

3 MATERIALS AND METHODS

3.1 *Escherichia coli* strains and culture media

E. coli strains DH5 α (GibcoBRL) and TOP10 (Invitrogen) were used for plasmid amplification. The *E. coli* strain HT115 was used for amplification and expression of RNAi plasmid constructs. *E. coli* transformants were cultured at 37°C in Luria-Bertani (LB) medium (Sambrook et al., 1989) supplemented with either 100 μ g/ml ampicillin or 50 μ g/ml kanamycin.

DH5 α	sup E44 Δ lacU169 (\square 80 lacZ Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i> (Hanahahn 1983; Beteshda Research Lab. 1986)
TOP10 F ⁻	<i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) \square 80lacZ \square M15 Δ lacX74 <i>deoR recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL</i> (Str ^R) <i>endA1 nupG</i> (Invitrogen)
HT115	W3110, <i>rnc14::Tn10</i> (Timmons, 2001).

3.2 Plasmids

3.2.1 Plasmid constructions

The plasmids used in this study are listed in Table 1, which also presents a description of the cloning strategy and the features of the plasmid.

Table 1) Plasmids

Plasmid	Features / Construction / Reference
PBluescript II SK (+/-)	2.961 kb; Amp (Stratagene)
PCR3band clone16 (A1) PCR10 clone 16.2 (A1) PCR3/10 (A3) PCR8 clone 6 (B)	<i>daf-12</i> cDNA, isoforms A1, A2 and B. 1 st round; 3.7 kb (<i>daf-12A1</i>), 3.6 kb (<i>daf-12A2</i>), and 2.2 kb (<i>daf-12B</i>) fragments were PCR amplified with the primer SL1+external adapter and F11A1,37088-; 2 nd round PCR, fragments were PCR amplified with the primers external adapter and F11A1, 38383-, restricted with BamHI/PstI and ligated with BamHI/PstI restricted pBluescript SK (+/-) (Antebi et al., 2000).
pALL-1	<i>daf-12A1</i> cDNA without mutations; 5.43 kb; Amp, 216 bp Nsi I/Stu I fragment of PCR3band1clone16 was ligated with Nsi I/Stu I restricted PCR10 clone 16.2; this work.
pALL-2	5.43kb; amp; 500 bp ApaI /BspE I fragment from PCR3band clone6 was ligated with ApaI /BspE I restricted pALL-1; this work.
pALL-A3	<i>daf-12A3</i> cDNA without mutations; 3.8 kb; <i>daf-12A3</i> cDNA, Amp, 0.3 kb Stu I/SspB I fragment from PCR3/10 was ligated with Stu I/SspB I restricted pALL-2 (this work).
pALL-12B	<i>daf-12B</i> cDNA without mutations; 3.8 kb; Amp, 0.7 kb BamH I/Aat II fragment from PCR10 clone16 was ligated with BamH I/Aat II restricted PCR8a clone6 (this work).
pBTM117c	.35 kb; LexA Trp-1 Amp CAN1 (Wanker et al., 1997).
pCR Blunt TOPO XL	3.5 kb; Kan, Zeo (Invitrogen).
pCR-BluntII-TOPO	3.519 kb; Kan, Zeo (Invitrogen)
pBTM117c-DAF-12HL	10.74 kb; DAF-12 fragment 879-2291bp was PCR amplified with the primer pBTM117cLP5 and pBTM117cLp3-2 from pALL-2 and Kpn I cloned into pBTM117c (this work).

