ALKB-8, a 2-oxoglutarate-dependent dioxygenase and S-adenosine methionine-dependent methyltransferase modulates metabolic events linked to lysosome-related organelles and aging in *C. elegans*

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## Abbreviations:

- **AlkB** – *Escherichia coli* Alpha-ketoglutarate-dependent dioxygenase AlkB
- **ALKBH1** – Alkylated DNA repair protein AlkB homolog 1
- **ALKBH8** – Alkylated DNA repair protein AlkB homolog 8 (a.k.a. ABH8)
- **alkb-8** – *C. elegans* gene coding for the orthologue of the ALKBH8
- **ALKB-8** – *C. elegans* orthologue of ALKBH8
- **FTO** – Fat mass and obesity associated protein
- **TRM9** – yeast TRna Methyltransferase 9, a SAM-dependent methyl transferase
- **SAM** – S-adenosyl methionine
- **RNAi** – RNA interference
- **LRO** – Lysosome related organelles
- **RRM** – RNA recognition motif

## Abstract:

ALKB-8 is a 2-oxoglutarate-dependent dioxygenase homologous to bacterial AlkB, which oxidatively demethylates DNA substrates. The mammalian AlkB family contains AlkB homologues denominated ALKBH1 to 8 and FTO. The *C. elegans* genome includes 5 AlkB-related genes, homologues of ALKBH1, ALKBH4, ALKBH6, ALKBH7 and ALKBH8, but lacks homologues of ALKBH2, 3 and 5 and FTO.
ALKBH8 orthologues differ from other AlkB family members by possessing an additional methyltransferase module and an RNA binding N-terminal module. The ALKBH8 methyltransferase domain generates the wobble nucleoside 5-methoxycarbonylmethyluridine from its precursor 5-carboxymethyluridine and its (R)- and (S)-5-methoxycarbonylhydroxymethyluridine hydroxylated forms in tRNA_{UCG}^{Arg}, and tRNA_{UCC}^{Gly}. The ALKBH8/ALKB-8 methyltransferase domain is highly similar to yeast TRM9, which selectively modulates translation of mRNAs enriched with AGA and GAA codons under both normal and stress conditions. In this report, we studied the role of alk8-8 in *C. elegans*. We show that downregulation of alk8-8 increases detection of lysosome-related organelles visualized by Nile red in vivo. Reversely, forced expression of alk8-8 strongly decreases the detection of this compartment. In addition, overexpression of alk8-8 applied in a pulse during the L1 larval stage increases *C. elegans* life span.

**Introduction**

The 2OG/Fe(II) (2-oxoglutarate- and Fe^{2+}-dependent) oxygenase superfamily possess an important position in-between oxygenases. The heme group is substituted in these enzymes by a protein module that coordinates Fe^{2+} and whose enzymatic activity is dependent on 2-oxoglutarate that serves as an electron donor and is consumed during the enzymatic reaction while converted to succinate and carbon dioxide. Unlike monooxygenases that are dependent on heme and which transfer one oxygen atom to
the substrate and reduce the other oxygen atom to water, 2OG/Fe(II) oxygenases
incorporate both atoms of molecular oxygen (O\textsubscript{2}) into the product(s) of the reaction and
are classified as dioxygenases. 2-oxoglutarate is a rate-limiting factor for enzyme
catalytic activity for its critical intracellular concentration level. Enzymes of this
category function in a wide spectrum of metabolic processes including posttranslational
modification of proteins, DNA repair, epigenetic modification of DNA and the
regulation of hypoxia responsive genes (Aravind & Koonin 2001; van den Born et al.
2011; Fedeles et al. 2015).

The AlkB family of dioxygenases encompasses homologues of AlkB from
*Escherichia coli* which is a DNA repair enzyme demethylating methylated DNA and
RNA bases (e.g. 1-methyladenine and 3-methylcytosine). Mammalian AlkB
homologues include 9 genes, named ALKBH1 to 8 and a fat mass and obesity
associated protein FTO originally identified as a gene localized at a chromosomal locus
associated with the rat fussed-toes phenotype (Peters et al. 1999; Gerken et al. 2007;
Fedeles et al. 2015). FTO gene received attention for its association with human obesity
(Frayling et al. 2007; Yajnik et al. 2009) later in part shown to be associated with a
homeobox gene IRX3 that is regulated by noncoding sequences within the *FTO* gene
(Smemo et al. 2014). This connection is conserved between fish and mammals. Besides
that, FTO has its own role in obesity as its global overexpression lead to hyperfagia and
obesity (Church et al. 2010).

ALKBH8 homologues have a special position among all AlkB proteins for possessing
two extra domains in addition to the dioxygenase domain, a methyl transferase domain
and an N-terminal RNA recognition motif that likely helps the AlkB domain in search
for specifically modified tRNAs (Songe-Moller et al. 2010; Pastore et al. 2012).
ALKBH8 has been shown to regulate the rate of protein synthesis from mRNAs that are
coded by codons for which there is a limited amount of tRNA through the modification of bases in the anti-codon region of tRNA especially the wobble base, the first base in the anti-codon place of tRNAs, that can following this modification recognize additional codons (Songe-Moller et al. 2010; van den Born et al. 2011). ALKBH8 was shown to have a role in urothelial carcinoma cell survival mediated by NOX-1-dependent ROS signals. Silencing of ALKBH8 induced JNK/p38/gammaH2AX-mediated cell death (Shimada et al. 2009). The role of human ALKBH8 as a tRNA methyltransferase required for wobble uridine modification and DNA damage survival is well documented. Fu et al. showed that the AlkB domain of mammalian ALKBH8 catalyzes hydroxylation of 5-methoxycarbonylmethyluridine at the wobble position of tRNA (Fu et al. 2010a; Fu et al. 2010b). The AlkB domain of ALKBH8 specifically hydroxylates mcm(5)U into (S)-mchm(5)U diastereomer in tRNA-Gly(UCC) (van den Born et al. 2011).

The ALKBH8 methyltransferase domain shows close relationship to a yeast methyltransferase TRM9. The function of the yeast TRM9 has been investigated (Kalhor & Clarke 2003; Deng et al. 2015). The enzyme catalyses the methylation of the wobble bases at position 34 in tRNA. U at this position can recognize all four bases while the modified uridine residues are more restrictive and limit the recognition to only A and G, or to only one of these residues. Codon-biased translation can be regulated by wobble base tRNA modification systems during cellular stress responses (Chan et al. 2010; Chan et al. 2012; Gu et al. 2014). This mechanism is conserved in plants. In Arabidopsis thaliana the Trm9 orthologue (AtTRM9, AT1G31600) and two other ALKBH8-like proteins AtTRM112a and AtTRM112b function in the formation of modified wobble uridines. AtTRM9 is responsible for the final step in mcm(5)U formation. The enzymatic activity of AtTRM9 depends on either AtTRM112a or
AtTRM112b. *A. thaliana* ALKBH8 orthologue AtALKBH8 is required for hydroxylation of mcm(5)U to (S)-mchm(5)U in tRNA(Gly)(UCC). Plants with mutant atalkbh8 have increased levels of mcm(5)U and of mcm(5)Um, its 2'-O-ribose methylated derivative, suggesting that accumulated mcm(5)U is prone to further ribose methylation by another mechanism (Leihne et al. 2011). Protozoan ALKBH8 oxygenases display both DNA repair and tRNA modification activities (Zdzalik et al. 2014).

ALKBH8 was shown to regulate selenocysteine-protein expression as a protective mechanism against damage by reactive oxygen species (Endres et al. 2015). *C. elegans* has two thioredoxin reductases, TRXR-1 and TRXR-2 (Buettner et al. 1999) but only one of them, TRXR-1 is a selenoprotein. Thioredoxin (TRX-1) is related to life span regulation and oxidative stress response in *Caenorhabditis elegans* (Jee et al. 2005; Miranda-Vizuete et al. 2006). TRXR-1 and TRXR-2 have differential physiological roles in *C. elegans* and localizations. TRXR-1 is a cytosolic protein. TRXR-2 is mitochondrial and protects mitochondria from oxidative stress, where reactive oxidative species are mainly generated, while cytosolic TrxR plays a role to maintain optimal oxido-reductive status in the cytosol. The cytosolic trxr-1 is highly expressed in pharynx, vulva and intestine. trxr-2 is mainly expressed in pharyngeal and body wall muscles and its defects cause a shortened life span and a delay in development under stress conditions. Deletion mutation of the selenoprotein trxr-1 results in decreased acidification of the lysosomal compartment in the intestine. Interestingly, the acidification defect of *trxr-1*(jh143) deletion mutant was rescued, not only by selenocystein-containing wild type TRXR-1, but also by a cysteine-substituted mutant TRXR-1. Both trxr-1 and trxr-2 were up-regulated when worms were challenged by environmental stress such as heat shock (Li et al. 2012).
A prominent feature of *C. elegans* enterocytes are lysosome-related organelles (LRO) called gut granules. Similarly as mature lysosomes, gut granules have internal acidic pH, contain hydrolytic enzymes and lack mannose-6-phosphate receptors. Gut granules are highly heterogeneous when analyzed by electron microscopy, display various level of birefringence in light microscopy and autoflorescence, which increases with animal age. In *C. elegans*, staining by Nile red applied on animals *in vivo* together with bacterial food allows highly reproducible functional determination of a specific subpopulation of lysosome-related organelles (Soukas et al. 2013). *In vivo* Nile red uptake may be used as an effective tool for identification of proteins that function at the level of specific LRO (Soukas et al. 2013).

In this report, we attempted to functionally characterize ALKB-8 in *C. elegans*. We show that *alkb*-8 downregulation by RNAi leads to slightly accelerated larval development and elevated values of *in vivo* Nile red compartment staining. The forced expression of *alkb*-8 downregulates this subcellular compartment. While downregulation of *alkb*-8 does not affect *C. elegans* longevity, forced expression of *alkb*-8 increases *C. elegans* life span by approximately 30%.

**Materials and Methods**

**Maintenance of *C. elegans* strains and transgenic lines**

All *C. elegans* strains were maintained as described (Brenner 1974). The wild type strain N2 (var. Bristol) was obtained from the *C. elegans* Stock Center (https://cgc.umn.edu/).
Transgenic lines were prepared by microinjections of plasmid DNA into gonads of young adult N2 hermaphrodites using an Olympus IX70 microscope equipped with Narishige microinjection system (Olympus, Tokyo, Japan). Injections were done as described (Fire et al. 1998; Tabara et al. 1999).

Synchronized populations of L1 larvae were prepared by the „Bleaching“ technique where the cultured nematodes are treated with alkaline hypochlorite solution, which destroys all larval stages except the embryos that are protected by egg shells. Embryos hatch in liquid solution without access to food, which prevents further development. The protocol is described in (Porta-de-la-Riva et al. 2012).

**Isolation of genomic DNA**

The genomic DNA used as a template for PCR reactions was isolated using High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). For isolation we used about 50 mg of washed wild-type animals of mixed developmental stages and we followed the manufacturer’s protocol “Isolation of Nucleic Acids from Mammalian Tissue”.

**Total RNA isolation and cDNA synthesis**

Total RNA was isolated from N2 animals kept on 2% agarose plates. Animals of the required developmental stage and feeding status were washed with water, pelleted by centrifugation for 5 min at 200 x g and frozen at -80 °C. The frozen pellet was quickly melted and resuspended in 0,5 ml of resuspension 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), vortexed for 1 min and incubated 60 min at 55 °C. RNA was isolated by phenol-chloroform extraction and ethanol precipitation and the pellet was dissolved in water. The sample was then treated with 1 unit of DNase I (New England Biolabs, Ipswich, MA) per 1 µg of total RNA for 30 min at 37 °C and purified by phenol-chloroform extraction and ethanol precipitation followed by RNA resuspension in DEPC water.

