# 3. Material and Methods

## 3.1. Genetics

### 3.1.1. Nematode Culture Conditions

Nematode strains were cultured on NG agar seeded with *E. coli* strain OP50 as described by Brenner and Sulston (Brenner, 1974; Sulston and Hodgkin, 1988). Strains were cultured at 20°C unless indicated otherwise. Lists of nematode strains isolated (Table A1.), obtained by injection (Table A2.), obtained by crosses (Table A3.) and strains used in this study (Table A4.) are found in the Appendices. The wild type strain used was N2.

| | | |
|---|---|---|
| NG-medium: | 6 g | NaCl |
| | 5 g | Peptone |
| | 33.7 g | Agar (Difco) |
| | 1.925 l | Bidest $H_2O$ |
| | 50 ml | 1 M $KPO_4$ |
| | 2 ml | 5 mg/ml Cholesterol in 95% EtOH |
| | 40' autoclaving | |
| | 4 ml | 0.5 M $CaCl_2$ |
| | 2 ml | 1 M $MgSO_4$ |

### 3.1.2. Microscopy

Nematodes were scored by Leica MZ8 dissection microscope. Slides were prepared as described by Sulston and Hodgkin (Sulston and Hodgkin, 1988). Nematodes were anaesthetized using 0.5 % isophenoxypropanol or 1 mM levamisole. Nematodes were scored by Normarsky DIC microscopy using a Zeiss Axioscope 2 plus microscope. Photographs were taken with a Sony progressive 3CCD camera and with a Hamamatsu orca ER digital CDD camera. Adobe PhotoShop was used to adjust brightness and contrast.

### 3.1.3. Mutant Isolation – EMS mutagenesis

The *dre-1* mutants were isolated from an F2 mutant screen for the enhancement of the heterochronic gonadal migration (Mig) phenotype of the *daf-12* null mutant *rh61rh411.* Mutagenesis was performed according to Sulston (Sulston and Hodgkin, 1988). For each screen, the P0 *daf-12(rh61rh411)* hermaphrodite culture (young adults) was mutagenized with 0.5 % EMS (Ethylmethanesulfonate) in 4 ml M9 buffer for 4 hours. Next nematodes were washed 4 times in 4 ml M9 buffer. The pellet was transferred to 50 seeded NG plates. After 12 hours, hermaphrodites were transferred to 35 cm x 6 cm plates (3 hermaphrodites/plate) and allowed to self by incubation at 20°C for 6 days (F1 generation). The L1 larvae of the F1 generation were harvested by washing them off with

M9 buffer and transferred to five seeded plates. After four days the approximately 127000 genomes were scored for Mig phenotypes using a dissecting microscope. After additional five days the Mig mutants were re-screened for true breedings (F2 generation). In three screens we isolated nine Mig mutants, eight alleles of *dre-1* (*dh99, dh172, dh190, dh278, dh279, dh280, dh284, dh292*) and one allele of *dre-2* (*dh184*).

### 3.1.4. Outcrossing of isolated mutants

All mutants obtained by EMS mutagenesis were outcrossed to the wild type N2 strain at least three times. Hermaphrodites of *daf-12(rh61rh411*) and *dre-1* or *dre-2* Mig double mutants were crossed by N2 males. F1 cross hermaphrodites were singled and allowed to self. F2 non-Mig hermaphrodites were transferred on *daf-12* RNAi plates and scored for the Mig phenotype in the F3 generation. Mig animals were transferred to normal plates and scored for non-Migs in the F4 generation (homozygous test).

### 3.1.5. Allele specificity test

*dre-1(dh99)* was crossed into *daf-12(m421)* and *daf-12(sa156)* strains. *dre-1(dh99)* males were crossed with *daf-12* hermaphrodites. F1 hermaphrodites were singled and scored for the Mig phenotype in the F2 generation.

### 3.1.6. Strain constructions

#### 3.1.6.1. Construction of *dre-1* and *dre-2* single mutants

All *dre-1* mutants were obtained as double mutants of *dre-1* and *daf-12(rh61rh411).* To obtain the *dre-1* single mutants, all *dre-1; daf-12* double mutants were crossed to wild type N2 males. In the F1 generation, nonMig cross hermaphrodites were selected and allowed to self. In the F2 generation, nonMig hermaphrodites were transferred to *daf-12* RNAi plates and the F3 generation was scored for the Mig phenotype. Mig animals were selected and the F4 generation was scored for nonMigs. Applying this strategy, we singled *dre1(dh99), dre1(dh172), dre1(dh190), dre1(dh278), dre1(dh279), dre1(dh280), dre1(dh284)*, *dre1(dh292)* and *dre-2(dh184).*

#### 3.1.6.2. Double mutants constructed for epistasis analysis

*dre-1(dh99);lin-29(n546)*

*dre-1* males were crossed to *lin-29/dpy-10(e128)unc-52(e444)* hermaphrodites. In the F1 generation, putative cross hermaphrodites were selected when cross males appeared on the mating plate. In the F2 generation, Mig animals with a protruding vulva were selected.

*daf-12(rh61rh411);lin-29(n546)*

*daf-12(rh61rh411)/+* males were crossed to *lin-29/dpy-10(e128)unc-52(e444)* hermaphrodites. In the F1 generation, putative cross hermaphrodites were selected when cross males appeared on the mating plate. In the F2 generation Mig animals with a protruding vulva were selected.

*dre-1(dh99);lin-4(e912)*

*dre-1(dh99)* males were crossed to *lin-4(e912)/mnC1 dyp-10(e128);unc-52(e444)* hermaphrodites. In the F1 generation, putative cross hermaphrodites were selected when cross males appeared on the mating plate and transferred to *daf-12* RNAi plates. In the F2 generation vulvaless, very long Mig animals were selected.

*daf-12(rh61rh411);lin-4(912)*

*daf-12(rh61rh411)/+* males were crossed to *lin-4(e912)/mnC1 dyp-10(e128);unc-52(e444)* hermaphrodites. In the F1 generation putative cross hermaphrodites were selected when cross males appeared on the mating plate and transferred to *dre-1* RNAi plates. In the F2 generation vulvaless, very long Mig animals were selected.

*dre-1(dh99);lin-14(ma135)*

First, a double mutant of *lin-14(ma135)* with the *dpy-11(e224) unc-42(270)* that are closely linked to *dre-1(dh99)* was constructed. *lin-14(ma135);DpyUnc* triple mutant hermaphrodites were then mated to *dre-1* males. In the F1 generation wild type hermaphrodites were selected. In the F2 generation, nonDpy nonUnc animals were selected. In the F3 generation, plates were selected that did not segregate DpyUnc animals.