Table 1) Plasmids, continued

Plasmid	Features / Construction / Reference
pBTM117c-DAF-12A1	11.61 kb; daf-12 DAF-12 fragment 1-2291bp was PCR amplified with the primer pBTM117cLP3-2 and pBTM117c-FL from pALL-2 subcloned into pCR-BluntII-TOPO and Kpn I cloned into pBTM117c (this work).
pBTM117c-DAF-12A3	11.60 kb; DAF-12 fragment 1-2243bp was PCR amplified with the primer pBTM117cLP3-2 and pBTM117c-FL from pALL-A3, sub-cloned into pCR-BluntII-TOPO, cut with Kpn I and ligated with Kpn I restricted pBTM117c (this work).
pBTM117c-DAF-12 LBD	10.85 kb; 1.5 kb fragment was PCR amplified with the primer pBTM 117c3-2 and daf-12-m419-c-rev-Kpn from pALL-2, subcloned into pCR-BluntII-TOPO and Kpn I cloned into pBTM117c (this work).
pBTM117c-DAF-12rh61	10.2 kb; 1.85 kb fragment was PCR amplified with the primer pBTM117c5-2 and daf-12f1-Brev-Kpn from pALL-2, subcloned into pCR-BluntII-TOPO, cut with Kpn I and ligated with Kpn I restricted pBTM117c (this work).
pBTM117c-DAF-12rh61-3	10.1 kb; 0.76 kb fragment was PCR amplified with the primer pBTM117c3-2 and daf-12-f1-Bfw-Kpn from pALL-2, subcloned into TOPO blunt, cut with Kpn I and ligated with Kpn I restricted pBTM117c (this work).
L4440	RNAi feeding vector; 2.790 kb, Amp, T7 promoters (A. Fire, 1999).
L4440-31,-32 -23, -55, -65, -59	Different sizes, pACT- II-I/Bgl II fragments from the yeast- twohybrid screen I (Figure 8, Table 5) were ligated with Bgl II restricted L4440 (this work).
L4440 -27-1, -140, -195, -170, -128, -73, -116	Different sizes, pPC86-III/Sal I/Not I fragments from the yeast- two hybrid screen II (Figure 8, Table 6) were ligated with Sal I/Not I restricted L4440 (this work).
L4440- V-3, - V-10	Different sizes, pACT- II-V/Bgl II fragments from the yeast- two hybrid screen III (Table 2, 7) were ligated with Bgl II restricted L4440 (this work).
L4440-VI-20, 33, 34, 38	Different sizes, pACT- II-VI/Bgl II fragments from the yeast- two hybrid screen IV (Table 2, 8) were ligated with Bgl II restricted L4440 (this work).
L4440-DIN1'3end	<i>din-1</i> fragments 29336- 30711; 29336- 30417; 29280- 30711; 29280- 30417 were PCR amplified, subcloned into pCR-BluntII-TOPO, cut with Sal I/Not I and ligated with Sal I/Not I restricted L4440 (this work).
Topo-DIN1-I Topo-DIN1-II	5.72 kb; kan, zeo, 1.15 kb fragment was PCR amplified from cosmid F07A11 with the primer mint.Cterm and mint.Cterm-not and subcloned into pCR-BluntII-TOPO (Topo-DIN-1-I). A 1.05 kb Fragment was PCR amplified with mint.3utrfor and mint.3utrev, cut with Not I/Xba I and ligated with Not I/Xba I restricted Topo-DIN-1-I (this work).
Topo-DIN-1::GFP	6.9 kb; a 1167 bp Not I fragment fragment from pCCM117 (gfp vector) was ligated with Not I restricted Topo-DIN1-II (this work).
pASZ-11	5,76 kb; ADE2 ; Amp; (Stotz and Lindner, 1990).
L3781	gfp vector; 3.781 kb; Amp.
L3781::din-1 promoter	11.38 kb; Amp, 8 kb fragment was PCR amplified from cosmid F07A11 with the primer AgeI-zk20.12055 and notI-zk20.20003, cut with Not I/Age I and ligated with Not I/Age I restricted L3781 (this work).
Topo 5'end Ex11	5.1 kb; kan, zeo, RNA isolated from N2 was amplified with the primer F07A11.28260; cDNA was amplified with the primers zk20.12055 and zk20.4845, 2.5 kb fragment was subcloned into pCR-BluntII-TOPO (this work).
Topo 3'end Ex11	8.7 kb; kan, zeo, RNA isolated from N2 was amplified with the primer F07A11.28260; cDNA was amplified with the primers F07A11.29280 and F07A11.cDNA2583/ApaI; 5.2 kb fragment was subcloned into pCR-BluntII-TOPO (this work).
Topo-din-1cDNA	11.2 kb; kan, zeo, 5.2 kb Xba I/Apa I fragment from Topo 3'end Ex11 was ligated with Xba I/Apa I restricted Topo 5'end Ex11 (this work).

Table 1) Plasmids, continued

Plasmid	Features / Construction / Reference
din-1 cDNA::GFP	11.18 kb; Amp, 7.7 kb Nhe I/Apa I fragment from Topo-din-1cDNA was ligated with Nhe I/Apa I restricted L3781 (this work).
Topo-din-1 D	8.5 kb; kan, zeo, 5 kb fragment was PCR amplified with the primers F07A11.33195 and F07A11.28230 from the cosmid T14A11 and subcloned into pCR-BluntII-TOPO (this work).

For plasmid construction the following compounds were used:

Restriction enzymes	New England Biolabs; Boehringer Mannheim
Polymerases :	Taq Polymerase (Perkin Elmer), Hot Start Turbo (Stratagene), Taq Plus long (Stratagene)
PCR system:	Failsafe (Epicentre Technologies)
Cloning:	Rapid DNA Ligation Kit (Roche), Shrimp alkaline phosphatase (USB), Qiaquick PCR purification kit (QIAGEN); Qiaquick Gelextraction kit (QIAGEN)

Restriction digestion, PCR and cloning steps were performed according to the user manuals, gelelectrophoresis was done according to Sambrook et al., 1989.

Kits for plasmid DNA isolation

QIAGEN Plasmid Mini Kit and Plasmid Maxi Kit (QIAGEN) were used to isolate plasmid clones from bacteria; QIAGEN Plasmid Midi Kit was used to isolate plasmid DNA, cosmids and YACs.

3.2.2 Plasmids isolated from cDNA libraries in yeast- two- hybrid screens

Table 2) Plasmids isolated from cDNA libraries in yeast- two hybrid screens with *daf-12* bait constructs