Complementary DNA (cDNA) was prepared with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) with random hexamers, according to manufacturer’s protocol.

Quantitative PCR

Total RNA from individual developmental stages (embryos, L1, L2, L3, L4 and young adult animals) and from fasted and fed animals was isolated and used for cDNA synthesis as described above. Quantitative PCR (qPCR) was performed using the Universal Probe Library technique (Roche Molecular Systems, Inc. Pleasanton, CA, USA). Primers and probes were designed with Universal Probe Library System Assay Design Software. Reactions were run on LightCycler 2.0 with the software LightCycler 4.1 (Roche Molecular Systems, Inc. Pleasanton, CA, USA) and the protocol described earlier (Vohanka et al. 2010) was used. The expression was normalized against ama-1. All samples were run in triplicates. The expression ratio (ΔΔCt) is calculated using the efficiency corrected model. In different developmental stages the resulting values mean fold change of expression compared to expression in embryos. In fasting experiments the result is fold change of expression compared to fed control animals.
RNA interference

For downregulation of *alkb-8* expression we used the RNAi feeding method where animals are fed on bacteria producing dsRNA as previously described (Timmons et al. 2001).

For preparation of the feeding vector we first cloned the whole cDNA sequence of *alkb-8* into pCR® II vector using TA Cloning® Kit Dual Promoter (pCR® II) (Invitrogen, Carlsbad, CA, USA). Primers used for the PCR reaction were 11/08 (5´ ATGTATTTCAATGAAGAAAAAGCGA 3´) and 10/08 (5´ TCAAATTTTCTTCGCAATAATAATAA 3´). Then the *alkb-8* sequence was recloned into the L4440 vector using enzymes Hind III and Xba I. The *E. coli* strain HT115 was transformed with *alkb-8::L4440* and empty L4440 control vector and one colony from each was inoculated to LB medium with Ampicillin (100 µg/ml final concentration) and let grown to OD$_{600}$ ≈ 0.4. Then isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to culture to a final concentration of 0.4 mM to induce dsRNA production. The culture was grown for 4 hours at 37 °C and then 300 µl was plated onto NGM plates supplemented with Ampicillin (100 µg/ml final concentration) and IPTG (0.4 mM final concentration). The plates were kept at room temperature overnight and the next day synchronized L1 larvae were placed on these plates.

Nile red staining

For the estimation of LRO compartment visualization by *in vivo* Nile red uptake and resulting fluorescence, the synchronized L1 larvae (control larvae, larvae inhibited for *alkb-8* by RNAi or larvae with forced overexpression of *alkb-8*) were transferred on
feeding culture + 50 ng Nile red / ml of culture. 300 µl of OP50 culture with Nile red was used per plate. Nematodes were kept at 22 °C for 48 h and then fluorescent pictures of young adults were acquired using identical settings and exposure times (magnification with 20x objective, exposure time 10 ms in RNAi experiments and 50ms in overexpression experiments). Resulting images were analyzed using the ImageJ program (https://imagej.net/). The total pixel intensity of the cytoplasmic area of the first two intestinal cells in images yielding highest fluorescence was determined and used for comparison.

**Overexpression of alkb-8**

The entire cDNA sequence of alkb-8 was recloned from pCRII vector into the expression vectors which contain heat shock inducible promoter pPD49.78 and pPD49.83 using restriction enzymes EcoRV and KpnI. Constructs were injected into N2 hermaphrodites (at a concentration of 50ng/µl) along with a positive selection marker, pRF4 plasmid (50 ng/µl), which encodes a mutant collagen (rol-6(su1006)) that induces a dominant "roller" phenotype. As control we used animals injected only with pRF4 plasmid.

Forced expression was induced in a synchronized population of L1 animals. Larvae were placed on plates seeded with OP50 bacterial culture and were left for 2 hours at RT for recovery and then subjected to 30 min heat shock at 34 °C, after which the animals were kept at 22°C and life span was determined. In case of Nile red staining experiment, the bacterial culture was supplemented with 15 ng of Nile red per plate. Pictures were taken after 50 hours using a constant setting.
Preparation of \textit{alkb-8::gfp} transgene regulated by CEOP3136 promoter and endogenous 3’ UTR

According to WormBase (WS263) \textit{alkb-8} is organized in a hybrid operon CEOP3136. This operon includes four genes, \textit{wdr-5}, \textit{dph-1}, \textit{alkb-8} and \textit{nrde-1}. Since the expression from a transgene regulated by the internal \textit{alkb-8} promoter is already known, we constructed an expression vector to prepare transgene expressing ALKB-8 tagged with GFP under the regulation of operon promoter and \textit{alkb-8} endogenous 3’ UTR. To achieve this, four amplified DNA fragments containing the operon promoter, \textit{alkb-8} genomic sequence, gene coding for GFP and the 3’UTR of \textit{alkb-8} were amplified (primer sequences are listed in Table 1) and assembled using GENEART® Seamless Cloning and Assembly Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacture’s protocol. The resulting product was verified by sequencing and used for preparation of transgenic lines (injected in a concentration of 50 ng/µl without pRF4 vector). The scheme of the construct is shown in Fig. 1.

Table 1. Primers used for seamless cloning and assembly of the \textit{P_{CEOP3136::alkb-8::gfp::alkb-8^3’UTR}} construct.

<table>
<thead>
<tr>
<th>Primer</th>
<th>sequence 5’→ 3’</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>11/80</td>
<td>AATTCGAGCTCGGTACGGATAAGGAAGATCATCAATGTTT</td>
<td>S CEOP3136 promoter</td>
</tr>
<tr>
<td>11/81</td>
<td>TCACACATATCTGAAATCACAGCAGAAAAATCAA</td>
<td>AS CEOP3136 promoter</td>
</tr>
<tr>
<td>Line</td>
<td>Sequence</td>
<td>Type</td>
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<tr>
<td>------</td>
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</tr>
<tr>
<td>11/82</td>
<td>TTCAGATATGTTGAGTTTCAATTTCAACCC</td>
<td>S</td>
</tr>
<tr>
<td>11/83</td>
<td>GGGTCCTCAATTTCCTTCGCAATAATAATA</td>
<td>AS</td>
</tr>
<tr>
<td>11/84</td>
<td>AGAAAATTGAGGACCTTTGAGGGTACCCTGTA</td>
<td>S</td>
</tr>
<tr>
<td>11/85</td>
<td>TAAAAAAACTATTGTATAGTTCCATCCATGCC</td>
<td>AS</td>
</tr>
<tr>
<td>11/86</td>
<td>ACAATAGTTTAAAAAGTTTTTCTATTGG</td>
<td>S</td>
</tr>
<tr>
<td>11/87</td>
<td>GCCAAGCTTGCATGCCTTTTAGCAGTTTGAGATCTGAA</td>
<td>AS</td>
</tr>
</tbody>
</table>
Fig. 1 Preparation of the transgenic line expressing *alkb-8::gfp* under the regulation of promoter of CEOP3136 and endogenous 3’ UTR. A – Organization of *alkb-8* on chromosome III. *alkb-8* is the third gene in operon CEOP3136 and has its own internal promoter. B – Strategy for preparation of transgene expressing ALKB-8 tagged by GFP at its 3’ end. Corresponding fragments of CEOP3136 promoter, *alkb-8* genomic sequence, gene coding for GFP based on pPD95.75 and *alkb-8* 3’UTR were amplified by PCR and assembled by Seamless Cloning Assembly Reaction.

**Developmental assay**

To estimate the timing of larval development of control *C. elegans* and animals with *alkb-8* downregulated by RNAi, the synchronized population of L1 larvae was prepared and equal volumes of liquid larval culture were transferred to control plates containing HT115 bacteria with empty L4440 plasmid and plates containing bacteria transfected with the same plasmid but containing the cloned insert of *alkb-8*. Both bacterial cultures were induced by IPTG. The experiment was done in quadruplicate from which one representative set was selected for more specific analysis. Equal surface of plates with experimental (photographed first) and control animals was photographed at the time when control animals started to first lay eggs (after 78 hours at 16 °C) and the number of animals and laid eggs was determined on the photographs. The pictures were taken on Olympus SD30 microscope (Olympus, Tokyo, Japan) with Panasonic DMC-TZ3 camera (Panasonic, Kadoma, Japan).

**Life span determination**
For determination of life span, a large scale of synchronized N2 L1 larvae was prepared and divided to control cultures and cultures subjected to \textit{alkb-8} dsRNA produced by bacteria that were fed to experimental animals and synchronized populations of transgenes containing \textit{rol-6} gene as control and experimental animals carrying extrachromosomal arrays containing \textit{rol-6} and \textit{alkb-8} cloned in heat shock vectors pPD49.83 and pPD49.78. For each experimental condition, 100 L1 larvae were selected and followed on a daily basis throughout their complete life span in the overexpression experiment. In RNAi experiments 60 animals were followed in each group.

**Microscopy**

Nomarski optics microscopy and fluorescence microscopy pictures were taken with Olympus BX60 microscope equipped with DP30BW CD camera (Olympus, Tokyo, Japan). Confocal microscopy was done using an inverted Leica SP8 TCS SMD FLIM system equipped with a 63 × 1.2 NA water immersion objective, a pulsed white light laser (470-670 nm), AOBS and two internal hybrid single photon counting detectors, and operated by Leica Application Suite X program (Leica Micosystems, Wetzlar, Germany).

**Results**

Expression of \textit{alkb-8} continues from embryonic stages through larval development to adults
We analyzed the gross expression of *alkb-8* during developmental stages using reverse transcription-quantitative PCR. The results were normalized for the expression of Polymerase II subunit *ama-1* and related to the expression observed in mixed stages embryos. The relatively high expression of *alkb-8* in embryos decreased in synchronized L1 larvae and steadily increased from L2 stage to young adults (Fig. 2a). We analyzed the effect of 6 hours fasting and feeding in synchronized L1 larvae on the level of expression of *alkb-8* (and *fat-7* and *asc-2*, for which the response to starvation is known). Starvation had no effect on *alkb-8* expression while *fat-7* and *asc-2* expression responded to fasting as expected.

![Bar chart A and B](image)

**Fig. 2** The expression profile of *alkb-8* gene analyzed by RT-qPCR. A-The expression of *alkb-8* during development. Results are shown in logarithmic scale and the values represent fold change of expression compared to expression in embryos. The expression drops in the L1 stage and gradually increases during development. B- The relative expression of *alkb-8* after six hours of fasting. The values represent fold change
of expression compared to fed control animals. The expression of \textit{alkb-8} is not affected by the feeding status. Genes previously reported to be affected by fasting (Van Gilst et al. 2005), \textit{fat-7} (expression decreases after fasting) and \textit{asc-2} (increases) were used for control.