*dre-1(dh99);let-7(n2853ts)*

The *let-7(n2853)* is a temperature-sensitive lethal allele. At the non-permissive temperature, (25°C) animals explode as young adults or are sterile. *dre-1(dh99)* males were crossed to *let-7(n2853)* hermaphrodites at 15°C. After two days, the mating plate was shifted to 25°C to distinguish between cross and non-cross progeny. Wild type appearing (non-exploding and non-sterile) hermaphrodites were selected and allowed to self at 15°C. *dre-1* like animals (long and clear) were selected and allowed to self. When plate was crowded, a small piece of the plate was transferred to a new plate and grown at 25°C. Plates with many exploders and steriles were selected and held at 15°C.

*dre-1(dh99);lin-42(n1089)*

*dre-1* males were mated to *lin-42* hermaphrodites. In the F1 generation, wild type animals were selected an placed on *daf-12* RNAi plates. In the F2 generation, Mig animals were selected and placed on NG plates. In the F3 generation, animals with strong precocious alae were selected.

*daf-12(rh61rh411);lin-42(n1089)*

*lin-42* hermaphrodites were mated to wild type males. Cross males were mated to *daf-12(rh61rh411)* hermaphrodites. In the F1 generation, wild type animals were placed on *dre-1* RNAi plates. In the F2 generation Mig animals were selected. In the F3 generation, Dpy (*lin-42* animals) were selected and in the F4 generation tested for precocious adult alae.

*daf-12(rh61);lin-42(n1089)*

*lin-42* hermaphrodites were mated to wild type males. Cross males were mated to *daf-12* hermaphrodites. In the F1 generation, wild type animals were selected. In the F2 generation, Mig animals were selected. In the F3 generation, Dpy (*lin-42* animals) were selected and in the F4 generation tested for precocious adult alae.

*dre-1(dh99);ajm-1::gfp*

*dre-1* males were mated to *ajm-1::gfp* hermaphrodites. In the F1 generation, roller animals were selected and placed on *daf-12* RNAi. In the F2 generation, roller animals that were Mig and GFP+ were selected.

*dre-1(dh279);ajm-1::gfp*

*dre-1* hermaphrodites were mated to wild type males. Cross males were mated to *ajm-1::gfp* hermaphrodites. In the F1 generation, roller animals were selected and placed on *daf-12* RNAi. In the F2 generation, roller animals that were Mig and GFP+ were selected.

*dre-1(dh99);lin-29(n546);ajm-1::gfp*

*dre-1(dh99);ajm-1::gfp* roller males were crossed to *lin-29/dpy-10(e128)unc-52(e444)* hermaphrodites. In the F1 generation, rolling hermaphrodites were selected. In the F2 generation, Mig animals with a protruding vulva that were rolling were selected.

*lin-29(n546);ajm-1::gfp*

*ajm-1::gfp* hermaphrodites were mated to wild type males. Cross males were mated to *lin-29/dpy-10(e128)unc-52(e444)* hermaphrodites. In the F1 generation, rolling cross

hermaphrodites were selected. In the F2 generation, GFP+ roller animals with a protruding vulva were selected from nonDpynonUnc plates.

*lin-4(e912);dre-1(dh99);ajm-1::gfp*

*lin-4(e912)/mnC1 dyp-10(e128);unc-52(e444)* hermaphrodites were mated to *dre-1;ajm-1::gfp* males. In the F1 generation, rolling cross hermaphrodites were selected from nonDpy;nonUnc plates and placed on *daf-12* RNAi plates. In the F2 generation, rolling Mig (*dre-1*) animals that were very long and vulvaless (*lin-4)* and GFP+ were selected.

*lin-4(e912);ajm-1::gfp*

*lin-4(e912)/mnC1 dyp-10(e128);unc-52(e444)* hermaphrodites were mated to *ajm-1::gfp/+* males. In the F1 generation, rolling cross hermaphrodites were selected. In the F2 generation, rolling hermaphrodites that were GFP+, very long and vulvaless (*lin-4)* were selected.

*let-7(n2853);dre-1(dh99);ajm-1::gfp*

*dre-1;let-7* hermaphrodites were mated to *dre-1;ajm-1::gfp* males at 15°C (permissive temperature for *let-7)* for two days. Then plates were shifted to 25°C (*let-7* non-permissive temperature) to distinguish between cross and non-cross progeny. In the F1 generation, rolling *dre-1* hermaphrodites were selected and shifted to 25°C (non-permissive temperature for *let-7).* In the F2 generation, roller animals were selected and grown at 15°C. In the F3 generation, animals were shifted to 25°C and tested for lethality in the next generation by comparing to *let-7* and *let-7;dre-1* controls. Young larvae from plates with lethals were selected and placed at 15°C.

*let-7(n2853);ajm-1::gfp*

*let-7* hermaphrodites were mated to *ajm-1::gfp/+* males at 15°C for two days and then shifted to 25°C to distinguish between cross and non cross progeny. Among the non exploding F1 generation, rolling hermaphrodites were selected and allowed to self at 15°C. In the F2 generation, rolling hermaphrodites were selected and placed at 15°C. In the F3 generation, a small piece of each plate was transferred to a new plate and tested for the segregation of lethals at 25°C. Young larvae from plates segregating lethals were selected.

*lin-42(n1089);dre-1(dh99);ajm-1::gfp*

*lin-42*;*dre-1* hermaphrodites were mated to *dre-1;ajm-1::gfp* males. In the F1 generation, rolling hermaphrodites were selected and placed on *daf-12* RNAi plates. In the F2

generation, Mig animals (*dre-1)* that rolled were selected. In the F3 generation, Dpy animals (*lin-42)* were selected and tested for wild type like adult alae.

*lin-42(n1089);ajm-1::gfp*

*lin-42* hermaphrodites were mated to *ajm-1::gfp/+* males. In the F1 generation, rolling hermaphrodites were selected. In the F2 generation, rolling hermaphrodites that were GFP+ and had precocious alae were selected.

*dre-1(dh99);daf-2(1368)* and *dre-1(dh99);daf-2(e1370)*

*daf-2* hermaphrodites were mated to wild type males. Cross males were mated to *dre-1(dh99)* hermaphrodites. In the F1 generation, wild type hermaphrodites were selected. In the F2 generation, daf-c (*daf-2*) animals were selected. In the F3 generation, animals with Mig blibs were selected.