Clones, isolated in the yeast- two hybrid system from a <i>C. elegans</i> cDNA library (R. Barstead), Screen I, III, IV.		Clones, isolated in the yeast- two hybrid system from a <i>C. elegans</i> cDNA library (GIBCO), Screen II	
Plasmid	Insert	Plasmid	Insert
pACT-II	7.65 kb, 2 micron ori; LEU2; Amp, ADH promotor activation domain	pPC86	7.093Kb; Trp1; Amp,ADH:Gal4A D; ARS- CEN
pACT-II-I-33, 64, 55	T13B5.1	pPC86-III134,135,10,89...	T07C4
pACT-II-I-B1-1, 31,32, 37	F07A11.6	pPC86-III-87	C14A4.3
pACT- II-I-59, 62	T09E8.1	pPC86-III-27-1	C44C10.1
pACT- II-I-65, 61, B3	B0228. 2	pPC86-III-195	M03F4.7
pACT- II-I-23	ZC8.2	pPC86-III-170	F09G2.2
pACT- II-V-3, 6	T05C1.6	pPC86-III-128	K10B3
pACT- II-V-10, 11, 17,19	M04B2.1	pPC86-III-102	R1075.5
pACT- II-VI-20, 21, 40	D2085.5	pPC86-III-73	Y6D1A
pACT- II-VI-33, 31	F39H11	pPC86-III-116	C53B4.4
pACT- II-VI-34, 36, 37	ZK270.1		
pACT- II-VI-38	R144.7		
pACT- II-VI-43	R03G5		

3.3 Primer

Table 3) List of the primer used for the plasmid construction.

Primer	Sequence
32073L	5'-ctgtgagaaagactgctcatcc-3'
AgeI-zk20.12055	5'-ggaccggctcacattgcaaaccgcgattc-3'
daf-12-fl-Bfw-Kpn	5'- ggggtaccaatgggcacaaatggaggag-3'
daf-12fl-Brev-Kpn	5'- ggggtaccattacccttaattgttggtctt-3'
daf-12-m419-c-rev-Kpn	5'- ggggtaccattaatacggatcagattgatattg-3'
external adapter	5'-aactgcagccgcgctcgag-3'
F07A11.28230.SpeI	5'-actagtgcagccgggactgtttctgt-3'
F07A11.29258	5'-tttgggttggtgaattggc-3'
F07A11 28921	5'-tgagaaagcgggtgtccaaaagaca-3'
F07A11.29280	5'-gggggcccaggaaataagctcttctcg-3'
F07A11.32602	5'-tgtccttgaccgatcattgcatg-3'
F07A11.cDNA2536/ApaI	5'-acgatgccgattgtcccaccgc-3'
F11A1, 38383 -	5'-cgggatccgttgtaggtattgaaggcat-3'
F11A1,37088-	5'ggctagattgagttgttgatggaggc-3'
L4440-as	5'-gcgactgacccttttgggaccg-3'
L4440-s	5'-acgactcactataggagaccgg-3'
mint.3utrfor	5'-gcgccgcctaatacaggaagaccctatttctctg-3'
mint.3utrev	5'-tctaga gttccggccatccgatttc-3'
mint.C-term	5'- ccgcgggaggtcgcggtgcacttttg-3'
mint.C-term.not	5'-gcgccgccttggtactgcttactggtggaac-3'
NheI- zk20.12055	5'-gggctagcatggtcaagtaccttctacaaactgta-3'
NotI/zk20.20003 r	5'-gggcggccgccgcaagaaactcacaatc-3'
notI-zk20.20003	5'-gggcggccgccgcaagaaactcacaatc-3'
pACT-2fw	5'-gtgcacgatgcacagttgaagtgaacttgcg-3'
pACT-2rev	5'-catac gatgtccagattacgctagc-3'
pBTM117c3-2	5'-gggtacctttgattttgaaaaattctctggcag-3'
pBTM117cDAF-12fl-5	5'-gggtaccaatgggcacaaatggaggga-3'
pBTM117cLP5	5'-gggtacctccattgacatccccagtc-3'
pPC86as	5'-gtaaatttctggaaggtag-3'
pPC86s	5'-tataacgcgtttggaatcact-3'
SL1	5'- ggttaattaccaagttgag-3'
SL1 + external adapter	5'aactgcagccgctcgagggttaattaccaagttgag-3'
zk20.4845	5'-cgccagatgtcatggactgcg-3'
NheI/zk20.12055	5'-gggctagcatggtcaagtaccttctacaaactgta-3'

Table 4) List of the primer for *din-1* sequence analyses.

Primer	Sequence
FO7A11.29758L /1R	5'-gcatcgcaaatctcactcc-3'
FO7A11.30266/2R	5'-cattcccctccactccgatg-3'
FO7A11.30712/3R	5'-ccgagatataagccgtcacag-3'
FO7A11.30732R4L	5'-ctgtgacggcttatatctcgg-3'
FO7A11.31489L/5R	5'-gtttttgtgctcggggggc-3'
FO7A11.332986L/8R	5'-gccagattgcaagggaagctg-3'
FO7A11.33007R/9L	5'-cagcttccctgcaaatctggc-3'
FO7A11.33583R	5'-cggtcgactcttatctg-3'
ZK20.1297L/13R	5'-cgatcctgttgattgtctgtg-3'
ZK20.1798L/ 14R	5'-ggcgtctgctctggcgattc-3'
ZK20.1817R/15L	5'-gaatgccagagcagacc-3'
FO7A11.35117/16R	5'-ctccccgagaggagtca-3'
ZK20.2869L/ 17R	5'-ctggtcctgttcttgggac-3'
ZK20.3392R/19L	5'-gcatcgtcaattagtgatcacg-3'
ZK20.3874L/20R	5'-ctgccgttctattctctcg-3'
ZK20.4556L/21R	5'-cgacgagctcagttccc-3'
ZK20.5390L/25r	5'-ctctcaatggatggctcaattg-3'
ZK20.5908L/26r	5'-ggtgtagtgcgagtagacag-3'
ZK20.6384L/27r	5'-cgcggggctcgtgaaacac-3'
ZK20.6930/29R	5'-ctcaccaccattagattcgtctc-3'
ZK20.7381L/30R	5'-cctgtatcttggtgccagtag-3'
ZK20.7845L/31R	5'-gagcaaaagtcagggccacc-3'
ZK20.7864R/32L	5'-gtggccgtcactttgctc-3'
zk20.9060	5'-cgtccaaccagctcatccgt
zk20.9114	5'-agcgttaattaccgacttcgatc
ZK20.6402R/28L	5'-gtgttccagagccccgcg-3'
ZK20.10334	5'-gtgatcattgctgaaggcaggt-3'
ZK20.10552	5'-gcctgtaataatggacgcgt gc-3'
ZK20.10831	5'-tgccgtggtgagacaaatcgta-3'