Tissue- and cell-specific expression of \textit{alkb-8} from the operon promoter

According to WormBase (WS263) \textit{alkb-8} is organized as a third gene in a hybrid operon CEOP3136 indicating that its expression depends partially on the operon promoter and partially on its own promoter. Expression of \textit{alkb-8} dependent on the internal promoter was described by Pastore and coworkers and revealed \textit{alkb-8} expression decreasing during later larval stages and the expression pattern was restricted to a small number of cells, especially several neurons (Pastore et al. 2012). For visualization of \textit{alkb-8} expression dependent on the operon promoter, we prepared lines carrying extrachromosomal arrays containing the transgene consisting of the CEOP3136 promoter, \textit{alkb-8} genomic sequence fused to \textit{gfp} and followed by the endogenous \textit{alkb-8} 3’-UTR. The transgene is expressed ubiquitously in embryos from approximately the 40 cell stage throughout the embryonic development. The expression continued in L1 larvae, although it was necessary to use longer time exposure for its visualization in accordance with the decreased expression observed in L1 larvae in the RT-qPCR experiment. The cytoplasmic expression of the transgene was strong in neurons, pharyngeal and body wall muscles, and other tissues such as somatic gonad and the egg-laying apparatus (Fig.3 and Fig. 4). We also observed diffuse expression in intestinal cells (Fig. 3).
Fig. 3 Expression pattern of ALKB-8::GFP in early stages of development using a transgenic line carrying an extrachromosomal DNA construct.

The construct composition is shown in Fig. 1. The GFP signal in embryos can be detected early after eggs are laid (around 40 cells stage) shown in panels A and B. The expression continues to be ubiquitous during embryonic development; panels C and D show an embryo at the end of the gastrulation phase, panels E and F an embryo at the 2-fold stage. Panels G to J show early L1 larvae where the GFP signal is detected in all cell types with similar intensity. In the L1/L2 developmental stage (panels K through N) the expression starts to be differentiated and the highest signal is seen in pharyngeal and neuronal cells in the head and tail areas. Strong signal is also detected in seam (arrows) and muscle cells (arrowheads) in panel K. In panels M and N the same animal as shown in panels K and L but with focus on a different layer. High expression is visible in intestinal cells (arrows), the distal tip cell (DTC) (arrowhead) and in the ventral nerve
Fig. 4 Expression of *alkb-8::gfp* from extrachromosomal arrays regulated by the promoter of CEOP3136 operon analyzed by confocal microscopy. Panel A shows the expression of *alkb-8::gfp* in the head of an adult animal. Strong signal is detected in
neurons (arrows), pharyngeal muscle cells (small arrows) and head muscle cells (arrowheads). Panel B – shows the same animal as panel A but in Nomarski optics. Panels C and D show the central part of the body of an adult animal with two freshly laid embryos. The embryo in the left is approximately in the 30 cells stage (arrowhead) and shows no expression of \textit{alkb-8::gfp}. In contrast, the embryo on the right is in the approximately 100 cells stage (small arrowhead) and shows ubiquitous cytoplasmic expression of the transgene. The canal-associated neuron (CAN) marked by arrow shows strong cytoplasmic expression of the transgene. Panels E and F show expression of \textit{alkb-8} in the spermatheca (arrow) and body wall muscles (arrowheads). Panels G and H show another focal plane of the same animal as showed in E and F. The arrow indicates strong expression in the CAN neuron and in another unidentified neuron (arrowhead). Panels I and J show the central part of the body of a L4 larva where high expression of \textit{alkb-8} is detected in cells of the somatic gonad and egg-laying apparatus indicated by small arrows (DTC- distal tip cell, ST- spermatheca, UT- uterus, VUL- vulva). Large arrow points to the CAN neuron, arrowheads point to body wall muscles. Panels K and L show the distant part of a L4 larva with many \textit{alkb-8} positive cells. Tail neurons (arrows), hyp cell (arrowhead) and rectal epithelial cells (small arrows) are indicated. Panels B, D, F, H, J and L shows the same picture as the fluorescent picture on their left in Nomarski optics. Bars represent 50 µm.

The effects of \textit{alkb-8} downregulation and forced overexpression on \textit{C. elegans} development

Downregulation of \textit{alkb-8} by RNAi using the protocol with bacteria producing dsRNA did not reveal any directly observable phenotype. In contrary, the larvae with
downregulated \textit{alkb-8} seemed to be in a very good feeding status and possibly slightly bigger than the controls fed with bacteria containing empty vector expressing short non-specific dsRNA. Since the observed difference was not causing delays in complete larval stages, we analyzed the onset of egg laying in control and RNAi treated cultures. This strategy revealed clearly observable difference in time given by the onset of egg laying by control larvae at which the larvae with downregulated \textit{alkb-8} laid already approximately 50 times more embryos (Fig. 5). No specific developmental defects were observed.

\textbf{Fig. 5 Analysis of the effect of alkb-8 downregulation by RNAi on \textit{C. elegans} larval development.} Equal amounts of synchronized L1 larvae were transferred on plates with control cultures (HT115 bacteria transformed with empty L4440 vector) and experimental plates seeded with bacteria transformed with L4440 vector containing \textit{alkb-8} cDNA. Both control and experimental plates were induced using IPTG and the cultures observed to the time point when control animals start to lay eggs. At this time, equal areas of plates with nematodes were photographed and the number of animals (and laid eggs) was determined. The experiment shows that inhibition of \textit{alkb-8} by the
feeding method that was used in this experiment doesn’t affect the larval development of *C. elegans*. In contrary, animals with downregulated *alkb-8* developed faster compared to control animals.

The effect of *alkb-8* downregulation and forced overexpression on the visualization of the Nile red-positive compartment

In order to assess a possible involvement of ALKB-8 in the function of lysosome-related organelles, we assayed the uptake of Nile red delivered to nematode synchronized cultures together with bacterial food. Animals with inhibited *alkb-8* showed markedly higher Nile red dependent fluorescence in enterocytes. In both experimental and control animals, the Nile red fluorescence was higher in proximal enterocytes compared to enterocytes of the middle part of the gut. We therefore analyzed the fluorescent signal in the first two proximal enterocytes. Densitometric analysis of Nile red-dependent fluorescence confirmed an approximately 30% increase of the Nile red positive signal in animals with inhibited *alkb-8* (Fig. 6).

![Image of fluorescence signals and bar chart](image-url)
Fig. 6 Detection of the signal in the in vivo Nile red stained compartment in control animals and animals with downregulated alkb-8. Panel A shows a Nile red derived fluorescence in a young adult control animal. Panel B shows a larva with alkb-8 inhibited by RNAi with the identical optical settings. Panel C shows the result of densitometric analysis of Nile red derived fluorescence in the two most proximal enterocytes of 23 animals with downregulated alkb-8 and 21 control animals. The results show a pronounced increase of approximately 30 % of Nile red derived fluorescence in animals with alkb-8 down regulated by RNAi compared to control animals. P < 0.001.

We also assayed if forced expression of alkb-8 affects the Nile red positive fluorescence in enterocytes. Two transgenic lines expressing alkb-8 from extrachromosomal arrays under the regulation of heat shock regulated promoter based on the plasmid pPD49.78 and pPD49.83 were prepared. Both plasmids lead to the transgene expression in a wide spectrum of cells and differ in the extent of the expression in intestinal cells, which is higher in case of pPD49.83. Both transgenic lines showed a strong decrease in the extent of Nile red positive signal in enterocytes (Fig. 7). Keeping with ALKB-8 intestinal role, the line based on pPD49.83 which leads to a strong intestinal expression of the transgene showed the lowest values for Nile red dependent fluorescence.
Fig. 7 The effect of *alkb*-8 forced overexpression on the signal of the Nile red positive compartment of LRO. Panels A, C and E show fluorescence images of young adult larvae stained in vivo with Nile red. Panel A- shows an animal from the control group, panel C- an animal from the group overexpressing *alkb*-8 from pPD49.78 vector, panel E- an animal from the group with *alkb*-8 in pPD49.83 vector. Panels B, D and F show the same pictures as the pictures next to them in Nomarski optics. Panel G shows the result of Nile red staining analysis after forced expression of *alkb*-8 calculated just as in the RNAi experiment. Overexpression of *alkb*-8 from pPD49.78 decreases Nile red staining in intestinal cells by 60% (marked as *alkb*-8_78) and from pPD49.83 (marked as *alkb*-8_83) by 70% compared to control animals. P < 0.0001
The effect of *alkb-8* overexpression on *C. elegans* life span

To determine if the effect of ALKB-8 on the Nile red positive compartment has a broader metabolic role, we assayed the life span of animals with downregulated *alkb-8* expression or pulse-overexpressed *alkb-8*. Downregulation of *alkb-8* expression (applied for the entire lifetime of the assayed animals) had no effect on the animal life span (Fig. 8). In strong contrast, pulse forced expression in animals during their L1 stage led to pronounced life span extension of experimental animals reaching 10 to 40%.
Fig. 8 Determination of the effect of *alkb-8* on the life span of *C. elegans*. Panel A – The effect of *alkb-8* downregulation on nematode longevity. Animals inhibited for *alkb-8* to the level that is affecting Nile red positive compartment staining has no effect on nematode longevity. Panel B – the effect of pulse overexpression in L1 stage on *C. elegans* longevity. Compared to controls, animals with forced expression of *alkb-8* have life span extended by 10 to 40%.

Discussion

Our results support ALKB-8 modulatory function in metabolic events linked to lysosome-related organelles and aging in *C. elegans*. Surprisingly, despite that *alkb-8*
being expressed strongly and ubiquitously from early embryonic stages to adulthood, its
downregulation by RNAi to levels that affect the detection of lysosome-related
organelles by in vivo Nile red staining do not harm embryonic development. This
suggests that the sensitivity of lysosome-related organelles to ALKB-8 levels is greater
than a possible involvement in developmental events. Keeping with the metabolic roles
of ALKB-8, its overexpression applied during the first larval stage markedly prolonged
life span. On the other hand, downregulation of alkb-8 by RNAi does not shorten their
life span. There are several factors that may cause this discrepancy. Firstly, RNAi is not
significantly affecting neuronal cells in wild type N2 C. elegans unless specific lines are
used for silencing experiments (Simmer et al. 2002) and thus a proportion of ALKB-8
responsible for the observed phenotypes may be unaffected in alkb-8 downregulation
experiments. The experiments with alkb-8 forced overexpression are likely to lead to
elevated levels of ALKB-8 in most cells, except in the gonads. It can be assumed that
the effects on the extent of detection of the in vivo Nile red positive compartment is at
least partially a result of ALKB-8 direct function in enterocytes. The effect on longevity
may be to a large extent based on neuronal functions of ALKB-8. In agreement with
this, in rrf-3 mutant animals, in which RNAi affects also neuronal cells, neuronal
inhibition of the autophagy nucleation complex extends life span of C. elegans. The
authors demonstrated that inhibition of the VPS-34/BEC-1/EPG-8 autophagic
nucleation complex as well as its upstream regulators strongly extend C. elegans life
span and that post-reproductive inhibition of bec-1 mediates longevity specifically
through the neurons (Wilhelm et al. 2017).

The positive effect of ALKB-8 on life span may be connected with the short-term
heat-shock that was applied to both control and experimental animals in order to induce
forced expression of the transgene. Nevertheless, the applied heat-shock lasted only 30
minutes in the L1 larval stage and the life span of control animals subjected to the short-term heat-shock did not differ from the normal life span of animals kept under similar laboratory conditions but not subjected to the experimental heat-shock. Involvement of ALKB-8 in other kinds of stress is supported by the known role of AlkB proteins in the stress response. The founding member of the protein family, the bacterial AlkB is involved in the DNA damage-induced stress (Fedeles et al. 2015) (Fedeles, Singh et al. 2015). ALKBH8 is known to regulate the rate of translation of thioredoxin reductase (Endres et al. 2015) which is one of the main enzymes important for dealing with oxidative stress (Li et al. 2012; Cunniff et al. 2014).