*dre-2(dh184);lin-42(n1089)*

*dre-2* hermaphrodites were mated to wild type males. Cross males were mated to *lin-42* hermaphrodites. Putative cross hermaphrodites (non Dpy) were placed on *daf-12* RNAi. In the F2 generation, Mig animals were selected (*dre-2*). In the F3 generation, animals were scored for precocious alae.

*dre-1(dh99);unc-42(e270)*

*dre-1* males were mated to *dpy-11(e224)unc-42(e270)* hermaphrodites. In the F1 generation, nonUncnonDpy animals were selected and placed on *daf-12* RNAi plates. In the F2 generation, Unc/Mig animals were selected. In the F3 generation, Unc animals from plates that did not segregate Dpy animals were selected.

*dre-1(dh99); unc-46(e177)*

*dre-1* males were mated to *dpy-11(e224)unc-46(e177)* hermaphrodites. In the F1 generation, nonUncnonDpy animals were selected and placed on *daf-12* RNAi plates. In the F2 generation, Unc/Mig animals were selected. In the F3 generation, Unc animals from plates that did not segregate Dpy animals were selected.

### 3.1.7. Staging of nematodes

For staging worms, plates with many adults were used. Larval and adult worms were killed using alkaline hypochloride, thereby setting eggs free (Epstein, 1995). Eggs were transferred in M9 medium containing cholesterol and allowed to hatch over night into L1 diapause. These were placed on seeded plates and allowed to grow until the required larval stage.

### 3.1.8. Molting experiments

For molting experiments, one 3 fold egg was placed on each NG plate containing a thin bacterial lawn of about 1 cm in diameter. After the time point of hatching was determined, the L1 larvae were allowed to grow for several hours. Then every plate was checked every 20 minutes if ecdysis occurred. Therefore, the bacterial lawn was searched for a shedded cuticle. After the time point of the L1 molt was determined, the worms were again allowed to grow for several hours and then checked every 20 minutes for ecdysis. The whole procedure was repeated for every single molt of each worm.

## 3.2. Mapping *dre-1* and *dre-2*

*dre-1* was mapped using SNP, three factor and deletion mapping techniques. Below, techniques are described in detail.

### 3.2.1. SNP mapping

SNP (Single Nucleotide Polymorphisms) mapping has been performed as described by Wicks and co-workers (Wicks et al., 2001). In the *C. elegans* Hawaiian wild type strain CB4856 over 6222 SNPs were predicted and 3457 snipSNPs were found to be evenly distributed over the genome. On average every 873 bp one SNP occurs with higher densities towards chromosome arms. Two types of SNPs are commonly used for mapping, snipSNPs (detectable by RFPL) or seqSNPs (detectable by sequencing). In this work, we used snipSNPs solely.

Chromosome mapping and fine mapping using bulked segregant analysis

To map *dre-1* and *dre-2* to a chromosome, we chose a set of three confirmed snipSNPs for each chromosome and used bulked segregate analysis. For fine mapping, we used confirmed and predicted snipSNPs for chromosome I (*dre-2*) and chromosome V (*dre-1*). A list of all SNP primers used (including primer name, primer sequence, position, restriction enzymes and restriction fragment lengths of N2 and CB4856) is found in the Appendices Table A6, A7 and A8. Males of the Hawaiian strain CB4856 were crossed with the *dre-1* or *dre-2* Mig mutant hermaphrodites, mutant and non-mutant F2 generation animals (Mig) were selected and allowed to self. DNA from the mutant and non-mutant (heterozygote) F3 generation was prepared as described in 3.3.2. An 1 µl aliquot of each mutant and non-mutant DNA was pooled and PCR was performed with the pooled DNA samples as a template. For chromosome mapping and fine mapping we used 50 recombinants of *dre-1* and 100 for *dre-2.*

To detect linkage of a marker we screened for the exclusion of CB4856 alleles. The proximity of a linked marker to the mutation was estimated by the relative proportion of each

form of the biallelic marker in populations of wild type and mutant genomes. The relative fraction of CB4856 DNA to N2 DNA was determined by deriving a ratio of the density of a CB4856-specific band and an N2-specific band for each of the Mig and nonMig bulked lysates. The map ratio was calculated by taking a ratio of Rm/Rw, where, Rw = Dw[CB4856]/Dw[N2], where, Dw[CB4856] is the density of the CB4856 form of the biallelic marker, and Dw[N2] if the density of the N2 form, each in the lane of digested wild type DNA and, Rm = Dm[CB4856] / Dm[N2], where Dm is the density of the indicated allele in the mutant lane (Wicks et al., 2001). Ratio determination was achieved through scanning of DNA gels with the FluorImager 575 of Molecular Dynamics. Densities of the N2 and CB bands were determined using the ImageQuant 1.1 software of Molecular Dynamics. Unlinkage was indicated by a map ratio of one showing that the amounts of each form of a biallelic marker in the two bulked lysates were equal. Linkage was indicated by a map ratio significantly below one suggesting that either the N2 form of the allele predominates in the nonMig lysate, or the CB4856 allele predominates in the Mig lysate, or both.

SNP chromosome mapping indicated that *dre-1* mapped to chromosome V and SNP fine mapping positioned *dre-1* to the center of chromosome V. Snip mapping of *dre-2* placed it to the left arm of chromosome I between the SNPs pKP1102(-15.242) and pKP1016(-12.101).

PCR-mix:

| | |
|---|---|
| 18.4 µl | $H_2O$ |
| 2.5 µl | 10x Taq PCR buffer |
| 0.75 µl | 50 mM $MgCl_2$ |
| 0.2 µl | 25 mM dNTPs |
| 1 µl | 10 mM Forward primer |
| 1 µl | 10 mM Reverse primer |
| 0.15 µl | Taq polymerase |
| 1 µl | template |

PCR – program:

| | | | |
|---|---|---|---|
| Step 1 | denaturation | 95°C, | 5 min |
| Step 2 | denaturation | 95°C | 40 sec |
| Step 3 | annealing | 58°C | 40 sec |
| Step 4 | elongation | 72°C | 40 sec |
| 30 cycles (step 1 to step 3) | | | |
| Step 5 | final elongation | 72°C | 10 min |

Restriction:

Enzyme mix added to PCR vial; each mix contains 5 U of restriction enzyme.

