* is controlled by DAF-12, the nematode homologue of the vitamin D receptor [15] that in turn is regulated by the sterol dafachronic acid. Unlike *Drosophila*, *C. elegans* does not synthesize cholesterol, so DAF-12-mediated signaling and many other events regulating molting depend on exogenous sterols [16]. These nematode pathways illustrate that many upstream components and mechanisms for the regulation for ecdysis, including the critical involvement of NHRs and sterol ligands, are evolutionarily conserved among ecdysozoa.

To determine if other elements of ecdysis-related events were similarly conserved, we searched in *C. elegans* for genes acting downstream of NHR-23 using genome wide expression microarrays. Synchronized L2 larvae treated with *nhr-23* RNAi by feeding from hatching were used as a source of mRNA for profiling. Among the potential NHR-23 target genes were those that were expected, including those encoding collagens, a principle component of the cuticle. However, we also identified several genes encoding hedgehog family-related proteins. Although, hedgehog-related proteins were previously identified as regulators of growth and molting [17–24], our results reveal the first clues about their co-regulation with collagens and their dependence on NHR-23. NHR-23 is likely a key positive regulator of molting and growth, orchestrating the expression of functionally linked epidermal genes in the nematode.

2. Materials and methods

2.1. C. elegans strains

Wild type (N2) *Caenorhabditis elegans* were obtained from the *C. elegans* Stock Center and maintained as described [25]. The mutant strain *nhr-23*(*tm1323*) was obtained from the National Bioresource Project, Tokyo, Japan. The mutant was backcrossed three times with N2 animals. The *nhr-23(tm1323)* deletion allele was confirmed by sequencing and the strain maintained as a heterozygote by scoring progeny mutant phenotypes.

2.2. RNA preparation

Synchronized populations of L1 larvae were plated with two sets of HT115 bacteria, one that had been transformed with the RNAi vector only (L4440 plasmid) and another that had been transformed with a vector targeting *nhr-23* (clone 5174) [13]). Worms were kept on 2% agarose plates for 21 hr at 20°C, collected, and approximately 200μl of worms resuspended in PBS were used in each individual experiment. Total RNA was isolated from frozen pellets using a Mixer-Mill (Miller-Mill 300) following an RNeasy Mini Kit (Qiagen, Germantown, MD) according to manufacturer protocol. Aliquots of cultures used for RNA isolation were kept on *nhr-23* RNAi plates to confirm the knockdown phenotypic changes occurred during subsequent molts.

2.3. Analysis of microarray results

C. elegans whole genome expression microarrays (Affymetrix, Santa Clara, CA) were used to profile gene expression from three independent, biological replicates for both experimental and control samples with all samples processed simultaneously. Microarray chip data was collected and analyzed by both Affymetrix MAS 5.0 suite software (≥1.6-fold change in mRNA expression) and Robust Multichip Average (RMA) (≥1.2-fold change in mRNA expression) as part of the Partek genomics suite software package, all with a p-value less than or equal to 0.05. Normalized data was further analyzed and visualized with Genespring software (Agilent Technologies, Santa Clara CA).

2.4. RT-qPCR

cDNA was prepared from 3μg of total RNA that was isolated as described above. Reverse transcription (RT) was performed as previously described [26] and quantitative PCR (qPCR) was performed using the Roche Universal Probe Library technique (Hoffmann-La Roche, Basel, Switzerland) [26]. Primers are given in Supplementary Table S1 and these primer sets interrogated the following transcripts: *dpy-2*, *dpy-3*, *dpy-7*, *dpy-8*, *wrt-1*, *wrt-2*, *wrt-4*. All samples were normalized against *ama-1*, the large subunit of RNA polymerase II as previously described [26].

3. Results

3.1. Identification of NHR-23 dependent genes

The microarray experiment evaluated 22,625 probe sets on the *C. elegans* whole genome expression arrays (Affymetrix) with triplicate RNA samples generated from wild type or an age-matched sample of *nhr-23* RNAi treated animals. From these, 331 probes were decreased in at least two out of three RNAi experiments including 90 probes that were decreased in all three RNAi experiments. Probe sets that showed decreased values in two RNAi experiments but increased values in the third RNAi experiment were not evaluated further. The resulting 266 unique down regulated genes were identified based gene annotations in WS190 (Supplementary Table S2). Gene ontology analysis using the DAVID tool [27, 28] identified 10 clusters with enrichment scores greater than 2 with the main GO terms including molting cycle, collagen and cuticle genes, regulation of growth, and development. Genes annotated as constituents of hedgehog and sterol sensing signaling pathways were identified separately as significantly enriched GO clusters (Supplementary Table S3). A similar analysis of *nhr-23* RNAi up-regulated transcripts yielded only 36 genes (not shown) that were grouped using DAVID software in only two GO clusters, both with enrichment scores less than 2.

