MFB-1, an F-box-type ubiquitin ligase, regulates TGF-β signalling

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TGF-β signalling regulates cell growth, differentiation, morphogenesis and apoptosis. MAFbx/Atrogin-1 has been identified as a regulator for skeletal muscle atrophy and encodes an F-box-type E3 ubiquitin ligase. However, little is known about how MAFbx/Atrogin-1 regulates cellular signalling. Here, we identify and genetically characterize MFB-1, a MAFbx/Atrogin-1 homologue from Caenorhabditis elegans. The mfb-1 deletion mutant significantly enhanced the dauer constitutive (Daf-c) phenotype caused by mutations in the DAF-7/TGF-β-like signalling pathway, but not the DAF-2/insulin receptor-like signalling pathway. Conversely, the Daf-c phenotypes of DAF-7 pathway mutants were partially suppressed by mfb-1 cDNA transgenes. Therefore, MFB-1 acts genetically downstream in the DAF-7 pathway. A mfb-1::GFP fusion was found to be expressed in the nervous system, hypodermis and intestine and overlapped expression of many DAF-7 pathway genes. We propose that MFB-1 is a novel F-box protein that negatively regulates dauer formation in concert with the DAF-7 signalling pathway in C. elegans.

Introduction

Members of the TGF-β superfamily regulate a variety of biological processes, including growth inhibition, differentiation, embryonic pattern formation and induction of apoptosis (Kingsley 1994; Moses & Serra 1996; Massague & Chen 2000). These secreted ligands bind to and activate heteromeric complexes of type I and type II serine/threonine kinase receptors. The activated receptors phosphorylate and activate intercellular SMADs, which then translocate into the nucleus and regulate the expression of target genes (Massague 2000).

In Caenorhabditis elegans, at least two TGF-β-related pathways have been genetically characterized (Patterson & Padget 2000): the DBL-1 pathway, which regulates body size and male tail development, and the DAF-7 pathway, which regulates the development of dauer larvae that arise in response to starvation or overcrowded conditions (Golden & Riddle 1984). Dauer-constitutive (daf-c) or -defective (daf-d) genes such as daf-1, daf-3, daf-4, daf-5, daf-7, daf-8 and daf-14 have been proposed to act in a common pathway in the regulation of dauer larva formation. DAF-7 is a TGF-β ligand (Ren et al. 1996), DAF-1 is a type I receptor (Georgi et al. 1990), DAF-4 is a type II receptor (Estevez et al. 1993) and DAF-8 and DAF-14 are SMADs (Inoue & Thomas 2000). Mutations in these genes can lead to a temperature-sensitive Daf-c phenotype, i.e. the formation of dauers even under favourable conditions (Riddle & Albert 1997). This Daf-c phenotype can be suppressed by mutations in DAF-3, which encodes another SMAD (Patterson et al. 1997) and DAF-5, which is a C. elegans homologue of Sno/Ski (Vowels & Thomas 1992; Thomas et al. 1993; da Graca et al. 2004). DAF2 encodes an insulin receptor family member and regulates the control of dauer development in parallel with DAF-7 signalling (Kimura et al. 1997).
Components of the DAF-2 insulin receptor-like pathway include the ligand DAF-28 (Li et al. 2003), the receptor DAF-2 (Kimura et al. 1997), AGE-1 as PI3K (Morris et al. 1996), PDK-1 as a homologue of mammalian PDK1 (Paradis et al. 1999), AKT-1/-2 as a homologue of mammalian AKT (Paradis & Ruvkun 1998), DAF-18 as PTEN (Ogg & Ruvkun 1998) and DAF-16 as a forkhead transcription factor (Lin et al. 1997; Ogg et al. 1997).

Recently, several groups have reported that some ubiquitin-dependent protein degradation systems regulate TGF-β superfamily signalling. Smurf1, a member of the HECT-type E3 ubiquitin ligase family, interacts with ligand-specific Smads (R-SMADs) in the BMP signaling pathway and mediates their ubiquitination and degradation (Zhu et al. 1999; Zhang et al. 2001). Smurf2 is also an HECT-type E3 ubiquitin ligase. Smad2 recruits Smurf2 to SnoN in a TGF-β-dependent manner, and thereby targets SnoN for ubiquitin-mediated degradation by proteasomes (Bonni et al. 2001). Moreover, the anaphase-promoting complex (APC), a type of RING-finger-containing E3 ubiquitin ligase, is involved in SMAD3-induced ubiquitination and degradation of SnoN (Stroschein et al. 2001). Therefore, TGF-β signalling is controlled by ubiquitin/proteasome-mediated proteolysis.