Digest mix :

| | |
|---|---|
| 3 µl | NEB Buffer |
| (3 µl | BSA) |
| 0.5-1 µl | Enzyme |

fill to 10 µl $H_2O$

Restricted PCR reactions were loaded on 2 % agarose gels in TAE. A 100 bp ladder was used as a marker.

### 3.2.2. Three factor mapping

To confirm SNP mapping data, we took a classical three factor mapping approach that was performed according to Sulston (Sulston and Hodgkin, 1988). Double mutants *unc-46(e177);dpy-11(e224)* and *unc-42(e270);dpy-11(e224)* were used. *dre-1(dh99)* males were crossed to the double mutants. In the F1 generation, cross hermaphrodites were singled. In the F2 generation, Dpy;nonUnc and Unc;nonDpy recombinants were selected. Recombinants were tested for *dre-1(dh99)* by scoring for the Mig phenotype on *daf-12* RNAi plates. Recombinants obtained from *dpy-11;unc-42* cross: 17/17 of the Unc;nonDpy recombinants, but none (0/12) of the Dpy;nonUnc recombinants picked up the *dre-1* mutation. Similar results were obtained for recombinants of *dpy-11;unc-46*: 13/13 of the Unc;nonDpy recombinants, but none (0/11) of the Dpy;nonUnc recombinants picked up the *dre-1* mutation. These data confirmed that *dre-1* mapped to the center of chromosome V, between the two markers *unc-46* and *unc-42* and close to *dpy-11.*

### 3.2.3. Deletion mapping

SNP and three factor mapping data argue that *dre-1* mapped to the center of chromosome V, close to *dpy-11*. However, three factor mapping data did not indicate of which side of *dpy-11 dre-1* mapped to. We therefore employed deletion mapping. Deletions used were *sDf26* ((-16.94) – (-0.09)), *sDf30* ((-0.9) – 0.6) and *sDf36* ((-0.44) – (-0.08)). *dre-1(dh99)* males were crossed to deletion hermaphrodites and the F1 cross males and hermaphrodites were scored for *dre-1* epidermal phenotypes. Deletions *sDf26* and *sDf30* failed to complement *dre-1* indicating that *dre-1* mapped within the deletion interval between (-0.44) and (-0.08). However, the deletion endpoints turned out to be wrong, since *dpy-11(e224)* was proved to be outside of the *sDf36* deletion, but *dre-1*, mapping to the right side of *dpy-11*, was still inside the deletion.

### 3.2.4. SNP mapping with single recombinants

To further narrow down the region to which *dre-1* maps, we employed single recombinant SNP mapping (SNP fine mapping). We therefore used *dre-1(dh99);unc-42(e270)* and *dre-1(dh99);unc-46(e177)* double mutants obtained from three factor mapping. The double mutant hermaphrodites were crossed by CB4856 males. The F1 nonUnc cross progeny was transferred to *daf-12* RNAi plates. In the F2 generation Unc;nonMig recombinants were selected. Since *dre-1;daf-12(RNAi)* double mutants are Mig, *dre-1* must be absent or heterozygous in nonMig animals. To completely eliminate *dre-1* F2 Unc;nonMig recombinants were again transferred to *daf-12* RNAi plates and scored for the absence of the Mig phenotype in the F3 generation. Unc;nonMig recombinants were subsequently tested for breakpoints close to *dpy-11* by snipSNPs. A list of all snipSNPs used for fine mapping is found in the appendix Table A7.

Isolation of genomic DNA from recombinant animals, PCR and restriction was performed as described in 3.2.1. and 3.3.2. For fine mapping confirmed and predicted SNPs were used. Predicted snipSNPs were selected from WormBase Release WS115. Primers were designed that meet the following criteria. First, they would amplify a PCR fragment of approximately 400 bp. Second, the snipSNP is contained at the left or right portion of the fragment yielding fragments of two different sizes.

By SNP fine mapping, we narrowed the mapping region down to the area spanned by the yeast artificial chromosome Y40G12.

For *dre-2,* we also employed snipSNP fine mapping. In contrast to *dre-1,* we searched for breakpoints among the recombinants selected for the bulked segregate analysis. Similar to *dre-1,* we also designed primers for the predicted snipSNPs (WormBase Release WS120).

### 3.2.5. Microinjections

Microinjections were performed as described in Mello (Mello et al., 1991). Yeast artificial chromosomes (YACs) were injected at a concentration of 100-200 ng/µl and cosmids at a concentration 5-15 ng/µl together with 75ng/µl pTG96 (*sur-5::gfp*) (Yochem et al., 1998) in young adults of *dre-1(dh99)* or *dre-1(dh99);daf-12(rh61rh411)*. Stable GFP+ lines were scored for the Mig phenotype of double mutants or of single mutants on *daf-12* RNAi. Additionally, GFP+ lines were scored for the epidermal alae gap phenotype.

For *dre-2(dh184),* YACs were injected at a concentration of 100-200 ng/µl together with 75 ng/µl pTG96(*sur-5::gfp*) (Yochem et al., 1998) in young adults of *dre-2(dh184);daf-12(rh61h411)* double mutants. Subsequently, stable GFP+ lines were scored for rescue of the Mig phenotype.

The *dre-1::gfp* construct was injected at a concentration of 5 -15 ng/µl together with *lin-15(+)* marker (75 ng/µl) into *lin-15(n765)* and *lin-15(n765);dre-1(dh99)* mutant animals.

A list of all strains obtained by injections is found in the Appendices Table A2.

## 3.3. Molecular biology

### 3.3.1. Sequencing of candidate genes

Since we obtained YAC rescue with Y40G12, but no rescue with the spanning cosmids, we sequenced predicted ORFs found in the YAC region. Therefore, genomic DNA of *dre-1(dh99)* was isolated (as described in 3.3.2.), appropriate primer designed, each ORF was amplified by PCR, gelelectrophoresis performed, concentration estimated on a 1% agarose gel (using the 100bp or 1kb marker of New England Biolabs), the appropriate DNA fragment extracted (as described in 3.3.5) and sequenced. Sequencing was performed by Seqlab (Sequence Laboratories Göttingen GmbH). A list of primer is found in the Appendices Table A9.