Our results as well as published data (Pastore et al. 2012) indicate the cytoplasm as the primary place of ALKB-8 action although a low level of nuclear ALKB-8 cannot be ruled out. \textit{alkb}-8 is organized in chromosome III in a hybrid operon CEOP3136. As such, it is trans-spliced with both SL1 and SL2 splice leaders indicating that part of the expressed forms of \textit{alkb}-8 depend on the operon promoter and the other part on the internal \textit{alkb}-8 promoter. The expressional pattern of the transgene expressed under the regulation of the operon promoter (used in our study) is very similar if not identical with the data reported for the internal \textit{alkb}-8 promoter (Pastore et al. 2012). Our experiments as well as the data reported by Wormbase (WS263) (Byrne et al. 2007) detected \textit{alkb}-8 expression in intestinal cells. It is therefore likely that the effect of \textit{alkb}-8 inhibition and overexpression is at least partially caused by intestinal ALKB-8.

ALKB-8 (from amino acid position 362 to the end) shows significant homology to a yeast methyl transferase TRM9 (TRM9\_YEAST) not only in the SAM binding part but also at the C-terminus. Deletion of TRM9 significantly increased life span in Saccharomyces cerevisiae (Fabrizio et al. 2010) suggesting that ALKB-8 may act in the
same pathway as the *C. elegans* orthologue of TRM9 (although in opposite ways). TRM9 is predicted to be important to protect cells against protein stress (Patil et al. 2012). In *C. elegans* (and in most sequenced animal species), there is another gene that is similar to AlkB8, that only has the methyl transferase domain, not the demethylase domain C35D10.12 (NP_497751.1) but nothing is known about its function.

Our study shows that ALKB-8 regulates the function of the intracellular compartment that can be visualized by *in vivo* Nile red staining (Ashrafî et al. 2003) that forms a distinct class of lysosome related organelles (Soukas et al. 2013).

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GEI-8, a Homologue of Vertebrate Nuclear Receptor Corepressor NCoR/SMRT, Regulates Gonad Development and Neuronal Functions in Caenorhabditis elegans

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Abstract
NCoR and SMRT are two paralogous vertebrate proteins that function as corepressors with unliganded nuclear receptors. Although C. elegans has a large number of nuclear receptors, orthologues of the corepressors NCoR and SMRT have not unambiguously been identified in Drosophila or C. elegans. Here, we identify GEI-8 as the closest homologue of NCoR and SMRT in C. elegans and demonstrate that GEI-8 is expressed as at least two isoforms throughout development in multiple tissues, including neurons, muscle and intestinal cells. We demonstrate that a homozygous deletion within the gei-8 coding region, which is predicted to encode a truncated protein lacking the predicted NR domain, results in severe mutant phenotypes with developmental defects, slow movement and growth, arrested gonadogenesis and defects in cholinergic neurotransmission. Whole genome expression analysis by microarrays identified sets of de-regulated genes consistent with both the observed mutant phenotypes and a role of GEI-8 in regulating transcription. Interestingly, the upregulated transcripts included a predicted mitochondrial sulfide:quinine reductase encoded by Y9C9A.16. This locus also contains non-coding, 21-U RNAs of the piRNA class. Inhibition of the expression of the region coding for 21-U RNAs leads to irregular gonadogenesis in the homozygous gei-8 mutants, but not in an otherwise wild-type background, suggesting that GEI-8 may function in concert with the 21-U RNAs to regulate gonadogenesis. Our results confirm that GEI-8 is the orthologue of the vertebrate NCoR/SMRT corepressors and demonstrate important roles for this putative transcriptional corepressor in developmental and neuronal function.


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Introduction
NCoR and SMRT are paralogous vertebrate proteins that were first identified as transcriptional corepressors interacting with unliganded thyroid and retinoid receptors [1,2]. Both NCoR (a.k.a. NCoR1, NCoR1) and SMRT (a.k.a. NCoR2, NCoR2) knockouts in mice are embryonic lethal suggesting that their regulatory roles are indispensable for normal development [3]. NCoR/SMRT function occurs through the assembly of a repressor complex composed of nuclear hormone receptors (NHRs), histone deacetylases (HDACs), and other components [4]. Chromatin remodeling depends on the formation of a stoichiometric complex between SMRT/NCoR and HDAC3 that is mediated by two SANT (a.k.a. MYB) domains located at the N-terminus of NCoR/SMRT. Such domains are present in many nuclear receptor corepressors and related proteins and consist of three alpha-helices folded around a core of three hydrophobic amino acids, which determines its characteristic spatial structure [5–7]. The N-terminus proximal SANT1 domain activates the HDAC3 deacetylase [8,9] and is referred to as the deacetylase activation domain (DAD). A prominent feature of all DAD domains is the absolutely conserved lysine residue (K449 in human SMRT) that blocks its subsequent acetylation [7,8]. While the SANT2 domain in NCoR/SMRT possesses all of the typical features of a general SANT domain, the presence and structure of the SANT1 domain is unique to NCoR/SMRT and its orthologues [10]. The SANT1 domain contains a characteristic irregular N-
terminal helix that is important for forming an additional surface hydrophobic groove that contributes to the interaction with HDAC3. Thus, there are multiple diagnostic domains and amino acid residues that can distinguish NCoR/SMRT orthologues from more general SANT domain-containing proteins.

Although homologues of NCoR/SMRT can be easily identified across vertebrate species, obvious homologues of these corepressors were difficult to identify by sequence homology in either *Drosophila* or *C. elegans*. This is surprising in light of the identification of clear sequence homologues for other NCoR/SMRT corepressor complex components in flies and worms such as the histone deacetylase complex associated factors NuRD and SIN3 [11,12]. We have taken a bioinformatics approach focusing on the unique features of NCoR/SMRT to identify GEI-8 as a possible NCoR/SMRT homologue in *C. elegans*; Yamamoto and colleagues came to the same conclusion while this work was in progress [13]. GEI-8 was originally identified as a GEX-3 binding protein based on yeast-two-hybrid assays [14]; no RNAi phenotypes or functions for GEI-8 have previously been described. We have analyzed the expression of *gei-8* and studied its function using a putative null allele with a large deletion in the *gei-8* coding sequence resulting in a truncated protein product due to a novel stop codon; this truncated product lacks the domain involved in binding of nuclear receptors (NR domain, a.k.a CoRNR box [15]). Our mutant studies demonstrate a role for GEI-8 in development and suggest it is specifically required for germinal development and proper cholinergic regulation. Our whole genome expression analysis demonstrates that GEI-8 is required for transcriptional regulation, consistent with its function and orthology to mammalian NCoR/SMRT corepressors.

**Results**

**Sequence Analysis**

In an effort to identify homologues of NCoR/SMRT in the *C. elegans* proteome, we performed BLAST and PSI-BLAST searches in multiple protein databases [16,17]. Searches with human NCoR and SMRT sequences returned the sequence annotated as GEI-8 (UniProt: GEI8_CAEEL, E value 2e-10), as the best hit. In the reciprocal BLAST, NCoR and SMRT appeared likewise as the best hits for GEI-8 within the human proteome. Although only a small fraction of the entire protein sequence (~7%) was retrieved by Blast searches, nearly complete protein sequences were recovered in PSI-BLAST after the third iteration. Six GEI-8-related proteins from other Nematoda species (*C. elegans*, *C. brenneri*, *C. briggsae*, *C. remanei*, *C. japonica*, *Loa loa* and *Brugia malayi*) were aligned and submitted as a query in PSI-BLAST (Figure 1). Sequences were extracted from databases UniProt, Wormbase and Ensembl. Entries for *C. japonica* and *X. tropicalis* were corrected according to NCBI nucleotide sequences using the GeneWise program [18]. An alignment of these nematode GEI-8-related proteins with human NCoR was obtained in the second iteration.

Multiple sequence alignments resulting from PSI-BLAST were further improved using the profile-to-profile alignment method (PSI-Coffee) [19], however, its quality remained ambiguous in several regions across the protein. All NCoR homologues contain long stretches of low complexity (e.g. 25% of amino acids in GEI-8 or 13% in human NCoR1) that are variable in length. The well conserved N-terminal region from representative *Metazoa/Fungi* NCoR/SMRT is shown in Figure 1. The sequence conservation in the C-terminal domains is much lower; all sequences contain many insertions, deletions, prolines, serines and oligoGlu residues that vary between species. This C-terminal variability is evident even within the alignment of the GEI-8-related proteins from the phylogenetically related *Caenorhabditis* species. We also used ClustalW2.0 for identification of putative interaction motifs near the C-terminus. NCoR and SMRT bind nuclear hormone receptors by NR-binding domains consisting of three and two CoRNR-box sequences respectively. The CoRNR-box sequence was previously defined as L.x.x.x.I.x.x.x.I/L [20]; I/L.x.x.x.I/L [21]; L/V.x.x.I/V.I [22]. We identified two putative CoRNR-box like sequences in GEI-8 (Figure 2A). The predicted GEI-8 sequence also contains two glutamine rich regions [23] that also might serve as interaction domains.

The most conserved N-terminal regions of the GEI-8-related sequences contain both the DAD and SANT domains with their location and the positions of the conserved helices shown in Figure 1. We noted that GEI-8 and related sequences preserve all features known to be essential for correct functioning of NCoR/SMRT as an HDAC-dependent transcriptional corepressor [10] (highlighted in Figure 1). These include the number of helices, their topology, the conserved amino acids needed for the integrity of the structure and for the interaction with HDAC and, most importantly, the K159 residue in the loop between helices H1 and H2 that is indispensable for the activation of HDAC3. The helix H0, known to be very irregular in human SMRT, is probably also present although it contains a two amino acid insertion between the second and third helical turn. Based on the sequence analysis, we concluded that GEI-8 bears all major features identified in other NCoR/SMRT orthologues in annotated genomes from other species and is the NR corepressor and NCoR/SMRT orthologue in *C. elegans*.

**The C-terminal Region of GEI-8 is Capable of Binding GST-NHR-60**

In order to confirm functional relatedness of GEI-8 with NCoR/SMRT, we performed a binding assay of the GEI-8 C-terminal domains to GST-NHR-60. NHR-60 is a member of a diversified subfamily of nematode receptors related to HNF-4 alpha and is important for embryonic and early larval development [24]. Mammalian HNF-4 alpha interacts both physically and functionally with SMRT [25] raising the possibility that NHR-60 may similarly interact with GEI-8. We divided the C-terminal region of *gei-8* into three domains: I. containing the NR1 binding site (position 2480–3485 in *gei-8a* isoform), II. containing the sequence between NR bindings sites (position 3413–4389) and III. containing the NR2 binding site (position 4274–5513). As expected from our sequence homology analysis of GEI-8 as it relates to NCoR/SMRT, the C-terminal region I of GEI-8 that includes the predicted NR1 binding site showed affinity to GST-NHR-60 but not to the control protein expressing the GST anchor used for pull-down experiments (Figure S1).