3.4 Yeast methods

Media for the growth of *Saccharomyces cerevisiae* were described previously (Guthrie and Fink, 1991). Minimal medium (YNB) was supplemented as appropriate with 20 µg/ml for adenine, uracil, tryptophan and histidine or 30 µg/ml for leucine and lysine. The yeaststrain used in the two- hybrid screen is L40ccu was kindly provided by Prof. Dr. Erich Wanker. For homologous recombination of GFP tagged *din-1* in yeast, the yeast

strain Y7G9 was used (Sanger Center). Growth and manipulation of yeast was carried out according to standard procedures (Guthrie and Fink, 1991). Yeast transformations were performed according to Ito et al., 1983.

3.5 Yeast- two hybrid screening

To identify DAF-12 interacting proteins, we used the version of the yeast two- hybrid-system previously described by Wanker et al., 1997 to screen c-DNA libraries. A LexA-DAF-12 hybrid containing full length DAF-12 and a truncated DAF-12, comprising aa 334-759, were used as "bait", and as "prey" we used *C. elegans* cDNA libraries (R. Barstead and GIBCO). A total of approximately 10^8 independent transformants were plated on minimal medium lacking histidine and uracil. After incubation for 5-9 days at 30°C, His⁺, Ura⁺ colonies were picked and tested for β-galactosidase activity. Plasmids from positively tested clones for expression of *URA3*, *HIS3* and β- galactosidase were isolated (QIAGEN), amplified in *E. coli*, retransformed into the two- hybrid tester strain, and tested for autoactivation and interaction with LexA. Inserts of candidates that specifically interacted with DAF-12 were compared by Hind III restriction digestion and sequenced with the primers pACT-2fw and pACT-2rev or pPC86-as and pPC86s. Sequence analyses were done by SEQLAB Sequence Laboratories Göttingen GmbH (cycle sequencing) and Dr. M. Meixner, FU Berlin.

3.6 *C. elegans* cDNA libraries

In the yeast- two hybrid screens, oligo dT and random primed mixed stage *C. elegans* cDNA libraries kindly provided by Robert Barstead were used. The cDNA library fragments had been cloned into the Xho I site of the vector λ ACT. cDNA clones were excised from phage stocks by mixing 1×10^8 plaque forming units from the library with logarithmically growing *E. coli* strain RBE4 (Elledge, 1993) and 10 mM MgCl₂. The mix was incubated at 30°C. After addition of 2 ml LB medium cells were incubated 1' with shaking. 200 µl of the cells were spread on each of 10 150 mm LB plates containing 50 µg/ml ampicillin and 0.2% glucose and incubated over night at 37°C. Colonies were resuspended in 10 ml LB/plate; pooled bacteria were used to inoculate 3 liters TY medium (Sambrook, 1989) containing 50 µg/ml ampicillin. The culture was grown to stationary phase and plasmid preps were done with QIAGEN Giga prep kit, according to the user

manual. Additionally, a commercially available oligo dT and random primed cDNA library provided by GIBCO was used.

3.7 Database analyses

To evaluate the candidate genes from the yeast- two hybrid screens for functions in the regulation of transcription, database searches were performed. Protein BLAST alignments (Altschul et al., 1997; www.ncbi.nlm.nih.gov/BLAST) of the complete targeted protein, as well as Protein BLAST alignments of the Two- Hybrid- interaction domain were performed. In addition, protein analyze programs like Pfam (Bateman et al., 1999; Sonnhammer et al., 1998; www.sanger.ac.uk/Software/Pfam/; 5-11-2002) and Psort (Horton and Nakai, 1996; psort.nibb.ac.jp/; 5-11-2002) were used to detect nuclear localization sites and functional domains typical of proteins involved in transcriptional regulation.

3.8 *din-1* cDNA clones

Yuji Kohara (National Institute of Genetics, Mishima, Japan) provided the following clones encoding *din-1* cDNA: YK501h2, YK297g11, YK132c3, YK96e12, YK38b7, and YK1121c03. YK clones were excised from phage stocks: XL-1 Blue cells were grown in TY medium containing 0.2% maltose and 10 mM magnesium sulfate to an $OD_{600}=1.0$. They were spinned at 1000 xg for 10'. Cells were resuspended in 10ml 10mM magnesium sulfate and mixed with 200 μ l XL1-Blue-cells, 200 μ l of phage stocks and 10 μ l of the helper phage VSCM13 and incubated for 15' at 37°C. After addition of 5 ml TY, the mix was incubated for another 3h with shaking and subsequently incubated for 20'at 70°C. The culture was spinned for 15' with 1000 xg and the phage supernatant was decanted into a sterile tube. After addition of 1ml TY and incubation at 37°C for 30', cells were spinned down and resuspended in 200 μ l TY. Cells were plated on LB plates containing ampicilin and grew over night. Finally, plasmids were isolated from bacteria (QIAGEN).