### 3.3.2. Isolation of genomic DNA of *C. elegans*

Isolation of *C. elegans* genomic DNA from various strains was performed according to Sulston and Hodgkin (Sulston and Hodgkin, 1988). Nematodes were washed off an overgrown plate with 1 ml TE in a 1,5 ml Eppendorf tube. They were allowed to settle for 5-10 minutes by gravity. The volume was reduced to 100 µl leaving the nematode pellet undisturbed. Worms were washed twice in TE, the volume adjusted to 50 µl and frozen at -80°C for 10 minutes. After thawing at room temperature, 50 µl 2x lysis buffer was added (whereby 0.5 µl of 20 mg/ml proteinase K was added freshly). After incubation at 65°C for 1 hour, proteinase K was inactivated by incubation at 95°C for 15 minutes. The lysate was spun for 5 minutes at 14000 rpm and the supernatant was transferred into a fresh tube. After addition of 5 µl RNAse (5 mg/ml $H_2O$), tubes were incubated for 20 minutes at 37°C. Isolated genomic DNA was stored at -80°C. For subsequent PCRs, 1-2 µl/reaction was used.

Lysis buffer contains :
20 mM Tris pH 8.3
100 mM KCl
10 mM EDTA,
0.9 % Tween,
0.9 % NP-40,
0.2 mg/ml Proteinase K (20 mg/ml stock in dd$H_20$)

### 3.3.3. Isolation of cosmid DNA

Cosmids spanning the YAC Y40G12 region (T05H4, ZK40, W01A11, H02BB2, F46E10, K04A8, C40C4, ZK1055, F44C4) were provided by Alan Coulson (Sanger Institute). Single

colonies were cultured in 25 ml TY medium containing ampicillin (120 µg/ml) or kanamycin (50 µg/ml) or chloramphenicol (170 µg/ml) depending on the resistance marker. DNA isolation was performed with the QIAGEN Plasmid Midi Kit, the insert was restricted with *HindIII.* To test cosmid DNA quality, DNA of eight different colonies was isolated for restriction pattern comparison.

### 3.3.4. Primer

Primers were designed using "Primer 3" software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The following parameters were used: optimal size 20 base pairs, CG content >40 %, CG clamp of at least 1 base pair, melting point between 52 and 63°C. A list of all primers designed and used in this study is found in the Appendices Table A6 (SNP chromosome mapping), Table A7. (SNP fine mapping), Table A8 (SNP primer (all)) Table A9 (Sequencing) and Table A10. (Cloning).

### 3.3.5. Cloning

The plasmids made in this studiy are summarized in Table 3.1. This table gives an overview of cloning strategies, primers and restriction sites used and insert size. A list of plasmids, cosmids and YACs used in this study is found in the Appendices, Table A10. For cloning the following reagents were used:

Restriction enzymes: New England Biolabs; Boehringer Mannheim; Roche

Polymerases: Taq (Perkin Elmer), Taq Plus Precision (Stratagene), Taq Plus Long (Stratagene), Pfu (Stratagene), Pfu Hotstart (Stratagene), Pfu Hotstart Turbo (Stratagene)

PCR systems: Failsafe (Epicenter Technologies)

Cloning: Rapid DNA Ligation Kit (Roche), T4 DNA ligase (New England Biolabs), Shrimp alkaline Phosphatase (USB), CIP (New England Biolabs), Qiaquick PCR purification Kit (QIAGEN), Qiaquick Gelextraction Kit (QIAGEN), TOPO TA-, TOPO Blunt- and TOPO XL PCR Cloning Kits (Invitrogen)

Plasmid isolation: For plasmid isolation from bacteria, QIAGEN Plasmid Mini Kit and Maxi Kit were used. For cosmid isolation, QIAGEN Plasmid Mini Kit and for YAC isolation QIAGEN Genomic DNA Kit were used.

Transformation: For transformations the Invitrogen TOP10 competent cells (TOP10 F-mcrA(mrr-hsdRMS-mcrBC) _80lacZ_M15 lacX74 deoR recA1 araD139 (ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG) were used.

Restriction digestion, PCR, cloning and transformation were performed as described in the user manuals. Gelelectrophoresis was performed according to Sambrook (Sambrook et al, 1989).

**Table 3.1. Plasmids made in this study**

| Name | Size | Resistence | Description |
|---|---|---|---|
| *daf-12* RNAi | 4.2kb | Amp, Tet (strain) | L4440/ insert: *daf-12* cDNA 871-2295 (primer: pBTM117c-LP5+pBTM117c-LP3-2)/ insert size: 1424bp/ *SalI/NotI*-restriction sites |
| *hbl-1* RNAi | 3.3kb | Amp, Tet (strain) | L4440/ insert*: hbl-1* (Exon3)/(primer: hbl-1-*Bgl2*-for2+*hbl-1-Bgl2*- rev2) insert size: 483bp/ *BglII*-restriction sites |
| *dre-1* RNAi | 3.3kb | Amp, Tet (strain) | L4440/ insert: *dre-1* (Exon3) K04A8. 2470-2957(primer: dre-1-RNAi (*Bgl2*)- for1+*dre-1*-RNAi (*Bgl2*)- rev1) / insert size: 488bp/ *BglII* restriction sites |
| BE000N10.3 RNAi | 3.3kb | Amp, Tet (strain) | L4440/ insert: BE000N10.3 (Exon10)/(primer: BE3N10.3R-f+BE3N10.3R-r)/ insert size: 514bp/ *BglII* restriction sites |
| *dre-1*promotor in TOPO XL | 7.3kb | Kan/ Zeo | TopoXL/ insert: *dre-1*promotor fragment K04A8 (7936-3967)/(primer:*dre-1* GFPprom(*Kpn1*)-for1+*dre-1* GFPprom(*Kpn1*)-rev-1) insert size: 3790bp |
| *dre-1*ORF+ 3`UTR in TOPO 2.1 | 8.8kb | Amp/ Kan | PCR 2.1Topo/ insert: *dre-1*ORF+ 3`UTR (K04A8. 3965-; F46E10. 25061+),primer: *dre-1* GFP(*Nhe1*)-for1+*dre-1* GFP(*Spe1*)-rev1, insert size: 4870bp |
| *dre-1*promotor::*gfp* | 7.5kb | Amp | L3781/ insert: *dre-1*promotor fragment K04A8 (7936-3967), insert size: 3790bp/ *KpnI* restriction sites |
| *dre-1*promotor::*gfp*:: *dre-1*ORF+3'UTR (*dre-1::gfp)* | 12.2kb | Amp | L3781/ insert: dre-1promotor cloned with *Kpn1*, GFP, *dre-1*ORF+ 3`UTR (*NheI/ SpeI*), insert sizes: 3790bp; 4870bp |
| *dre-1* in pBTM117c | 12.3kb | Kan | pBTM117c/ insert: *dre-1*-2Hybrid fragment (K04A8. 21-3965)/primer:*dre-1*- 2Hyb- for1-*Kpn1*+*dre-1*- 2Hyb- rev1-*Not1*, insert size: 2811bp/ *KpnI/NotI*-restriction sites |
| *dre-1* in pBTM117c in yeast | 12.3kb | Kan, -Trp | pBTM117c/ insert: *dre-1*-2Hybrid fragment (K04A8. 21-3965)/ insert size: 2811bp/ *KpnI/NotI*-restriction sites |
| *lin-29* in TOPO 2.1 | 5.3kb | Amp/ Kan | PCR 2.1Topo/ insert: *lin-29* cDNA full length/ insert size: 1376bp |
| *lin-41* in TOPO 2.1 | 7.4kb | Amp/ Kan | PCR 2.1Topo/ insert: *lin-41* cDNA full length / insert size: 3441bp |
| *vit-1* cDNA clone 12 | 6.9kb | Amp | pT7T3D-Pac I/ insert: *vit-1*longest isoform (isoform1)/ insert size: 4kb/ *HindIII/XhoI*-restriction sites source: rzpd |