**gei-8 Expression**

The *C. elegans* *gei-8* gene is located on chromosome III and gives rise to three predicted isoforms with mRNAs ranging from 5.3 to 5.6 kb (WormBase WS195). All predicted isoforms contain two SANT domains that could provide DNA and HDAC interaction functions (Figure 2A and B). Using primers based on predicted cDNA sequences of *gei-8* isoforms, we cloned three overlapping regions corresponding to *gei-8* cDNAs and confirmed the expression of predicted isoform *gei-8a* containing both SANT domains and two putative CoRNR-box like motifs (Figure 2A). The *gei-8a* cDNA clones also revealed that exon 12 can be removed and exon 16 is modified by alternative splicing (Figure 2A); a spliced region of the same location and size as our cDNA clone was also detected by polyA mRNA expression profiling [26]. Depending on the presence or absence of exon 12,
the size of gei-8 cDNA is 5043 bp (gei-8d) and 5292 bp (gei-8e), giving rise to either a 1680 or 1763 amino acid long GEI-8 isoforms. We have not cloned the region containing the complete predicted protein encoded by inclusion of exon 16, however, polyA mRNA expression profiling data suggest that this variant is expressed. We confirmed the transcription of the gei-8a 5’ untranslated region (5’ UTR) and its splicing to SL1 by PCR assays [27]. Expression of gei-8b and gei-8c was not detected using primers directed at predicted exons 1 to 3; our results are consistent with polyA mRNA expression profiling data generated by modENCODE (Figure 2B) [26].

We quantified gei-8 expression in individual embryonic and larval stages by real-time qPCR using cDNA prepared from synchronized populations of wild-type animals. We separately analyzed a region common for all predicted isoforms (gei-8a, b, c) as well as a gei-8a-specific region. We detected expression after probing both regions in all developmental stages at constant relative levels with the exception of the fourth larval (L4) stage where we observed a 2-fold increase for both (Figure 3). We concluded that gei-8a was expressed throughout development, with its late larval increase possibly reflecting expression in the maturing germline.

The spatial expression pattern of gei-8 was studied using three different gei-8::gfp constructs based on the predicted start of transcription for gei-8b (promoter 1), the detected start of transcription for gei-8a (promoter 2), and an overlapping region covering both promoters (promoter 3) (Figure 2C). pPD95.69 and pPD95.67 promoterless, nuclear localization signal-containing vectors were used for the promoter 1 and promoter 2 constructs,
respectively. Expression from promoter 3 was studied by the PCR fusion-based SOEing approach [28].

The promoter 1 reporter gene consisted of 1.8 kb upstream of the predicted gei-8b start codon and 222 bps of predicted exon 1. Its expression started in embryos at the comma stage in a ubiquitous pattern and was present in all larval stages. In larvae, the expression was detected in pharyngeal and tail neurons, intestinal cells, egg-laying muscles and the anal depressor (Figure 4). The promoter 2 reporter gene construct consisted of 2.3 kb upstream of the predicted gei-8a start codon and included exon 1 and 64 bp of exon 2. The expression of this reporter gene was observed in all larval stages starting at the L1 stage and continuing through adulthood where expression was primarily observed in neurons of the pharyngeal nerve ring, head neurons, tail neurons and the egg-laying muscles. The promoter 3 reporter gene construct contained 6.2 kb upstream of the predicted gei-8a start codon, covering both promoter regions 1, 2 and exons 1, 2 and a part of exon 3; GFP sequences were derived from pPD95.75 by SOEing [28] and did not contain a nuclear localization signal. Expression of this reporter gene started at the embryonic comma stage. Larval expression was detected in pharyngeal neurons, ventral and dorsal nerve cords, tail neurons, egg-laying neurons, and egg-laying muscles. In males, GFP was observed in male-specific tail ganglia and rays. Typical examples of GEI-8::GFP cell- and tissue-specific expression are shown in Figure 4. Taken altogether, our reporter gene expression results defined multiple

Figure 2. Expression analysis of gei-8. (A) Schematic representation of the predicted gei-8a isoform consisting of 17 exons compared with detected expression. cDNA clones 7320, 7323 and 7324 are indicated with their exons (open rectangles). Expression of exon 12 and 16 is not constant. Exon 12 in cDNA clone 7323 and 45 bp from exon 16 in cDNAs 7323 and 7324 were removed by alternative splicing (bottom two lines). The location of predicted SANT and glutamine-rich interaction domains is marked by lines above the gei-8a diagram. Location of gei-8(ok1671) mutation used in expression analysis is marked by a line below the gei-8a diagram. Two regions identified as putative CoRNR nuclear receptor binding motifs are indicated in the exon 8 and 15 (NR). (B) Schematic representation of predicted gei-8 isoforms a, b and c. (C) GFP reporter gene constructs #1, #2 and #3 used for expression analysis. (D) Overlapping regions of gei-8 gDNA used for rescue. The size of the overlapping region is 191 bp.

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and distinct cis-acting regulatory regions of gei-8 that drive similar expression patterns that are present throughout development and predominantly in neurons. Expression in the germline would not be revealed by this strategy because transgenes are usually silenced in the germline [29]. However, we noted that gei-8 expression in the germline has been detected by Y. Kohara’s in situ hybridization results accessible in the Kohara in situ database NEXTDB (http://nematode.lab.nig.ac.jp).

Loss of gei-8 Results in Mutant Phenotypes

We obtained the VC1213 strain harboring a gei-8(ok1671) deletion allele generated by the C. elegans Knockout Consortium. The mutation was initially characterized as a 1095 bp deletion/45 bp insertion affecting exons 7 and 8 of gei-8a, removing the intron between them. We verified the size and location of the deletion by PCR genomic amplification from mutant animals and showed that the inserted sequences are identical to a 45 bp region from exon 7 starting at position 1550 of the predicted gei-8a isoform cDNA sequence. Sequencing the gei-8(ok1671) cDNA revealed a stop codon present in the gei-8(ok1671) transcript at position 663, giving rise to a predicted protein containing SANT1 and SANT2 domains, but missing the majority of the putative NR interaction sites at the C-terminus of the protein. The mutant mRNA was detected in homozygous gei-8(ok1671) animals using RT-PCR at levels similar to wild-type animals, suggesting the premature stop codon is not efficiently recognized by nonsense mediated decay [30]. Thus, truncated GEI-8 protein may be present in homozygous mutant larvae.

The homozygous gei-8(ok1671) animals had obvious phenotypes, including a progressive defect in locomotion starting at the L2 stage that was marked by a delayed response to prodding and a low pharyngeal pumping rate (Figure 5). Compared to wild-type animals of the same age, mutants were also characterized by a shorter maximum body length (750.25 μm, n = 6, SD = 50.59 μm), a convoluted intestine, gonadogenesis defects including loss of the spermathecae, sterility, and arrest at the L4 stage of development (Figure 6 C and D). After outcrossing the original mutant strain to wild-type animals, the heterozygous mutant strain segregated 26.2% (SD = 2.4; n = 2656) affected progeny as described (Table 1). To verify that the observed phenotypes were caused by the ok1671 deletion allele of gei-8, we performed rescue using intact gei-8 genomic DNA. This method has been used previously to generate transgenic animals and to rescue mutant animals [31–34]. Overlapping PCR regions containing a 6 kb putative promoter region plus the complete coding region of gei-8a (Figure 2D) were injected into heterozygous gei-8(ok1671) animals along with pRF4 injection marker, rollers were selected and their progeny were screened for locomotion defects as defined as impaired responses to prodding.

The wild-type gei-8 genomic sequences were able to reduce the percentage of affected mutant progeny segregating from heterozygous hermaphrodites from 26.2% to 18.3% (SD = 3.4; n = 7883); this difference was significant using the Student’s t-test (p<0.001; SD = 3.16) (Table 1). Importantly, all other mutant phenotypes also showed improvement in the presence of wild-type genomic sequences leading us to conclude that most, if not all, of the defects we observed in gei-8(ok1671) animals were due to disruption of GEI-8 activity.

We scored 20 gei-8(ok1671) mutant animals for germline development defects using Nomarski optics and DAPI (4’,6-diamidino-2-phenylindole) staining of fixed animals. In 19/20 mutant animals examined, distal tip cell (DTC) migration stopped short, reaching only two thirds of its normal length of migration on the dorsal side of the animal (Figure 6C and D). In homozygous mutant animals, both gonad arms were underdeveloped, containing fewer meiotic nuclei and germ cells compared to wild-type and heterozygous gei-8(ok1671) control animals. We also failed to detect spermathecae, sperm, or embryos in any mutant animals. We concluded that gei-8(ok1671) mutant germlines are arrested at the L4 stage, before complete gonad elongation and spermatheca development, although some somatic markers of early young adult stages were already present (adult alae, adult vulva). The arrested animals also had a shorter lifespan than wild-type controls. The average lifespan of gei-8(ok1671) mutants at 20°C was 11 days (n = 21, SD = 3.4), which was significantly shorter than the average lifespan of wild type controls (17.4 days, n = 12, SD = 3.9).

Two muscle-related phenotypes were observed in homozygous gei-8(ok1671) mutants; decreased locomotion on plates and decreased pharyngeal pumping rates. The locomotion defects we observed for gei-8(ok1671) animals on plates prompted us to carry out a thrashing assay. When placed in liquid, wild-type animals bend back and forth moving their head and tail relative to the midbody of the animal in a thrashing motion that can be easily quantitated [35]. In the gei-8(ok1671) mutant strain, this natural thrashing behavior is impaired and deteriorated over the course of development. Unlike wild-type controls, homozygous gei-8(ok1671) mutants at the L4 stage were not able to perform smooth thrashing. Instead, their movements were spastic and irregular, averaging only 0 to 6 bends per minute at the L4 stage compared to about 250 bends per minute for wild-type animals (n = 10). Similarly, assays of pharyngeal pumping revealed irregular and reduced contraction rates in the homozygous mutants that became progressively worse with age. The average pumping rate in gei-8(ok1671) homozygous animals was 31.8, 17.5 and 3.3 pumps per minute at L2, L3 and L4 stages, respectively (n = 10 for each larval stage), compared to 250 pumps per minute for wild-type animals.

The movement and pharyngeal mutant phenotypes could be due to defects in the functioning of muscle, nerves, or both. To investigate muscle structure, we performed immunostaining using phalloidin and anti-MY03 antibody directed against contractile apparatus components. Phalloidin stains actin filaments whereas the anti-MY03 probe recognizes myosin heavy chain-3 [36,37].
In immunostaining revealed no obvious structural differences between *gei-8(ok1671)* mutants and wild-type controls (not shown). Yamamoto et al. reported increased mitochondrial oxidative function in *C. elegans* after *gei-8* inhibition by RNAi [15]. We confirmed that finding using MitoTracker Red to visualize the mitochondrial oxidative state; homozygous *gei-8(ok1671)* mutants had an average mean density of staining that was more than 2.7 times greater (p < 0.001) than that observed in wild-type larvae (Figure S2). Elevated MitoTracker staining could also be visualized in heterozygous *gei-8(ok1671)* mutants compared to wild-type N2 worms, but was not statistically significant in densitometric analysis of randomly selected progeny of heterozygous *gei-8(ok1671)* animals with a normal phenotype (which included both heterozygous mutants as well as wild-type animals (Figure S3).

The absence of obvious muscle defects in *gei-8* mutants suggested that the locomotion and pharyngeal pumping defects might be due to problems in neurotransmission. We investigated synaptic transmission by assaying animal sensitivity to either aldicarb or levamisole [38,39]. Aldicarb is a reversible acetylcholinesterase inhibitor that increases the accumulation of acetylcholine in the synaptic cleft causing whole body paralysis and inhibition of pharyngeal pumping. Homozygous *gei-8(ok1671)* mutants (n = 64) and wild-type animals (n = 75) at the L4 stage were incubated on NGM plates with 1 mM aldicarb and scored over time for paralysis in three separate experiments. The onset of paralysis occurred significantly earlier in *gei-8(ok1671)* mutants than in wild-type controls (Figure 7A). Levamisole is a cholinergic agonist that also results in animal paralysis. We performed two experiments with homozygous *gei-8(ok1671)* mutants (n = 40) and wild-type animals (n = 40) at the L4 stage on NGM plates with levamisole at a concentration of 1 mM. As in the aldicarb assay, the onset of paralysis occurred significantly earlier in *gei-8(ok1671)* mutants compared to controls (Figure 7B). Taken together, these results indicate that the *gei-8(ok1671)* mutation results in abnormal cholinergic signaling, however, it does not distinguish between post-synaptic versus pre-synaptic transmission defects.