The cosmid clones T14A11, F07A11 and ZK20 and yeast strain Y7G9 were provided by the Sanger Center, Hinxton, UK.

3.9 Preparation of *C. elegans* mix-stage RNA

To isolate RNA from different *C. elegans* strains, standard protocols were applied, described by Krause et al., 1995. Worms were grown on 20x10 cm NG plates coated with 2% agarose and OP50 bacteria at 20°C for 3-4d. They were washed from the plates in M9 buffer and after settling for 1.5h by gravity, the pellet was washed with M9, spinned for 10' at 4K and washed again in M9. 1ml of acid-washed sand and 3ml-triazol reagent was added to the pellet, then it was vortexed for 10'. After settling for 5', 0.6ml chloroform was added and the mix was spinned down for 15' at 4°C at 14000 rpm. The aqueous phase was transferred into a fresh tube and 1.5 ml isopropanol was added. After 10' of settling, the probe was spinned for 10' at 14000 rpm, pellet was washed with 75% ethanol, air-dried and resuspended in 100µl diethyl pyrocarbonate (DEPC) treated water. The resulting RNA was used for RT-PCR.

3.10 Full length *din-1* cDNA

din-1 cDNAs were obtained by RT-PCR as described (Antebi et al., 2000). To synthesize the *din-1* 5'end, RNA isolated from *C. elegans* N2 strains was amplified with the *din-1* specific primer F07A11.28260 using Super ScriptTM First Strand Synthesis System. For RT-PCR (Invitrogen) cDNA was amplified with the primers NheI/zk20.12055 5'-gggctagcatg gttcaagtaccttctacaaactgta-3' and zk20.4845 5'-cgccagatgcatggactgcg-3'. The 2.5 kb PCR fragment contained *din-1* cDNA from exon 11 (position 4845 according to cosmid zk20) to the *din-1* 5'-end. It was subcloned into pCR-BluntII-TOPO. The resulting clone, Topo5'end Ex11 was analyzed by sequencing with the primers M13 forward, ZK204556, ZK209114 and M13 reverse.

To obtain the *din-1* 3' end, RNA isolated from N2 strains was amplified with the *din-1* specific primer F07A11.28260. cDNA was amplified with the primers F07A11.cDNA 2536ApaI 5'-acgatgccgattgtcccaccgcc-3' and F07A11.29280 5'-gggggcccaggaaataa-3'. The 5.2 kb PCR fragment contained *din-1* cDNA from exon 11 (position 5090 according to cosmid zk20) to the *din-1* 3'-end. The PCR fragment was subcloned into pCR-BluntII-TOPO vector and the resulting plasmid, Topo3'end Ex11 was analyzed by sequencing with the primers M13 forward, M13 reverse, FO7A11.30266/2R, FO7A11.30732R4L, FO7A11.32492L/7r, FO7A11.33007R ZK20.1297 L/13R, FO7A11.35117/16R, ZK20.3392R/19L.

A *din-1A* full length 7.7 kb cDNA containing clone was generated by ligating a Xba I/ Apa I fragment from Topo3'end Ex11 into Xba I/ Apa I restricted Topo5'end Ex11, resulting in the plasmid Topo-din-1cDNA. For the determination of the 3' end of *din-1* isoform D, RNA isolated from N2 strain was amplified with the *din-1* specific primer 29258 5'-tttgggttggtgaattggc-3'. Subsequently, exon 18 was transspliced with an external adapter/SL1 leader sequence (5'-aactgcagccgagg ctgaggggttaattaccaatttgag-3' and the *din-1d* specific primer 32073L 5'-ctgtgagaaagact gctca tcc-3' and 32602 5'-tgccttgaccgatcattgcatg-3', resulting in two fragments of 1.2 and 1.7 kb and 0.6 and 1.1 kb.

3.11 Nematode culture

Except where noted otherwise, nematodes were cultured at 20°C on NGM media inoculated with *E. coli* strain OP50 (Sulston and Hodkin, 1988).

NG-medium:	6g	NaCl
	5g	Peptone
	33.7g	Agar (Difco)
	1.925 liter	Bidest H ₂ O
	50 ml	1 M KPO ₄
	2 ml	5 mg/ml Cholesterol in 95% EtOH
40' autoclaving,	4 ml	0.5M CaCl ₂
	2 ml	1M MgSO ₄

For RNAi plates: 100 µg/ml ampicilin, 15 µg/ml tetracyclin.

3.12 *C. elegans* strains

The *C. elegans* strains used in this study were wild type N2 Bristol, *daf-2 (e1368)*, *daf-2(e1370)*, *daf-2(m41)*, *daf-7(e1372)*, *daf-9(rh50)*, *daf-11(m47)*, *daf-12(rh274)*, *daf-12(rh61)*, *daf-12(rh61rh411)*, *daf-12(rh273)*, *lin-15(n765)*, *dre-1(dh99)*, *daf-12(rh61)*, *dpy-10(e128)*, *daf-12(rh61 rh411)*; *daf-2 (e1370)*.