**Table 3.1. Plasmids made in this study continued**

| Name | Size | Resistence | Description |
|---|---|---|---|
| *dre-1*promotor::*gfp* :: *vit-1*ORF | 9.8kb | Amp | L3781/ insert: cDNA *vit-1*longest isoform (isoform1)/primer:*vit-1-gfp(Nhe1)*-for1+*vit-1*- GFP(*Spe1*)-rev1-neu, insert size: 2.5kb/ *NheI/SpeI*-restriction sites |
| *ubc-3* RNAi | 3.3kb | Amp, Tet (strain) | L4440/ insert: *ubc-3*/primer:*ubc3*-RNAi-Bgl2-f+*ubc-3*-RNAi-B*gl2*-r/ insert size: 500bp/ *BglII*-restriction sites |

### 3.3.6. *dre-1* cDNA clones

The following cDNA clones encoding *dre-1* cDNA were provided by Yuji Kohara (National Institute of Genetics, Mishima, Japan): yk1132 and yk87b4. yk1132 was a plasmid clone in *E. coli* and consisted of vector pME18S-FL3 (Genbank Accession No. AB009864) with the cDNA insert. Single colonies were cultured in 2 ml LB medium containing ampicillin (100 µg/ml), DNA isolation was performed with the QIAGEN plasmid Mini Kit, the insert was excised by restriction with *BamHI.* Intron/exon borders for both clones were determined by sequencing. Primers used were *dre-1*cDNA-check-for1, *dre-1*cDNA-check-rev1, K04A8.6left2, K04A8.6left7 and are found in the appendices Table A9.

#### 3.3.6.1. Phage stock preparation

yk87b4 was a phage clone. To isolate the cDNA, a phage stock had to be prepared, followed by excision of the phagemids (as described in 3.3.6.2.). First, an over night culture of XL-1 blue cells was grown in YT medium containing 0.2 % maltose and 10 mM $MgSO_4$. The next day, 50 ml of YT was prewarmed to 37°C, 0.5 ml of 20 % maltose was added (to obtain a final concentration of 0.2 %). To this medium 2.5 ml of the XL-1 blue over night culture was added and incubated at 37°C for 2-3 hours until an $OD_{600}$ of 1 was reached. Then YT-TOP-Agar was melted and cooled down to 47°C. At the same time LB plates were prewarmed to 37°C. Cells were spun down at 4000 rpm for five minutes and the supernatant was discarded. The cells were resuspended in 20 ml 0.01 M $MgSO_4$. 3 ml of YT TOP agar was poured into glass tubes and 100 µl of cell suspension added. The solution was immediately plated onto prewarmed LB-plates covering the whole surface. After solidification, 2 µl of the phages, $ddH_2O$ and control phage was added on labeled areas and grown over night. On the third day 0.5µl of SM (phage solution) was transferred into an 1.5 µl Eppendorf tube and 20 µl of chloroform was added. Then a phage plaque was taken from the surface of the plate with the back of a 1000 ml pipette tip from (without the solid agar) and added to the eppendorf tube and gently vortexed. This was left at room temperature for 1-2 hours to allow phage particles to diffuse out of the agar. The phage stock was then stored at 4°C.

#### 3.3.6.2. Excision of Phagemids of Lambda Zap II-phages

An overnight culture of XL-1 blue cells was grown in YT medium containing 0.2% maltose and 1 mM $MgSO_4$. On the second day, 50ml NZY containing 0.5 ml maltose was inoculated with 5 ml of the over night culture and grown for 2.5 hours until an $OD_{600}$ of 1 was reached. After the cells were spun down at 1000 x g for 10 minutes, they were resuspended in 10 ml 10 mM $MgSO_4$. After 200 µl XL-1 blue cells, 200 µl phage stock and 10 µl helper phage VSCM13 ($1 \times 10^7$) were mixed in an Falcontube and incubated at 37°C for 15 minutes. Afterwards 5 ml of 2 x YT was added, incubated at 37°C for 3 hours and then shifted to 70°C for 20 minutes. After a spinning step at 1000 x g for 15 minutes, phage supernatant was decanted into a sterile tube and stored at 4°C. On the third day an overnight culture of XL-1 blue was grown in YT containing 0.2 % Maltose and 1 mM $MgSO_4$ . 50 ml NZY with 0.5 ml Maltose was inoculated with 5 ml over night culture and grown for 2.5 hours to an $OD_{600}$ of 1. Afterwards, cells were spun down at 1000 x g for 10 minutes and resuspended in 10 ml 10 mM $MgSO_4$. Two LB plates (containing ampicillin) were prewarmed per sample. Two mixes for each sample were prepared in an 1.5 ml Eppendorf tube. Mix 1 contained 200 µl XL1-blue cells and 100 µl of the phage supernatant; mix 2 contained 200 µl of XL1-blue cells and 10 µl of the phage supernatant. These were incubated at 37°C for 15 minutes, 1 ml YT was added to each sample and incubated at 37°C for 30 minutes. Afterwards, the suspensions were spun down at 1000 x g for 10 minutes. The pellets were resuspended in 200µl YT medium. 50 µl and 100 µl were plated onto LB plates (containing ampicillin) and grown over night at 37°C. On the forth day, an over night culture was grown in LB medium containing ampicillin. On the fifth day, plasmid DNA was isolated and restricted with appropriate enzymes and the positive clone was chosen for sequencing.