The list of *nhr-23* RNAi down-regulated genes included four *wrt* genes, three *grd* genes, four *grl* genes, five *ptr* genes and the genes *ptc-3* and *qua-1*. Additional genes known to be involved in molting regulation were also identified as NHR-23 dependent genes including *mlt-8*, *mlt-9*, *mlt-10* and *mlt-11*. Interestingly, several collagen genes that are known to be co-expressed showed a dependence on NHR-23, including *dpy-2*, *dpy-3*, *dpy-7*, *dpy-8*, *dpy-10*, as did the *dpy-5* gene. Finally, the hedgehog-related genes *wrt-1*, *wrt-2* and *wrt-4*, which result in a Molt phenotype when mutated, were among those dependent on NHR-23. To validate the expression array data, selected collagen and hedgehog-related genes were analyzed by RT-qPCR in independent *nhr-23 RNAi* experiments. As shown in Fig. 1, these assays done at 21hr and 24hr of larval development confirmed that these genes were indeed down-regulated when NHR-23 activity was decreased by RNAi feeding. Thus, our expression array analysis of genes down-regulated by *nhr-23* RNAi identified both known and unexpected genes involved in molting as downstream targets of NHR-23 regulation.

Previously we have shown that NHR-23 binds the core DNA sequence AGGTCA and we provided evidence that NHR-23 likely functions as a transcriptional activator [12]. We analyzed the putative promoter regions, defined as 2000 bp upstream of the start codon, of the putative NHR-23 target genes encoding collagens and hedgehog-related factors that were down-regulated in response to *nhr-23* RNAi. Specifically, we looked for the NHR-23 binding site sequence, which is identical the ROR alpha site from mammals, that consists of the half-site PuGGTCA preceded by the AT-rich consensus sequence (A/G/T)(T/A)(A/T)(T/A)C(A/T) [29]. The program TFsearch [30] that is based on the Transfac database [31] identified monomeric NHR-23/ROR alpha binding sites in *dpy-2*, *dpy-3*, *dpy-7*, *dpy-8*, *dpy-10*, *wrt-1*, *wrt-2* and *wrt-4*. To determine if these binding sites were evolutionarily conserved between multiple nematode species, we used the tool EvoPrinterHD [32]. The NHR-23/ROR alpha sites for all of these genes were conserved in five nematode species (*C. elegans, C. briggsae, C. brenneri, C. remanei* and *C. japonica*) with the exception of *dpy-10*, which is conserved only in four species (not in *C. japonica*) and *wrt-4*, which contains the site only in *C. elegans* (Fig. 2). Analysis of all promoter regions from genes down-regulated in *nhr-23* RNAi experiment identified potential NHR-23/ROR alpha binding sites in 150 of the 266 genes (56 %). A similar analysis of the promoters for the up-regulated genes following *nhr-23* RNAi identified 12 of 36 total genes (33%) with the NHR-23/ROR alpha site. Of these, two genes had conserved binding sites in all five nematode species (F53B3.5 and ZK1290.11) and one (*alh-9*) is partially conserved (one base is not conserved). As a control for this analysis, we chose two sets of genes. One set consisted of 100 genes unrelated to molting that showed no change in expression in our analysis. The other was a set of 151 collagen genes that also showed no significant change in expression in our microarray experiment. The control set of 100 genes unrelated to molting had the NHR-23/ROR site in 33% of the promoters compared to 38% among the 151 unchanged collagen genes. We concluded that the promoters associated with genes down-regulated after nhr-23 RNAi had a significantly ($p=0.0003$) higher frequency of potential NHR-23/ROR alpha binding sites than control sets and that these sites were most often evolutionarily conserved.

3.2. Validation of expression data in the nhr-23(tm1323) deletion mutant

To further confirm that changes in gene expression were dependent on NHR-23, we turned to a mutant strain harboring the *nhr-23* deletion allele *tm1323* kindly provided by the National Bioresource Project, Tokyo, Japan. As reported, and confirmed by our independent sequencing, this mutant allele has an in-frame deletion that eliminates the coding region for the central part of the NHR-23 resulting in protein product lacking the entire DNA binding domain and a small portion of the adjacent parts of the A/B and hinge regions (Fig. 3). This allele was out-crossed three times with wild type animals prior to further characterization and Western blot analysis confirmed production of the predicted mutant protein product (data not shown).