In this study, the *C. elegans* strains *din-1(dh118)*, *din-1(dh127)*, *din-1(dh128)*, *din-1(dh149)*, *din-1(dh181)* have been isolated, another *din-1* strain, *din-1(sa1262)* was provided by Jie Li, University of Washington, Seattle, USA.

The *din-1* strains were crossed according to Sulston and Hodkin, 1988. The resulting double mutant strains are: *din-1(dh127)*; *daf-2(e1368)*, *din-1 (dh127)*; *daf-2(e1370)*, *din-1 (dh127)*; *daf-9(dh6)*, *din-1(dh127)*; *daf-12(rh61)*, *din-1(dh127)*; *daf-12(rh273)*, *din-1(dh127)*; *daf-12 (rh61)*, *din-1 (dh128)*; *daf-12(rh61)*, *din-1 (dh118)*; *daf-12(rh61)*, *din-*

1(dh127); daf-2(m41). All new strains were frozen as described by Sulston and Hodkin, 1988. Animals were scored and handled using a Leica MZ8 dissection microscope.

3.13 RNAi

3.13.1 RNAi plates

The plasmid clones of interest were digested with appropriate restriction enzymes, cloned into the multiple cloning site of the RNAi expression vector L4440 (Fire, 1999) and transferred into competent *E. coli* HT115 cells.

The transformed HT115 cells were grown in LB Medium with ampicillin (100 µg/ml) and tetracyclin (15 µg/ml) to an OD₅₉₅ = 0.4. T7 promoter activity was induced by supplementation of 4 µg/ml IPTG. The cells were grown at 37°C for 4h and supplemented with another 100 µg/ml ampicillin, 15 µg/ml tetracyclin and 4 µg/ml IPTG. Then the culture volume was reduced to 10 ml; cells were seeded on 10 cm NG plates containing 100 µg/ml ampicillin and 15 µg/ml tetracyclin.

3.13.2 RNAi feeding assays

To assess worms to RNAi, 3-5 young adults were transferred on plates seeded with HT115 bacteria expressing L4440 constructs. After incubation at 15-25°C for 1-5 d, both parameters vary with experimental conditions; the phenotypes of the F1 generation were scored by DIC and dissection microscopy.

3.14 DIC-microscopy

Worm slides were prepared according to Sulston and Hodkin, 1988. Animals were scored by Normarsky microscopy using a Zeiss Axioscope 2 plus microscope. Photographs were taken with a Sony progressive 3CCD digital camera and a Hamamatsu orca ER digitale CDD camera. Adobe Photoshop was used to adjust brightness and contrast.

3.15 EMS mutagenesis screens

The *din-1* alleles *dh118*, *dh127*, *dh128* and *dh149* were isolated after ethylmethane sulfonate (EMS) mutagenesis in F₂ screens for reversion of *daf-12(rh61)* mutants with

distal tip cell migration phenotypes (Antebi et al., 1998). The *din-1* allele *dh181* was isolated in a similar screen for reversion of *daf-12(rh274)* mutants. Approximately 10^5 genomes were mutagenized with 0.5% EMS in 4 ml M9 buffer; incubation time was 4 h15'. After the mutagenesis, worms were washed 3 times in 4 ml M9 buffer. The pellet was transferred to 10 seeded NG plates. 12 h later, 20 young adults were transferred to each of 35x 6cm NG plates and incubated at 20°C for 7 days (F1 generation). Four portions of each plate were distributed to fresh plates. After another 4 days, 2 portions of each plate were transferred to fresh plates. The resulting 280 plates (F2 generation) were scored for non-Mig phenotypes. Maximal 2 worms were cloned from each candidate plate. To test, if the non-Mig animals were linked to the X chromosome, candidate lines were outcrossed to *+dpy-10* males (Figure 6). Cross males with Mig phenotype were extragenic, cross males with non-Mig phenotypes were intragenic suppressers of the Mig phenotype. 10-15 hermaphrodites of extragenic lines were cloned, and the F1 generation was scored for segregation of Dpy phenotype, identifying cross hermaphrodites. From these plates, 24 Migs were cloned and the next generation was scored for segregation of Dpy, Migs, and non- Migs. Non- Migs were cloned, those that only segregated non-Dpy were saved and backcrossed to *daf-12(rh61)* to double check suppression. The localisation of the mutations was analyzed by sequencing.

3.16 Worm Mini-prep

Mutant strains were tested for mutations in the *din-1* locus by sequence analyses. Therefore, genomic *C. elegans* DNA was isolated and amplified with *din-1* specific primers, following the protocol described by Sulston and Hodkin, 1988. Worms were washed off a plate that had been grown for 3-4 d at 20°C with 1 ml TE and settled for 5-10' by gravity. for 10'. After removing the supernatant, worms were washed in TE and frozen at -80°C. After thawing at room temperature, 50 µl 2x lysis buffer (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 diH2O) was added and worms were frozen again for 10' at -80°C. The tube was placed at 65°C for 1h, than proteinase K was inactivated by 5' incubation at 95°C. The lysate was spinned in a microfuge 5' at 14000 rpm, the supernatant was transferred into a fresh tube and incubated for 20 'at 37°C after RNase (5 mg/ml diH2O) was added. For