### 3.3.7. *dre-1::gfp* constructs

Two different GFP constructs were made. One *dre-1promotor::gfp* construct and one contruct containing the *dre-1* promotor, ORF and 3'UTR. For the promotor construct, a 3972 bp region upstream of the start codon was amplified using the K04A8 cosmid as a template. Primers used were *dre-1*GFPprom(KpnI)for1 and *dre-1*GFPprom(KpnI)rev1. The obtained PCR-fragment was cloned into pCR-XL-TOPO vector. The promotor fragment was excised from the pCR-XL-TOPO vector with the restriction enzyme *KpnI* and cloned it into *KpnI* digested Fire vector L3781 (Fire vector kit 1997). This resulted in the *dre-1promotor::gfp* plasmid containing the *dre-1* promotor with the *gfp* fused to its 3' end. For the *dre-1promotor::gfp::dre1*ORF+3'UTR (*dre-1::gfp*) construct, we amplified a fragment from cosmid W01A11 containing the *dre-1* ORF (3945 bp) and the *dre-1* 3'UTR region (924 bp) using primer pairs *dre-1*GFP(NheI)for1 and *dre-1*GFP(SpeI)rev1. This fragment was subcloned into the pCR2.1-TOPO vector. The *dre-1* ORF/3'UTR fragment was excised from

the pCR2.1-TOPO vector with restriction enzymes *NheI* and *SpeI* and cloned into the *NheI* and *SpeI* digested plasmid *dre-1promotor::gfp*. This resulted in the *dre-1promotor::gfp::dre1*ORF+3'UTR plasmid containing the *dre-1* promotor, *gfp* fused to the 3' end of the promotor and *dre-1* ORF/3'UTR fused in frame to the 3' end of the *gfp*.

### 3.3.8. Preparation of *C. elegans* mixed stage RNA and cDNA

Isolation of *C. elegans* RNA from the wild type strain N2 was performed according to Krause (Krause, 1995). Nematodes were grown on 20x10 cm NG plates coated with 2 % agarose and seeded with OP50 bacteria for 3-4 days at 20°C until bacteria lawn on plates almost cleared. Then they were washed off the plates with 3 ml DEPC treated sterile M9 buffer and pooled into 50 ml polypropylene tubes (e.g. Falcon tubes). Nematodes were placed on ice and allowed to settle for 1.5 hours by gravity. The supernatant (not containing any worms) was carefully removed and the pellet was washed with ice cold DEPC treated M9 buffer two times with a spinning step (10 minutes at 4K at 4°C). The supernatant was removed and to the 0.5 to 1 ml worm pellet 1 ml acid-washed sand and 3 ml triazol reagent was added. After vigorous vortexing for 10 minutes, the mix was allowed to settle at room temperature. Then 0.6 ml of chloroform was added and after brief vortexing, the mix was allowed to settle for 15 minutes at room temperature. After the mix was spun down at 15 minutes at 4 K at 4°C, the aqueous top phase was transferred to a fresh tube. 1.5 ml isopropanol was added and allowed to settle at room temperature for 15 minutes. Then it was spun at 8000 rpm and the pellet was washed with 1.5 ml 75% ethanol by brief vortexing and an additional spin at 4°C for 10 minutes. The pellet was air dried and then dissolved in 100µl deionized DEPC treated water by heating it at 55 to 60°C for 5 to 10 minutes. For determination of RNA concentration the $OD_{260/280}$ was determined.

The RNA obtained was subsequently used as a template for cDNA preparation by RT-PCR. The SuperScript First-Strand Synthesis System was used for RT-PCR and was performed as described in the user manual. The cDNA was used as a template in various PCR reactions amplifying fragments for various constructs (e.g. *hbl-1* RNAi construct).

### 3.3.9. Deletion of *dre-1*

The deletion of *dre-1(hd60)* was isolated and obtained from Harald Hutter (MPI Heidelberg) and performed as described (McKay and Jones, 2002). The poison priming system was used to screen for the *dre-1* deletion by PCR. Primers used were External-Left (*dre-1*-dx4) ACACATACAGCTGCACCATTTC, External Right (*dre-1*-dx3) TTACATCTCCACCACCCTCTCT, Internal Left (di4) TTCAATATATGGCATTGCATCC, Internal Right (*dre-1*-di3) ATGCGTTTCTTGGTTTCTCAAT, Poison Left (*dre-1*-dp4) CAGCTTGAACTTTTTCATCGTG, Poison Right CGAAGCTCGAGAGTGAGTTGAT.

*dre-1(hd60)* deleted 1262 bp removing exons 1-3. For deletion detection by PCR primers *dre-1*-di3 and *dre-1*-di4 were used. PCR amplification using wild type as a template yielded a PCR fragment of 2020 bp whereas templates containing the *hd60* yielded fragments of 759 bp in size. PCR was performed using Taq polymerase and the PCR program used was:

| | | |
|---|---|---|
| Step 1 | 94°C | 30 seconds |
| Step 2 | 92°C | 30 seconds |
| Step 3 | 61°C | 30 seconds |
| Step 4 | 72°C | 45 seconds |
| Step 5 | 72°C | 10 minutes |

Step 2 to 4 were repeated 34 times.

*dre-1(hd60)* deletion was likely to be heterozygote, because the wild type and deletion band appeared when PCR amplified using the *dre-1*-di3 and *dre-1*-di4 primers. We therefore balanced the deletion over the *dpy-11(e224);unc-42(e270)* that were closely linked to *dre-1* and tested for heterozygousity. Dpy;Unc hermaphrodites were crossed to wild type (N2) males and the +/Dpy;Unc males were mated to the deletion hermaphrodites. In the F1 generation, wild type hermaphrodites were selected and allowed to self. In the F2 generation we looked for seggregation of DpyUnc animals and tested for the deletion by PCR. All plates segregated DpyUnc animals indicating that deletion animals on their own were lethal and only survived as heterozygotes. Plates segregating DpyUnc animals were checked for deletion by PCR.