We further characterized the *nhr-23(tm1323)* mutant because little was previously known other than it was homozygous inviable with larval arrest. We found that almost all homozygous mutants die during the embryonic or L1 larval stages, with a very small proportion of homozygous mutant larvae developing into the L2 stage prior to arrest (n= 2678, affected progeny 23.74% SD=2.07, embryonic lethality 11.37% SD=3.15, L1 arrest 8.52% SD=3, L2 arrest 3.54% SD=1.96). As we showed previously by *nhr-23* RNAi [12, 13], homozygous *nhr-23(tm1323)* embryos had a range of phenotypes with arrest and death from the comma stage to 3-fold stage with severe morphological defects common. Arrested homozygous L1 larvae also had variable phenotypes, but often included a Dpy phenotype and/or variable bulges and constrictions along the length of the body (Fig. 4). The small number of homozygous larvae that reached the L2 stage also had a severe Dpy phenotype

(Fig. 4). To confirm that these phenotypes were indeed due to the loss of NHR-23 activity, heterozygote hermaphrodites were injected with an amplified genomic sequence containing the wild type *nhr-23* gene (6,253 bp) in a rescue assay commonly used [33–36]. We observed rescue of the embryonic and early larval lethality in approximately 50% of offspring (Fig. 4 G), demonstrating that the mutant phenotypes described were due to loss of NHR-23 activity.

To validate changes in gene expression resulting from *nhr-23* RNAi, we analyzed transcript abundance in homozygous *nhr-23(tm1323)* animals compared to wild type controls. This analysis was limited to only a few genes because of the difficulty of isolating large populations of homozygous progeny. Therefore, we decided to focus on a few of the hedgehog-related genes as their deregulation as assayed by microarrays was unanticipated. From *nhr-23(tm1323)* heterozygote parental animals, we picked separately 200 L1 animals displaying either a wild type or Dpy phenotype, isolated total RNA, and assayed gene expression by RT-qPCR. Whereas mRNA corresponding to genes *wrt-1*, *wrt-2* and *wrt-4* was easily detectable in animals with a wild type phenotype, these genes were decreased or undetectable in the Dpy animals (Fig. 4 H) relative to the *ama-1* positive control. Thus, although very limited, our analysis of gene expression in homozygous *nhr-23* mutants was consistent with whole genome expression array results following *nhr-23* RNAi.

4. Discussion

C. elegans NHRs form a very large family of transcription factors encoded by approximately 300 genes. This gene family includes a small set (< 20) that is conserved between various animal phyla and a large set (>250) that are likely products of intensive multiplication of an ancestor gene related to hepatocyte nuclear factor 4 (HNF4). NHR-23 is a member of the small set of conserved NHRs and shares many functional similarities with its *Drosophila* homolog (DHR3), including the regulation of molting and ecdysis in the nematode and fly, respectively. Our current work identifies several genes dependent on NHR-23 for proper regulation that extends this evolutionarily conserved pathway to the hedgehog-related genes, further underscoring the ancient nature of this growth and developmental regulatory module.

NHR-23 appears to be a powerful regulator of genes required for embryonic and larval development. RNA interference applied at various stages of *C. elegans* development reveals the critical requirement of NHR-23 for late embryogenesis, growth and molting during all four larval stages [12, 13]. The morphological characterization of a putative null allele of *nhr-23* in the present study agrees well with the previously reported effects of *nhr-23* RNAi in the embryonic and L1 larval stages and together demonstrate the essential role for this factor. Thus, the identification of NHR-23 target genes is important for providing mechanistic insight into the function of this conserved regulatory pathway. One such insight comes from the co-regulation of functionally linked collagen genes that has been studied previously in some detail [37]. Our current work strongly suggests that NHR-23 may be a critical master regulator that orchestrates the expression of such specific gene groups throughout development. Presumably NHR-23 is acting in concert with other transcription factors that dictate which subset of NHR-23 targets are activated during specific molting cycles.

Another insight from our work is the connection between NHR-23, molting, and the hedgehog-related genes. Hedgehog signaling is an ancient metazoan pathway that employs sterols as structural molecules and sterol transport for signaling mechanisms in development [23]. Nematodes have orthologues of many hedgehog signaling proteins (encoded by *wrt*, *grd*, *grl*, and *qua* genes), and sterol-sensing receptors homologous to Patched proteins (the

ptr genes) [21] while some proteins of the hedgehog signaling pathway seem to be absent. Decreased activity of many of the *C. elegans* hedgehog-related genes result in growth and molting phenotypes as does sterol restriction [18–20, 24]. Our current work provides a strong connection between sterol signaling, the hedgehog-related pathways and NHR-23, thereby linking the signaling and structural components of molting with a common and evolutionarily conserved NHR regulator.