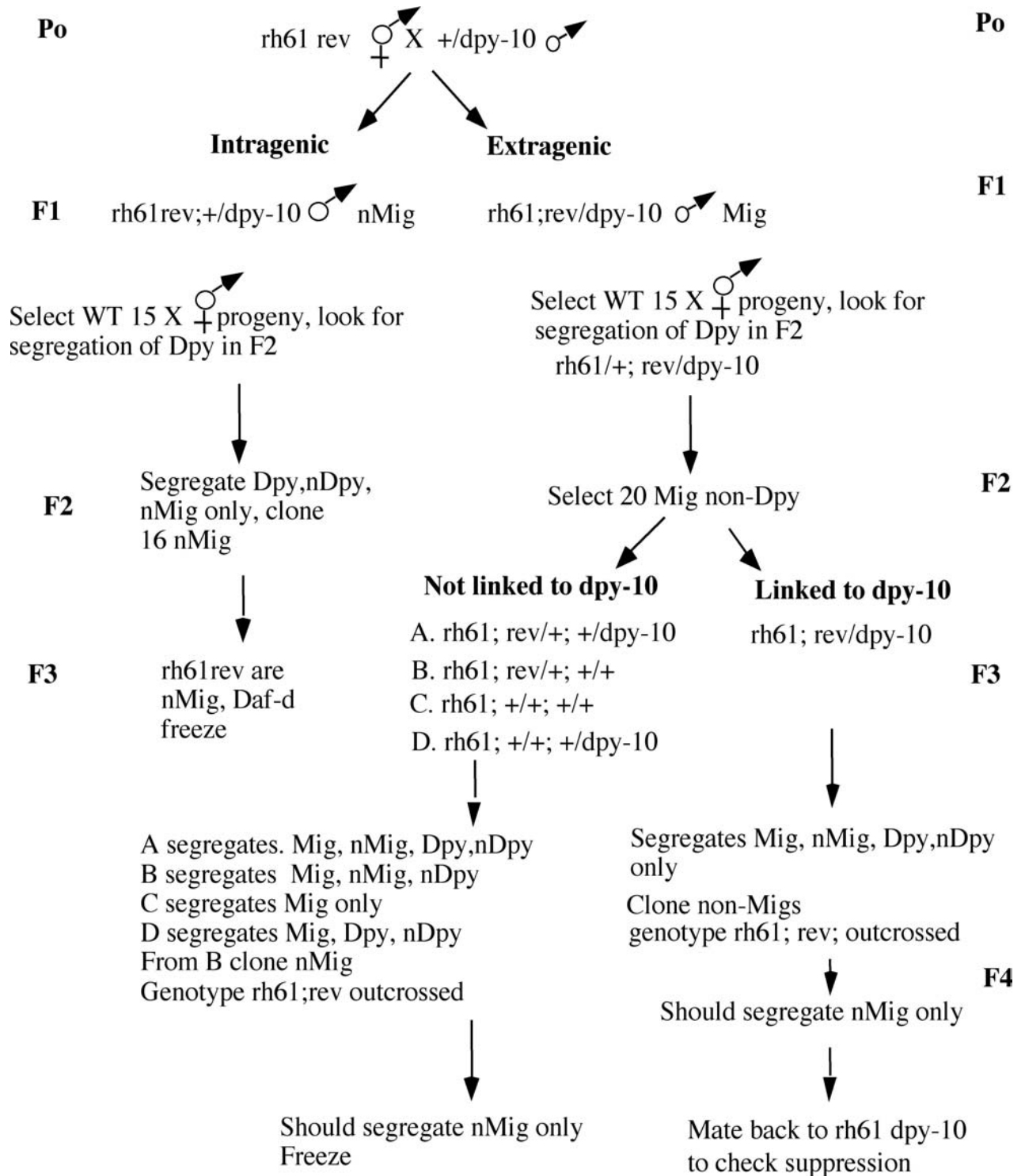


Figure 6) *daf-12(rh61)* reversion scheme.

subsequent PCR, 2 µl/reaction were used. The PCR fragments were subcloned into pCR-BluntII-TOPO and/or sequenced with appropriate primers listed in table 4.

3.17 Phenotypic analyses

To synchronize worm cultures, 10 gravid adult animals per plate were laid eggs for 4 h and removed. Progeny was scored after 4 d at 25°C.

Brood size

To compare brood sizes of *din-1* mutants and *din-1; daf-12(rh61)* double mutants with those of wild type, synchronous populations were grown at 15°C until the late L4 stage. Ten animals were placed singly on plates and shifted to 20°C. These P0 animals were transferred to new plates every 24 h until the end of the reproductive period. Each plate was examined daily to follow development to a terminal phenotype. Any adults or L4 larvae were counted and removed.

L3 seam: N2, *din-1* and *din-1; daf-12(rh61)* double mutants were scored at L3 for the number of seam cells by DIC microscopy.

Adult seam: N2, *din-1* and *din-1; daf-12(rh61)* double mutants were scored as adults by DIC microscopy for the gaps in adult alae.

Dauer formation: Synchronous populations were scored for dauer formation after 4 d of growth at 25°C.