### gei-8 Loss of Function Leads to Transcriptional Deregulation

Effects of the *gei-8(ok1671)* mutation on gene expression were studied with whole genome microarrays (Affymetrix). Changes in gene expression were defined as increased or decreased if statistically significant compared to wild-type controls in at least 2 out of 3 biological replicates. Deregulated genes were analyzed for Gene Ontology (GO) term enrichment and clustered according to functional classification using DAVID 6.7 [40] and KEGG pathway tools [41].

Expression microarray analysis revealed 756 probe sets with decreased expression, corresponding with 690 unique Wormbase IDs (Table S1). DAVID classification tools [40] identified 645 IDs using medium classification stringency. GO analysis resulted in 32 clusters with an enrichment score greater than 2 and P < 0.05. The list was enriched in spliceosome (29 genes), proteasome (13 genes), cysteine and methionine metabolism (7 genes), and RNA polymerase genes (6 genes) as identified by KEGG pathway analysis. Among specific genes involved are RNA polymerase II and III (Pol II subunits B4, B7, B9 and Pol III subunits AC2 and F097.3), spliceosome components (U1 to U6 snRNAs, hel-1 helicase and others), and proteasome subunits (psa-3, pas-4, pbs-1, pbs-3, pbs-4, pbs-6, pbs-7, ptf-1, ptf-2, ptm-2, ptm-5, ptm-8, ptm-12). The most common functional categories over represented by the changes in gene expression were growth, embryonic or larval development and development of reproductive structures. Other clusters include multiple histones and histone-like genes, mitochondrial membrane proteins, sperm structural proteins and hedgehog-like family genes. Interestingly, the set of genes down-regulated in *gei-8* mutants included several genes required for proper muscle function, including *unc-52* (myofilament assembly and/or attachment of the myofilament lattice to the cell membrane), *unc-27* (tropomycin I family), *unc-54* (myosin class II heavy chain), *pat-10* (body wall muscle troponin C), *lev-11* (tropomyosin), *mlc-2* (myosin light-chain), and *tm-1* (tropomin 1). It is unclear if such changes in muscle gene expression contribute to, or are the result of, the defective movement phenotypes we observed in *gei-8(ok1671)* mutant animals. Depletion of NCoR1 function specifically in mouse muscle resulted in increased muscle mass and mitochondrial function [13], a phenotype opposite to what we observed in worms with reduced *GEI-8* activity in all tissues.

Microarray analysis revealed 296 probe sets with increased expression, corresponding to 275 unique Wormbase IDs (Table S2). GO analysis identified 7 clusters with an enrichment score greater than 2 and P < 0.05. Enriched clusters included gene
Figure 6. Development of the germline in gei-8(ok1671) mutants and additional phenotypic changes induced by RNAi targeted against Y9C9A.16 (sqrd-2) in homozygous gei-8(ok1671) mutants. (A) The reproductive structures of a wild-type larva at the L4 stage is shown. The vulva is indicated by an arrowhead and formation of the uterus is visible next to vulval structures. The position of the lead migrating cell for the gonad (distal tip cell) during the larval L4 stage is indicated by arrow. (B) Development of the gonad in a young adult N2 animal. The distal gonad arm continues in growth beyond the position of the vulva (marked by arrowhead) and makes contact with the proximal gonad arm (arrow). (C) gei-8(ok1671) mutant gonadogenesis by Nomarski optics. The arrested gonad arm in a position similar to wild type L4 larva is indicated by arrow. The vulva is marked by an arrowhead. (D) A gei-8(ok1671) mutant with arrested growth of the gonad as visualized by DAPI staining. The distal tip of arrested gonad is marked by an arrow and the vulva by an arrowhead. (E, F, G, H, I and J) Additional phenotypic changes induced by RNAi targeted against Y9C9A.16 (sqrd-2) region including three 21U-RNAs: 21ur-2020, 21ur-11733 and 21ur-9201 in gei-8(ok1671) homozygous mutant animals. (E) A gei-8(ok1671) mutant treated with sqrd-2 RNAi shows growth of the gonad beyond the usual arrest point, reaching the position of the vulva (marked by arrow and arrowhead, respectively). (F) Additional phenotypes of gei-8(ok1671) animals treated with sqrd-2 RNAi. Nomarski optics view of homozygous gei-8(ok1671) larva treated with sqrd-2 RNAi revealing frequent growth defects, including irregular body shapes, (distention of proximal part of the body and thin elongation of the distal part of the body) and extended growth of the distal part of the gonad. The gonad is visualized by DAPI staining in panel G (distal arm of the gonad is marked by right arrow, proximal arm of the gonad is marked by left arrow). Arrowhead indicates the position of vulva in panels E, F and G. (H) Additional growth defects induced by sqrd-2 RNAi in homozygous gei-8(ok1671) worms including a Pvul phenotype (arrowhead), accumulation of gonadal cells with a possible incomplete second vulva formation (left arrow) and a distal arm of germline that fails to turn and instead continues to grow in the direction of the thin and elongated tail (right arrow). (I) A mutant animal with germline growth directional changes of both gonad arms induced by sqrd-2 RNAi: anterior gonad arm makes an incomplete turn dorsally and continues to grow in the anterior direction (left arrow) while the posterior gonad arm fails to turn and continues in additional growth towards the tail (right arrow). The position of vulva is indicated by arrowhead. (J) A homozygous gei-8(ok1671) mutant developing a convoluted irregular accumulation of cells of distal gonad arm in the position of gonad turn (marked by arrows). The position of vulva is indicated by arrowhead. Scale A, B, D and F 50 μm, C, E, G, H and J 100 μm. doi:10.1371/journal.pone.0058462.g006
annotations for life span and aging, lipid transport and vitellogenin genes, stress response (heat shock and cellular stress), metabolic genes (sugar metabolism, glycolysis), and neuropeptide signaling (including genes coding for neuropeptide like proteins nlp-27 to nlp-32). The KEGG pathway analysis identified six groups including genes involved in glycolysis (8 genes), cystein methionine metabolism (4 genes), galactose metabolism (3 genes), pentose phosphate pathway (3 genes), fructose and mannose (3 genes) and tryptophan metabolism (3 genes).

One of the most significantly affected genes in the gei-8(ok1671) homozygous mutants was Y9C9A.16, encoding a predicted mitochondrial sulfide:quinone oxidoreductase, which had an averaged 7.6-fold increase in expression compared to wild-type controls; this increase was confirmed by RT-qPCR. The Y9C9A.16 region is assayed by Affimetrix probe set 184710_at and, interestingly, includes three 21U-RNAs; 21ur-2020, 21ur-11733 and 21ur-9201. To determine if disruption of expression of Y9C9A.16 affected development, we performed RNAi targeted to the spliced mRNA covered by the Affymetrix probe set (184710_at) or only the regions that include 21ur-2020, 21ur-11733 and 21ur-9201. Progeny of parental animals injected with dsRNA targeting the specific regions were scored using Nomarski optics and fluorescent microscopy (DAPI stained). We were not able to identify any specific phenotype of Y9C9A.16 knockdown in wild type animals. However, because the expression from Y9C9A.16 showed a dramatic response to loss of GEI-8 activity, we thought there might be a biological connection between them. We predicted that knockdown of the expression from Y9C9A.16 locus in gei-8 (ok1671) homozygous mutants might revert or modify some of the observed phenotypes; the latter was observed. RNAi-mediated knockdowns targeted to the region covered by the 184710_at probe set and the region containing 21ur-2020, 21ur-11733 and 21ur-9201 induced additional phenotypes in the gei-8(ok1671) homozygous mutant background. Additional phenotypes included severe distal tip cell migration defects, irregular gonadal nuclei tumor like accumulation of germline cells and vulval protrusions observed in 13.9% of homozygous gei-8(ok1671) animals treated with Y9C9A.16 RNAi (n = 481) (Figure 6E, F, G, H, I and J and Table 2). Interestingly, Y9C9A.16 has a paralogue in the C. elegans genome, the gene sqrd-1 (sulfide:quinone oxidoreductase). This gene encodes a protein that is identical in size (361 aa) to Y9C9A.16 sharing 266 identical amino acids in its sequence although the genes share very little DNA homology. SQRD-1 expression is regulated by hif-1 in response to H2S and HCN [42], is involved in innate immunity and is associated with numerous 21U-RNAs. RNAi targeted to unique regions of the sqrd-1 coding region, including four 21U-RNAs, resulted in changes in gonad arm migrations and an accumulation of germline cells (4.5% affected, n = 198) that were similar, although less severe, as those observed after Y9C9A.16 RNAi. We concluded that the paralogues encoded by Y9C9A16 and sqrd-1, and perhaps their associated 21U-RNAs, have overlapping roles during development of the germline that can be exacerbated by loss of GEI-8 activity.

**Discussion**

**GEI-8 is a NCoR/SMRT Orthologue with Developmental Roles in C. elegans**

Our results demonstrate that GEI-8 is the C. elegans orthologue of the vertebrate NCoR/SMRT corepressors and that it has essential developmental and transcriptional functions throughout development. GEI-8 has the critical structural motifs necessary for corepressor functions, including the domains for HDAC interaction and activation. Moreover, it is able to interact physically

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**Table 1. Rescue experiment of gei-8(ok1671) with overlapping amplified regions of genomic DNA injected into the gonads of parents.**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Number of scored progeny</th>
<th>Affected larvae</th>
<th>%</th>
</tr>
</thead>
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<tr>
<td>Non-injected ok1671</td>
<td>2656</td>
<td>696</td>
<td>26.2</td>
</tr>
<tr>
<td>gei-8 rescue after injections</td>
<td>7883</td>
<td>1443</td>
<td>18.3</td>
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</table>

DOI:10.1371/journal.pone.0058462.t001

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**Figure 7. Analysis of neuromuscular function of gei-8(ok1671) mutant (VC1213).** Aldicarb and levamisole sensitivity assays revealed increased sensitivity of gei-8 mutants towards the acetylcholinesterase inhibitor aldicarb (A) and levamisole (B) suggesting a synaptic defect in cholinergic transmission.

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responsible for this reduced pharyngeal rate given that it is a semi-

consistent with the late developmental defects seen when other

absence of GEI-8 activity, development and/or cellular processes

We further show that GEI-8 is required for normal development in C. elegans based on our studies of a gei-8 deletion allele that severely truncates or inhibits the protein product. Although expressed, at least at the mRNA level, this mutant allele is predicted to lack the GEI-8 nuclear receptor interacting sites while expressing an mRNA that codes for the domains necessary for HDAC binding and activation. There is no evidence for dominant negative activity of this truncated product as heterozygous animals appear completely wild-type and the introduction of wild-type gei-8 coding regions in transgenic animals partially rescue multiple mutant phenotypes. Therefore, the mutant phenotypes likely represent the loss of function effects for gei-8. Given the early and widespread onset of gei-8 reporter gene expression in embryos, which is also detected by RT-qPCR, it is very likely that GEI-8 functions throughout development and in most, if not all, tissues. The lack of embryonic or early larval defects in homozygous mutants likely reflects the maternal load of gei-8 gene products in the embryo. It is also possible that GEI-8 has multiple functions requiring different amount of GEI-8 activity, with demands for higher levels post-embryonically, including germline development.