Regulation of molting by NHR-23 in *C. elegans* shares several features with its vertebrate homologues, the RORs. In addition to their conserved DNA response element sequence and predominant function as transcriptional activators [12], there appears to be conservation of downstream target genes. For example, hedgehog signaling was found in the ROR alpha regulatory pathway [38] and our current works links hedgehog-related pathways to NHR-23. Interestingly, hedgehog signaling is also involved in the execution of tissue specific developmental regulation in amphibian metamorphosis, perhaps reflecting a distant evolutionary link to ecdysis and molting in flies and nematodes, respectively. Once again we see that there is a limited repertoire of molecular mechanisms regulating signaling and morphogenesis that have evolved to control a variety of growth-related processes in animals. Understanding the details of these mechanisms in each model system will, therefore, shed light on all.

Highlights
> NHR-23 is a critical regulator of nematode development and molting. > The manuscript characterizes the loss-of-function phenotype of an *nhr-23* mutant. > Whole genome expression analysis identifies new potential targets of NHR-23. > Hedgehog-related genes are identified as NHR-23 dependent genes. > New link between sterol mediated signaling and regulation by NHR-23 is found.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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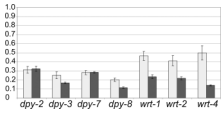

Figure 1.

Analysis of the expression of selected collagen and hedgehog-related genes by RT-qPCR at 21 hours (grey columns) and 24 hours (dark columns) of larval development. Mean values of quadruplicates (21 hours) and triplicates (24 hours) are expressed as the ratio of the normalized expression values relative to the *ama-1* gene in RNAi inhibited cultures compared to controls. SD are indicated by vertical bars.

dpy-2 -1570 tggccgtgggtagtgcaaaaaaa**GATTTATAGGTCAAAAAACTtTcTTT**tgttttcgctttttttcggtgt

dpy-3 -192 actaa**CGGAGATA**ttgaggat**AGATAATCCAGGTCA**c**TC**ag**T**c**GT**ta**TCTAATTAGGTC**gtatccacttat

dpy-7 -245 **CGA**t**AATTGGGTTATTGTGTCGAT**aaa**GA**c**TGACCTA**t**TTAACAGATAC**actccaccttcatccaccttt

dpy-8 -76 **CCTAATCGC**gc**CTTATC**g**AGTTTTACCCA**c**TGACCTATAT**c**C**gct**GCTTATAAGT**atcc**G**g**ATGGA**atgca

wrt-1 -102 **AGA**tgcga**AAGC**t**TCT**caccat**CTCGATCCAGGTCA**c**CAACTCGTTTTCCTATCACTTTCACCTGGCTT**ga

wrt-2 -67 **AT**ag**AGGAAAAGC**agcaaaa**AGAC**at**TTTGATGACCTTATTTTC**TTGTC**G**t**GT**c**G**agacggccatt

wrt-4 -1881 cgaacggattgccgcacagatgttatgctg**tgacct**aaaatttagtatgaaaataactt

Figure 2.

Analysis of predicted promoters of selected genes. The program TFSearch [30] identified monomeric ROR alpha response elements (grey shading) in promoters of the genes indicated to the left. Analysis with the program EvoprinterHD [32] was used to determine the conservation of the NHR-23/ROR alpha sites and flanking regions across five nematode species (see text). Conserved bases in five out of five nematode species, indicated by capital letters, reveal extensive sequence conservation within and around the putative NHR-23/ROR alpha binding sites.

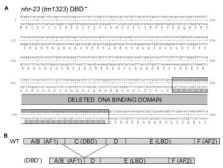

Figure 3.

Schematic representation of the *nhr-23(tm1323)* allele. A – The deletion extending from exons 3 to 5 creates a novel exon and results in an encoded protein that lacks the complete DNA binding domain and small adjacent portions of domains A/B and D. B – Schematic representation of the wild type (WT) and mutant NHR-23 (*tm1323*) DBD^{-}.