3.18 Life span assays

3.18.1 *din-1(dh118)*

Adult life span assays were performed as described (Gems et al., 1998). Day 0 corresponds to L4 stage. 200 hermaphrodite N2, *din-1(dh118)*, *daf-2(e1368)*, *daf-2(e1370)*, *daf-12(rh61rh411)*, *daf-2(e1370); daf-12(rh61rh411)* *din-1(dh118)*; *daf-2(e1368)*, *din-1(dh118)*; *daf-2(e1370)* were grown at 15° were picked (10/plate) and transferred to fresh plates seeded with *E. coli* OP50 bacteria every 2 days until they stopped reproduction. Afterwards they were transferred every 7 days until death.

Animals were scored as dead if they failed to respond to a tip on the head and tail with a platinum wire. Worms with internal hatching, exploders or animals that crawled off the

plate were excluded. Statistical analyses were performed with the Excel 98 Student's test and Mann-Whitney test.

3.18.2 *din-1(dh127)*; *din-1RNAi*

For N2 and *din-1(dh127)*, 100 hermaphrodites, for *daf-2(e1368)* and *din-1(dh127)*; *daf-2(e1368)* 200 hermaphrodites, for *din-1(dh127)*; *daf-2(e1370)* 300 hermaphrodites and for *daf-2(e1370)* 400 hermaphrodites (10/plate) were picked and put on plates seeded with *E. coli* HT115 bacteria either expressing empty L4440 RNAi feeding vector or L4440 *din-1RNAi*. They were transferred to fresh plates every 2 days until they stopped reproduction. Afterwards they were transferred every 7 days until death.

3.19 *DIN-1::gfp* construction

3.19.1 Genomic *din-1::gfp*

For *din-1::gfp* construction, a 1.15 kb genomic fragment containing the *din-1* 3' end was amplified from cosmid F07A11 with primers mint.C-term 5'-ccgcgagggtcgcggtgcacttttg-3' and mint.C-term.not 5'-gcgccgccctgtactgcttactggtggaac-3', and subcloned into pCR-Blunt II-TOPO vector, resulting in the plasmid Topo I. Next, a 1.054 kb fragment PCR amplified from F07A11 with the primers mint.3utrfor 5'-gcgccgccctaatcaggaagagcc ttatttctctg -3' and mint.3utrrev 5'-tctagagttgcccggccatccgatttc-3', was restricted with Not I/Xba I and ligated with Not I/Xba I restricted Topo I, resulting in the clone Topo-DIN-1 II. A Not I Fragment from containing the *gfp::sup4o* coding region from pCCM117 vector was ligated with Not I restricted Topo II. The resulting plasmid, Topo II *din-1::gfp* was Sac II/Bgl II digested. 5 µg of the gel-extracted fragment were transformed into the yeaststrain Y7G9, containing a YAC encoding genomic *din-1*. The transformants were screened for homologous recombination of the inserted fragment by selection on -ADE medium and DNA of positive clones was isolated (QIAGEN). Y7G9 *din-1::gfp* (10 ng/µl) and *lin-15(+)* coinjectible marker (Clark et al., 1994) (75 ng/µl) were injected into *lin-15(n765)* animals following the protocols described by Kimble et al., 1982 and Fire, 1986.

3.19.2 *din-1* Promotor region::gfp

An 8 kb fragment of the *din-1* promotor region was amplified from the cosmid F07A11 with the primers Age I-zk20.12055 5'-ggaccggtctcacattgcaaaccgcgatttc-3' and Not I-zk20.20003 5'-ggcgccgccgcaagaaactcacaatc-3', the obtained fragment was subcloned into TOPO XL vector (Invitrogen). The fragment was restricted with Not I/Age I and

ligated with the Not I/Age I restricted gfp vector L3781, fusing gfp in frame to the *din-1* promoter region. The resulting plasmid, L3781::*din-1* promoter (10-30 ng/μl) and *lin-15(+)* pL15.EK (15 ng/μl) were microinjected into the oogonia of *lin-15(n765)*. Transgenic F2 lines containing *lin-15(+)* extrachromosomal array were scored for GFP expression.

3.19.3 *din-1A* cDNA::*gfp*

To fuse *din-1* isoform A cDNA to GFP, a 7.7 kb Nhe I/Apa I fragment from Topo-*din-1*cDNA was ligated with Nhe I/Apa I restricted L3781, resulting in the clone L3781*din-1*cDNA::*GFP*. 10-30 ng/μl were microinjected with GFP(+)(10 ng/μl) coinjectible marker pTG96 into the oogonia of wild type animals. Transgenic F2 lines containing GFP(+) extrachromosomal array were scored for GFP expression.

3.20 *din-1 D* genomic construct

To get a genomic clone of the *din-1* isoform D, a 5kb PCR fragment was amplified from the cosmid T14A11 with the primers F07A11.33195.NheI-5'-gctagcgaggcagcaactgtaatggcag-3'and F07A11.28230.SpeI 5'-actagtgagccggtgactgtttctgt-3'.

The PCR fragment was subcloned into TOPO blunt vector revealing the plasmid Topo-*din-1D*. 10-30 ng/μl Topo-*din-1d* were micro injected with pTG96 GFP(+)(10ng/μl) coinjectible marker into the oogonia of *din-1(dh127);daf-2(e1368)* dauer constitutive animals. Transgenic F2 lines containing GFP(+) extrachromosomal array were scored for GFP expression and suppression of the Daf-c phenotype.