The most significant phenotype observed in gei-8 mutants is the late-L4 larval arrest, as revealed by the extent of gonadogenesis and DTC migration. One possibility is that this arrest reflects the depletion of maternally loaded gei-8 products and that in the absence of GEI-8 activity, development and/or cellular processes fail to be executed properly. This interpretation would be consistent with the late developmental defects seen when other essential, maternally provided gene products are exhausted, such as the G1 cell cycle regulators [46–48]. The second most pronounced phenotype in the gei-8(ok1671) homozygous mutants is reduced pharyngeal pumping. It is unclear what defect(s) is responsible for this reduced pharyngeal rate given that it is a semi-

autonomous action of the pharyngeal muscles that can be stimulated, but does not require neuronal input [49,50]. One possibility was that food sensation mechanisms were compromised in the gei-8(ok1671) mutants; in the absence of food, the pumping rate of wild-type worms is similar to the rate we observed in the homozygous mutants. However, when tested we found that gei-8(ok1671) mutants exhibited spontaneous chemotaxis towards OP50 lawns until the mutants terminally arrested late in development demonstrating that food sensation mechanisms were intact. Another explanation of reduced pharyngeal pumping is diminished activity of the MC pharyngeal cholinergic neuron and/or its receptor, EAT-2 that regulate pharyngeal pumping in response to food [51,52]. Such reduced cholinergic signaling is consistent with the sensitivity we observed for gei-8(ok1671) mutants to levamisole and aldicarb. Further experiments are needed to determine exactly which pathways are perturbed and the molecular basis for these aberrations.

GEI-8 Regulates Transcription

Whole genome transcriptional analysis indicates that GEI-8 is required for proper gene expression. Its loss-of-function allele resulted in altered expression of a wide range of genes; genes with elevated expression as well as with decreased expression were identified. However, while GO annotation of many genes that showed decreased expression correlated with the observed phenotypes, genes with increased expression (that could be potentially de-repressed) failed to show an obvious correlation. It is interesting to note that among the set of genes that were decreased in mutant worms were several muscle specific genes. Thus, while bodywall muscle was normal by gross observations with normal appearance, there may be defects in this tissue in homozygous mutants.

The set of deregulated (increased) genes included neuropeptide-like protein genes (nlp-27 to nlp-32). The neuropeptides that are generated from these genes fall in the subfamily characterized by the sequence YGGW [53] and are related to Aplysia APGW neuropeptides that regulate male reproductive functions [54]; the functional consequences of this, if any, are currently unknown. However, in agreement with results reported by Yamamoto et al. for mice [13], we have detected numerous metabolic genes in the set of genes with increased expression in homozygous mutant worms.

The set of increased genes included several clusters of metabolic genes involved in lipid transport, sugar metabolism, glycolysis, and amino acid metabolism. Several nuclear receptors may be involved in this metabolic regulation. The majority of C. elegans

<table>
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<tr>
<th>Target gene</th>
<th>Screening method</th>
<th>Hermaphrodites Injected</th>
<th>Homozygous larvae scored</th>
<th>Larvae with additional gonad and body shape defects</th>
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<tbody>
<tr>
<td>sqrd-2</td>
<td>Nomarski optics</td>
<td>24</td>
<td>211</td>
<td>40</td>
</tr>
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<td>270</td>
<td>27</td>
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<tr>
<td>Total for sqrd-2</td>
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<td>39</td>
<td>481</td>
<td>67 (13.9%)</td>
</tr>
<tr>
<td>sqrd-1</td>
<td>Nomarski optics</td>
<td>30</td>
<td>151</td>
<td>5</td>
</tr>
<tr>
<td>sqrd-1</td>
<td>DAPI staining</td>
<td>10</td>
<td>47</td>
<td>4</td>
</tr>
<tr>
<td>Total for sqrd-1</td>
<td>Nomarski optics+DAPI staining</td>
<td>40</td>
<td>198</td>
<td>9 (4.5%)</td>
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</table>

doi:10.1371/journal.pone.0058462.t002
NRs show similarity to HNF4a but some may support metabolic functions dependent on PPARs in vertebrates, as shown for NHR-49 [55]. Moreover, GEI-8 loss of function may be similar in metabolic regulation as shown for SMRT with a single disabled NR site [DR1 or mDR1, respectively] [56,57]. Interestingly, one of the genes that was increased in gei-8(ok1671) mutants (Y9C9A.16) encodes a sulfide:quinoine oxidoreductase that we name sqrd-2. Our results demonstrate that sqrd-2 and gei-8 functions are genetically linked; inhibition of sqrd-2 in homzygous mutants gei-8(ok1671) induces partial reversal of gei-8 mutant phenotypes as well as additional phenotypic changes. We also demonstrated similar reduction-of-function phenotypes for the sqrd-2 parologue, sqrd-1. Both sqrd genes are associated with 21U-RNAs scattered throughout the non-coding regions. It is intriguing to speculate that the gene expression pattern of gei-8 loss of function may be dependent on this class of regulatory RNAs. 21U-RNAs have been shown to be critical for sperm development and transposon silencing [58]. Both sqrd genes may be linked to their associated non-coding 21U-RNAs that may be localized in mitochondria as part of piRNA biosynthesis [59]. Changes in the mitochondrial compartment induced by gei-8 inhibition as reported by Yamamoto et al. [13] and observed in our experiments on gei-8(ok1671) mutants may also involve piRNAs mediated regulation. The connection to the regulation by 21U-RNAs is supported by our findings that additional changes in the phenotypes of homozygous mutants gei-8(ok1671) are induced by RNAi targeted at sqrd-1 gene. One of the three isoforms of sqrd-1 is predicted to code for a protein with the same length as the protein derived from sqrd-2 and both proteins show 74% identity in amino acid sequences suggesting that these proteins may substitute for each other in function. 21U-RNAs located in sqrd-2 show approximately 50% identity in the conserved cores formed by 16 or 17 bases with piRNAs found in sqrd-1. The levels of these non-coding RNAs, like the activity of sqrd-1 and -2, may be critical for gonad and/or germline development and metabolism. The early embryonic lethality of mice lacking NCoR1/SMRT as well as NCoR2 prevents us from assessing whether the role of GEI-8 in gonadogenesis is an evolutionarily conserved feature [3,60]. Interestingly, it was recently found that individual 21U-RNAs are regulated by fork-head transcription factors [61]. Moreover, the fork-head factor FoxP1 regulates development in concert with SMRT [62]. These results raise the intriguing possibility that GEI-8 might be directly involved in the transcriptional regulation of 21U-RNAs.

Materials and Methods

Worm Strains

All strains were maintained as described [63] and were grown on standard NGM plates or, in case of RNA isolation, on NGM plates cappled with 2% agarose. Wild-type animals were N2 (var. Bristol). The VC1213 strain, kindly provided by the C. elegans Gene Knockout Consortium, carried the gei-8(ok1671) allele over a bli-4 and GFP-marked balancer chromosome; homozygous gei-8(ok1671) mutants are lethal. Prior to experiments, we outcrossed the VC1213 strain three times to wild-type males. gei-8::gfp transgenic lines were constructed by injecting gei-8 promoter constructs into N2 hermaphrodites as described previously [64].

Total RNA Isolation and cDNA Preparation

Wild-type C. elegans animals were grown on 2% agarose-capped NGM plates, washed with water and frozen at −80°C. After thawing the pellet was resuspended in 0.5 ml of resuspension buffer (0.5% SDS; 5% 2-mercaptoethanol;10 mM EDTA; 10 mM Tris/HCl (pH 7.5) with 15 µl of proteinase K (20 mg/ml)), vortexed for 60 s and incubated at 35°C for 60 min. The sample was phenol-chloroform extracted and ethanol-precipitated, 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. After phenol-chloroform extraction and ethanol-precipitation RNA was resuspended in DEPC water. cDNA was synthesized from the isolated RNA using Roche Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland) with poly-T and gene specific primer 6242 from the 3’t untranslated region (UTR) of gei-8, or using Superscript II kit (Invitrogen, Carlsbad, CA) with random hexamer primers, all according to protocols by manufactuers.

RNA Interference

Y9C9A.16 dsRNA was synthesized using a 774 bp region of gDNA containing exons 2 to 6 amplified by primers 7501 and 7502 and cloned into pCRII vector (Invitrogen). Prior to in-vitro transcription by T7 or Sp6 polymerases, the construct was linearized. dsRNA was prepared by incubating ssRNAs at 70°C for 10 min and at 37°C for 30 min, followed by phenol-chloroform purification, ethanol-precipitation and dilution in DEPC water. dsRNA was injected into gonads of N2 wild-type hermaphrodites, heterozygous gei-8(ok1671) mutants, and homozygous wild-type progeny of heterozygous gei-8(ok1671) mutant parents. sqrd-1 RNAi was prepared as mentioned above using primers 7605 and 7606.

Immunostaining

L4 stage homozygous VC1213 mutants and N2 control animals were put on slides coated with poly-L-lysine, fixed in 5% paraformaldehyde, covered by a cover glass and incubated in a wet chamber for 30 minutes at room temperature. Freeze crack was performed after freezing the sample on dry ice for 5 minutes. Samples were placed in −20°C methanol followed by series of rehydration in methanol:TBBS (9:1, 7:3, 1:1 and 1:4 10 minutes each). Staining of actin filaments was done using phalloidin labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR). Samples were incubated with phalloidin (1:500 dilution) for 40 min and then washed in TBBS three times. Samples were mounted with fluorescent mounting medium (DakoCytomation, Copenhagen, Denmark) and coated with nail polish.

Immunostaining of myosin filaments was performed using anti-MYO3 antibody [65]. After rehydration samples were incubated with anti-MYO3 mouse antibody (1:200 dilution) for 24 hours at 4°C, then incubated with anti-mouse IgG antibody labeled with Alexa Fluor 568 (1:1000 dilution). Each incubation with antibody was followed by three TTBS washes. Samples were mounted as described above.

Staining with 4’,6-diamidino-2-phenylindole (DAPI)

L4 stage homozygous gei-8(ok1671) mutants and N2 control animals selected from progeny of injected mothers were put on slides coated with poly-L-lysine and 20 µl of water, covered by a cover glass and put on dry ice. Freeze crack was performed after freezing the sample on dry ice for 5 minutes. Samples were kept in −20°C methanol for 10 minutes, then stained with DAPI (20 µl, 1:1000 dilution of 1 mg/ml) and mounted with fluorescent mounting medium (DakoCytomation, Copenhagen, Denmark) and coated with nail polish.
Longevity Assays

Longevity assays were performed as described [66] with modification. Adult hermaphrodites were allowed to lay eggs for 4–5 hours. Homozygous gei-8(ok1671) mutants (n = 21) and N2 controls (n = 12) were cultured at 20°C and transferred to a new plate every second day. The vitality of the animals was checked once per day. Death was defined as cessation of pharyngeal pumping or lack of response to prodding.

Cloning

We used primers designed according to predicted sequences of gei-8 isoforms a, b and c (WormBase WS195). Multiple regions were amplified by Accuprime polymerase (Invitrogen), cloned into TOPO pCRYI, pCR4 or XL vectors (Invitrogen) and sequenced (Avant 3100). Selected clones are displayed in Figure 2A and C. Primer sequences are as follows: 107, 7149, 7144, and 6242. The sequences of all primers are in Table S3.