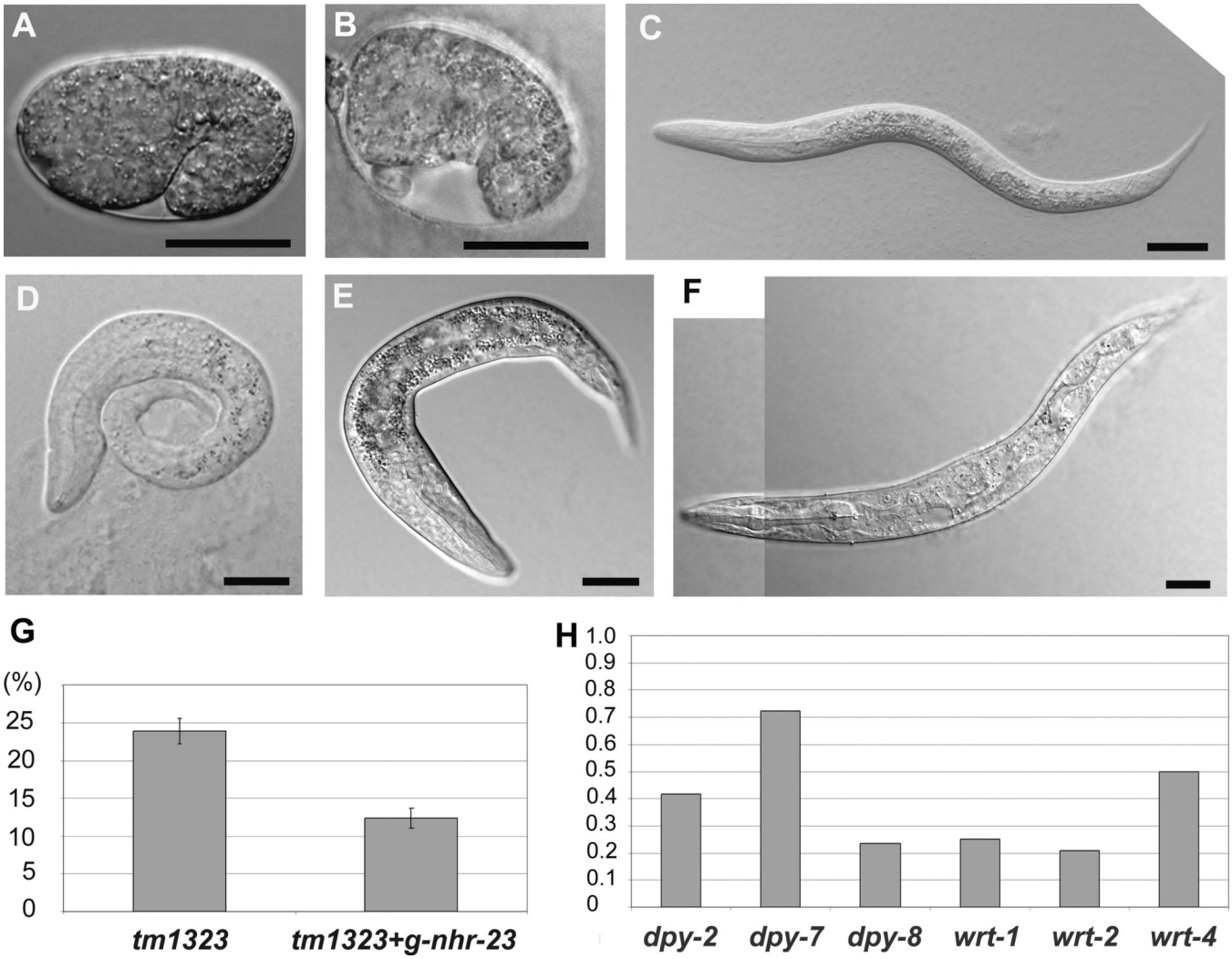

Figure 4.

Phenotypic characterization of homozygous *nhr-23(tm1323)* mutants. A – A wild type embryo at the 1.5 fold stage of development. B – An embryo arrested at the comma stage shows the abnormal positions for cells on the ventral side of the embryo and other morphological defects. C – Wild type L1 larva. D to F – *nhr-23(tm1323)* mutant larvae. Panel D shows the mutant L1 Dpy phenotype and irregular body shape with constrictions and bulges. Panel E shows an L2 larva with a Dpy phenotype, vacuoles in the head and irregular bulges. Panel F shows an L2 larva at the arrest point with numerous vacuoles throughout the body. Bars represent 20 μm. G – Rescue of the mutant lethality with a wild type transgene. Transgenic animals had a significantly decreased penetrance of *nhr-23* loss-of-function phenotypes scored as the percentage of L1/L2 arrest (n = 2307) compared to non-rescued controls (n = 6,125). Mean value of the percent affected progeny and SD are indicated, $P < 0.0001$. H – RT-qPCR of *dpy-2*, *dpy-7*, *dpy-8*, *wrt-1*, *wrt-2* and *wrt-4* expression in 200 manually selected mutated or control larvae. Results are shown as the ratio of the normalized expression values relative to the *ama-1* gene in *nhr-23* (*tm1323*) mutant larvae compared to controls.