The *dre-1(hd60)* strain was outcrossed three times. Therefore, the balanced deletion *hd60/DpyUnc* hermaphrodites were mated to wild type males and in the F1 generation, wild type hermaphrodites were selected. In the F2 generation, plates were chosen that did not segregate DpyUnc animals and tested for the deletion by PCR. *hd60/*+ hermaphrodites were then re-balanced by DpyUnc markers. Therefore, *hd60/*+ hermaphrodites were mated to wild type males. Subsequently, males from this cross were mated to Dpy Unc hermaphrodites. In the F2 generation all plates were tested for the deletion by PCR. Deletion heterozygotes were cloned and tested for segregation of DpyUnc animals in the F3 generation. The outcrossed balanced deletion strain was subsequently used for further outcrossing.

The *dre-1(hd60)* deletion phenotype was partially rescued by the *dre-1::gfp* construct of the deletion by the *dre-1promotor::gfp::dre1*ORF+3'UTR construct. The *dre-1::gfp Ex346* array was crossed into the balanced *dre-1(hd60)* deletion strain. Next, *dre-1::gfp Ex346* hermaphrodites were mated to wild type males. The GFP+ cross males were mated to DpyUnc/*dre-1(hd60)* hermaphrodites. In the F2 generation, only GFP+ hermaphrodites from plates segregating nonDpy;nonUnc were selected and allowed to self. In the F3 generation, we tested for the *hd60* deletion by PCR. To homozygous the deletion, GFP+ animals from positive plates were cloned. The *dre-1promotor::gfp::dre1*ORF+3'UTR construct rescued the

deletion as in the next generation GFP+ animals were viable and GFP- animals were embryonic lethal.

## 3.4. Database analyses

To identify DRE-1 conserved domains and reveal orthologs of DRE-1, we performed various database searches. Protein BLAST alignments of DRE-1 were performed using programs at the NCBI website (www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1997), at the Ensembl website (http://www.ensembl.org/Multi/blastview), and at the WormBase website, (http://www.WormBase.org), release WS115 (Harris et al., 2003). Protein analyze programs like Pfam (www.sanger.ac.uk/Software/Pfam/), NCBI conserved domain search (www.ncbi.nlm.nih.gov/BLAST) and Psort (Horton and Nakai, 1996) were performed for further protein domain search and detection of signals for intracellular localization. Multiple sequence alignment was done with ClustalW using the Bioedit 5.0.9 software.

## 3.5. miRNA search

To search for miRNA binding sites within the *dre-1* 3'UTR, we used the reverse complement of the mature miRNA and looked for these antisense sites in *dre-1* 3'UTR region. We manually searched for matches. Sequences used were CTCACACTTGAGGTCTCAGGGAAC for *lin-4,* ACTATACAACCTACTACCTCA for *let-7,* TCGCATCTACTGACCCTACCTCA for *mir-48*, TCTACACTTTTTAATTTTCGA for *mir-69*, TACAATATTACATACTACCTCA for *mir-84* and TCATTTCTCGCACCTACCTCA for *mir-241*.

## 3.6. Search for *daf-12* binding sites

*daf-12* DNA binding sites, the response elements, were identified in an *in vitro* approach by Shostak (Shostak, 2002). DAF-12 binds with high affinity to an oligonucleotide containing the AGTGCA response element. We searched for this binding site in the *dre-1* promotor region (1 kb upstream of the start condon) and introns of *dre-1.*

## 3.7. RNAi

RNAi is a well established technique to knock down target gene expression post-transcriptionally through destruction of the target message (Fire et al., 1998). In *C. elegans,* three methods are used for application of RNAi: Feeding, soaking or injecting. For our RNAi studies, we used feeding, since it is the most convenient method. Nematodes were fed with *E. coli* strains expressing siRNAs directed against targets. RNAi constructs of *dre-1, daf-12, hbl-1 ubc-3* and BE0003N10.3 were obtained by cloning. Plasmids containing the insert of interest were digested with certain restriction enzymes, cloned into the multiple cloning site

of the L4440 feeding vector (Fire Kit) and transformed into the *E. coli* strain HT115 (for details see Table 3.1 in 3.3.5). Other constructs were selected from the RNAi Library of the Ahringer lab (available from MRC geneservice (Kamath and Ahringer, 2003)). A list of used RNAi strains of the Ahringer lab is found in the Appendices Table A5. In both cases the *E. coli* strain HT115 contained the L4440 expression vector with an insert of the target gene. Selected strains were grown on tet/amp plates (100 µg/ml ampicillin, 12,5 µg/ml tetracycline) overnight at 37°C. RNAi experiments were performed as described by Kamath (Kamath et al., 2001).

For larger screens testing many different genes, bacteria (HT115) were grown over night at 37°C in 5 ml LB medium including 50 µg/ml amp. After a 10 minute spin at 3000 rpm (4°C), the culture volume was reduced to 1 ml, the pellet resuspended and seeded onto amp/IPTG NG agar plates (50 µg/ml ampicillin, 1 mM IPTG). Induction of dsRNA production was achieved over night at room temperature.

When a greater amount of plates of one gene was needed and/or to achieve a stronger induction of dsRNA, a different protocol was used. HT115 were grown in 25 ml LB medium including 100 µg/ml ampicilin and 15 µg/ml tetracycline to an $OD_{595}$= 0.4 at 37°C. Induction of siRNA production was achieved through addition of 20 µl IPTG (0.5M) to each culture. Then bacteria were grown for additional 4 h. Afterwards, each culture was supplemented with 100 µg/ml ampicilin, 15 µg/ml tetracycline and 20 µl IPTG (0.5M). After a 5 minute spin at 2000rpm, the culture volume was reduced to 7.5 ml, the pellet resuspended and bacteria were seeded onto tet/amp plates (100 µg/ml ampicilin, 15 µg/ml tetracycline).

For RNAi standard experiments 2 L4 larvae of wild type, the RNAi sensitive strain *rrf-3(pk1426)* and/or chosen mutant strains were transferred onto the RNAi plates and grown at 20°C or 25°C depending on experiment. The empty vector L4440 was used as a negative control. The F1 generation of RNAi animals were scored depending on phenotypes between the second and fourth day after transfer by dissecting microscope or DIC.

## 3.8. Life span assays

Adult life span assays were performed at 20°C as described by Gems (Gems et al., 1998). Day 0 corresponds to L4 stage. Nematodes were scored for survival every second day. They were counted as dead when they failed to respond to touch or prodding. If they crawled off, exploded or died as bags of worms, they were censored at the last day of life. Nematodes were grown under standard conditions (see above) and transferred to fresh plates every second day until egg laying ceased. Afterwards they were transferred every 7 days until death.