Sequencing the gei-8(ok1671) Allele

The mutation in strain VC1213 was confirmed by single-worm PCR with primers 107 and 307 producing bands of expected sizes for mutant and wild-type worms. The nature of gei-8(ok1671) deletion was confirmed by sequencing these PCR fragments (Avant 3100).

Aldicarb and Levamisole Sensitivity Assays

L4 gei-8(ok1671) homozygous mutants and L4 N2 wild-type animals were scored for aldicarb or levamisole sensitivity on NGM plates with 1 mM aldicarb [38] or 1 mM levamisole [39]. The assays were performed at room temperature and scored every 30 minutes until complete paralysis of all animals. Paralysis was defined as cessation of pharyngeal pumping and lack of response to prodding. The score was plotted as the ratio of moving animals to the total number of all animals on the plate.

Locomotion Assays

Thrashing assays were performed in 15 µl of 1× PBS solution on non-adhesive slides. One thrash was defined as a complete swing of the head, for example from left to right and left again, L4 stage VC1213 mutant animals and N2 controls were compared. All worms were allowed to acclimate to the solution for one min prior to scoring. The total number of thrashes was counted in one minute intervals. The pharyngeal pumping rate was counted per minute in well-fed worms in the presence of food at 20°C and VC1213 and N2 controls were compared at the I2, L3 and L4 stages (n = 10 for each stage).

Real-time PCR

Two regions of the gei-8 gene were analyzed for expression using the LightCycler 480 and the LightCycler® 480 SYBR Green I Master kit (Roche Diagnostics, Basel, Switzerland). Region 1 was amplified by primers 6200 and 05/153. Region 2 was amplified by primers 6168 and 01/042. We performed two independent reactions for each region. Reaction conditions were as follows: 5 min pre-denaturation at 95°C followed by 45 cycles amplification (10 s at 95°C, 15 s at 59°C, 15 s at 72°C) and melting curve analyses (5 s at 95°C, 1 min at 65°C and then continuously increasing temperature up to 97°C (temp rate 0.2°C/s)). Data were processed by the LightCycler® 480 software version 1.5. Efficiency values reflected standard curve dilution series, which corresponded to gel-purified ethanol-precipitated PCR products. The Cp values of studied genes were normalized relative to the constitutive gene ama-1 encoding the large subunit of RNA polymerase II [67,68].

Mutant Rescue

The gei-8 promoter and coding sequence was amplified in two overlapping PCR products RES1 and RES2 with primers 6174 and 6173; 6158 and 6243, respectively. The size of the overlapping region was 191 bp. Both PCR products were mixed together to a final concentration 250 ng/µl and injected with the pRF4 (rol-6[sa106]) dominant marker at 100 ng/µl in VC1213 mutant or wild-type adult hermaphrodites. Rollers were selected from the progeny of injected mothers and kept individually per plate at 16°C until they finished laying eggs. Total progeny were counted and scored for embryonic lethality and the number animals carrying the mutant phenotype [31–34].

GFP Reporters

Transgenic lines expressing gei-8::gfp from putative promoter 1 contains 1382 bp upstream of the translational start codon and 222 bp of predicted exon 1 of gei-8 and c. Putative promoter 2 contains 2300 bp upstream of ATG. Promoter 1 was amplified using primers 01/021 and 4938. Promoter 2 was amplified using primers 5056 and 5060. Promoter fragments were cloned into the GFP vectors pPD95.69 and pPD95.67, respectively and injected into L4 hermaphrodites. Both constructs contained a nuclear localization signal.

Transgenic lines expressing gei-8::gfp from putative promoter 3 were created by a PCR fusion-based approach described by Hobert (2002). A 6.2 kb long putative promoter region of gei-8 was amplified by primers 6228 and 6230. Primer 6230 contains an overhanging complementary to the gfp sequence of the pPD95.75 vector. The second product, containing gfp and unc-54 sequences was amplified by standard primers 6232 and 6233 using the pPD95.75 vector as template. The overlapping fusion PCR product was obtained by diluting the two products with water to 10–50 ng/µl and using a 1:1 mixture as a template for a subsequent PCR reaction with nested primers 6229 and 6234. The PCR fusion product was diluted to a final concentration of 50 ng/µl mixed with the injection marker rol-6 at 50 ng/µl and injected in N2 adult hermaphrodite animals. GFP expression was selected for until stable expressing lines were established.

Microarrays

C. elegans whole genome expression microarrays (Affymetrix, Santa Clara, CA) were used to profile gene expression in three independent replicates based on manually selected homozygous gei-8(ok1671) mutants and matched N2 wild-type larvae at the earliest stage when mutants can be easily recognized based on their movement phenotype. Microarray chip data was analyzed by 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) and Partek genomics suite software package, all with a p-value less than or equal to 0.05. The microarray data has been deposited in the NCBI's GEO database (http://www.ncbi.nlm.nih.gov/geo) accession number GSE40127.

Detection of Mitochondrial Activity by MitoTracker Labeling

Manually selected worms were transferred to a 10 µl drop of 10 µM MitoTracker Red CMXRos (Invitrogen, Molecular Probes) for 2 hr at room temperature (21°C) in PBS and were kept in dark. Worms were transferred to a drop of 20 µl PBS to...
NGM plate seeded with OP50 bacteria and kept in dark for 2 hr. Worms were then manually transferred to microscopic slides with agarose layer for fluorescent microscopy. For densitometric analysis, L4 larvae were analyzed using the Olympus BX60 microscope with a DP50 camera and pictures recorded at constant settings. For densitometric analysis, pictures of 12 larvae were used (four for each group, N2 wild-type larvae, mutant larvae gei-8(ok1671) determined by their moving phenotype and worms from the progeny of heterozygous gei-8(ok1761) that appeared normal). The total area of 149 000 μm² was analyzed in total 40 selected areas (excluding areas for determination of background values) using the computer program ImageJ Version 1.42q (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2011).

**GST Pull-down Assay**

The complete coding cDNA of NHR-60 [24] (with the exception of the first methionine codon) was amplified by PCR using primers 10/44 and 10/45 and cloned in pGEX-2T vector (Amersham Pharmacia Biotech, Amersham, UK). The glutathione-S-transferase (GST) fusion protein was expressed in Escherichia coli (BL-21-strain). For control experiments, the protein domain of GST was expressed from the pGEX-2T empty vector. Overnight cultures of transformed bacteria obtained from a single bacterial colony were cultured in 400 ml of Luria-Broth culture medium containing 100 μg/ml ampicillin at 37 °C overnight. Cultures with O. D. (600 nm) = 0.8 were induced using 1 mM isopropylthiogalactopyranoside (IPTG) and the cultures were cultivated at 20 °C for an additional 5 hr prior to harvesting by centrifugation at 3500 × g in a swing out rotor at 4 °C for 10 min. The bacteria were washed twice in phosphate-buffered saline (PBS) and resuspended in 5 ml of PBS. Bacteria were lysed in 6 ml of Lysis buffer, (Bio-Rad Laboratories, Hercules, CA) supplemented with protease inhibitor (1 × COMPLETE, Roche, Basel, Switzerland), incubated on ice for 10 minutes with intermittent vortexing and sonicated four times 10 sec. at 80% intensity (Sonicator UP100H, Hielser Ultrasonics, Teltow, Germany). The lysates were centrifugated at 11,800 × g/4 °C/10 min. The supernatant was removed and filtered using a 0.22 μm filter. Glutathione-agarose (Sigma-Aldrich, Saint Louis, MO) was prepared by swelling 0.1 g of beads in 1 ml PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.45 mM KH2PO4, pH 7.5). The beads were collected by sedimentation and swelling completed by repeating the swelling step for an additional 5 min. The beads were then resuspended in 100 μl of PBS. The resulting slurry was used for binding of GST or GST-NHR-60. For purification of GST-NHR-60 and GST, 100 μl of slurry (containing 0.01 g of beads), and 300 μl of bacterial lysates were incubated for 30 minutes at 4 °C with intermittent mixing (approximately every 4 minutes). The beads were washed four times in 1 ml of PBS Triton X-100 (1%) (Sigma-Aldrich, Saint Louis, MO). Beads were collected by sedimentation and resuspended in 500 μl of PBS. The resulting slurry was divided to four aliquots of 100 μl that were used for the binding assay.

The C-terminal domains of gei-8a coding for regions with the putative NR binding sites were amplified by PCR from cDNA and cloned in three constructs in pCR4 or pCRII TOPO-TA cloning vectors (Invitrogen, Carlsbad, CA). The three constructs marked as I, II, III were prepared using the following primers and positions in gei-8a isoform (Construct I: 7749, 7750; position 2480–3485, construct II: 7751, 7752; position 3412–4389; construct III: 7753, 7754; position 4274–5513). Constructs I and III included the predicted NR sites, NR1 and NR2 binding sites, respectively. (B) Pulled-down radioactivity determined by scintillation detection in the fractions shown in panel A. GST-NHR-60 binds in vitro translated domain I of GEI-8 (lane 4 in panel A and the corresponding bar in panel B) supporting the functional similarity between GEI-8 C-terminal region and NCoR/SMRT. The figure presents one of two experiments that gave similar results.

**Supporting Information**

**Figure S1** Binding analysis of C-terminal domains of GEI-8 including the predicted NR binding sites to GST-NHR-60. (A). Pull-down experiment of GST (lanes 1 to 3) and GST-NHR-60 (lanes 4 to 6) with in vitro translated proteins covering the C-terminal domains of GEI-8 (GEI-8*) cloned in three constructs: domain I (position 2480–3485 in gei-8a, lanes 1 and 4), domain II (position 3412–4389) lanes 2 and 5 and domain III (position 4274–5513) lanes 3 and 6. The domains I and III contain the predicted NR1 and NR2 binding sites, respectively. (B) Pulled-down radioactivity determined by scintillation detection in the fractions shown in panel A. GST-NHR-60 binds in vitro translated domain I of GEI-8 (lane 4 in panel A and the corresponding bar in panel B) supporting the functional similarity between GEI-8 C-terminal region and NCoR/SMRT. The figure presents one of two experiments that gave similar results.

**Figure S2** Elevated mitochondrial activity in mutant gei-8(ok1671) larvae. Control wild-type N2 L4 larvae and progeny of heterozygous mutant in the same developmental stage were stained using MitoTracker staining as described (Materials and methods). Panels A, D, G, and J show N2 control animals; panels B, E, H, and K show progeny of a mutant heterozygous parent that lack the mutant phenotype and represent heterozygous or wild-type larvae; panels C, F, I, and L show homozygous mutant larvae, from the same parent. Exposure time was 50 ms in panels A, B and C and 100 ms in all other panels. Panels A to F show the proximal part of larva; panels G to I show the middle part of the larval bodies and panels J to L show the distal part of larva. Elevated activity in homozygous animals is apparent in panels C, F, I and L. The scale bar represents 100 μm.

**Figure S3** Densitometric analysis of MitoTracker staining expressed in arbitrary units. N2 wild-type animals, and the progeny of heterozygous mutant parents divided according to the mutant phenotype to phenotypically normal heterozygous (+/−) and phenotypically homozygous (−/−) animals were analyzed. Elevated staining by MitoTracker in homozygous mutant larvae is statistically significant in paired Student’s T-test compared to both N2 and morphologically unaffected progeny of heterozygous parents (p<0.01).
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