MAFbx (Bodine et al. 2001)/Atrogin-1 (Gomes et al. 2001) has been identified as an F-box protein, which are components of the SCF family of E3 ubiquitin ligases. MAFbx/Atrogin-1 is required for skeletal muscle atrophy and appears to control muscle protein degradation through a ubiquitin-proteasome pathway, although its exact mechanism of action is unknown. We have recently identified MAFbx/Atrogin-1 as a TGF-β-inducible gene (H. Ide, S. Higuchi and T. Akiyama, unpublished results). Here, we report the isolation of mfb-1 (MAFbx-1), a C. elegans homologue of MAFbx/Atrogin-1. Genetic analysis showed that deletion of the mfb-1 gene significantly enhanced the Daf-c phenotype of DAF-7 pathway mutants. Conversely, over-expression of mfb-1 cDNA partially suppressed the Daf-c phenotype, but did not affect the DAF-2 pathway. Our results suggest that MFB-1 is a novel F-box protein involved in the DAF-7/TGF-β pathway, and that it negatively regulates dauer formation through the ubiquitin-proteasome system.

**Results**

**Isolation of mfb-1**

We searched a C. elegans genome database (AceDB) for genes with high homology to human MAFbx/Atrogin-1 and identified a single gene, DY3.6, which we designated as mfb-1. We isolated the mfb-1 cDNA from a C. elegans cDNA library by PCR, using primers based on the predicted mfb-1 coding sequence from the C. elegans genome database. The full-length mfb-1 cDNA consists of a 29-bp 5′ UTR, followed by a spliced leader 1 (SL1) sequence, and sequences encoding a 379-amino acid protein that is 26% identical to MAFbx/Atrogin-1 (Fig. 1A, Gomes et al. 2001). In addition to the MAFbx/Atrogin-1 homology, mfb-1 contains a predicted F-box domain and a Class II PDZ-binding motif in its carboxyl terminal region (Fig. 1B).

**MFB-1 regulates DAF-7 signalling pathway-mediated dauer formation**

To investigate the in vivo function of MFB-1, we screened for deletions in the mfb-1 genomic region from a library consisting of −7.5 × 106 mutagenized animals, and isolated a single mutation of mfb-1. The mfb-1 mutant allele deletes a 728-bp genomic region, including the entire first exon (Fig. 1B). A homozygote mfb-1 deletion mutation has no apparent effect on development: it is viable, healthy, fertile and has normal lifespan.

We tested whether MFB-1 is involved in the DBL-1 or and DAF-7 pathway(s), both of which are TFG-β-related

**Figure 1** Structural analysis of mfb-1. (A) The primary structure of MFB-1 aligned with the human MAFbx/Atrogin-1. Identical residues are shaded. Gaps were introduced with dashes. The asterisk denotes the stop codon. The predicted F-box domain is boxed. The deletion region in the mfb-1 mutant is indicated by an underline. The PDZ domain is double-underlined. (B) Schematic representation of the mfb-1 genomic structure, deletion site, the construct mfb-1p::mfb-1::gfp and mfb-1p::gfp. mfb-1 corresponds to the DY3.6 ORF. Exons are indicated by the box. Shaded boxes denote the region encoding the F-box domain. White boxes indicate the 5′- and 3′-UTR regions. The positions of the ATG and TAA stop codons are shown. The extent of the 728-bp deletion in mfb-1 mutant is indicated.
pathways in *C. elegans*. The *mfb-1* mutant did not exhibit any similarity to Sma and Mab phenotypes (data not shown), did not exhibit a Daf-c phenotype at 25 °C or 27 °C (Ailion & Thomas 2000) and did not exhibit a Daf-d phenotype when treated with a crude pheromone (data not shown). However, double mutants combining *mfb-1* with mutations in Daf-c genes from the DAF-7 pathway, *daf-1*, *daf-4*, *daf-7* and *daf-14*, revealed significant enhancement of the Daf-c phenotypes at 15 °C and 20 °C compared with the single mutants (Fig. 2A and 2B). Moreover, RNAi inhibition of MFB-1 function in any of these mutants also produced a phenotype (data not shown) similar to the synthetic Daf-c (Syn-Daf-c) phenotype (Ailion & Thomas 2000). As another dauer pathway is mediated by DAF-2 signalling, we examined the genetic interaction of *mfb-1* with *daf-2*. A double mutant of *mfb-1* with *daf-2* showed no enhancement of dauer formation at 20 °C, 22.5 °C or 25 °C (Fig. 2C). The *mfb-1;daf-2* double mutant also had a lifespan similar to that of the *daf-2* single mutant (data not shown). These results indicate that MFB-1 is genetically involved in the DAF-7, but not DAF-2 signalling pathway and negatively regulates dauer formation.

**Requirement for F-box domain in MFB-1 function in vivo**

To confirm that the enhancement of the Daf-c phenotypes observed in the *mfb-1* mutant is indeed as a result of a defect in MFB-1, we constructed *hsp16-2p: mfb-1*, a gene that places *mfb-1* gene under the control of a heat-shock promoter. This gene, or a control vector (pPD49.78), was introduced as an extrachromosomal array into a *mfb-1;daf-7* double mutant. The *hsp16-2* promoter is inducible in many tissues including neurones, intestine and hypodermis. We found that the enhancement of the Daf-c phenotypes of these transgenic animals were unchanged in the absence of heat treatment. However, following heat treatment, the rate of dauer formation was significantly reduced in the *mfb-1;daf-7* double mutant, and was similar to that of the *daf-7* single mutant (Fig. 3). Control worms transformed with the control vector appeared unchanged (Fig. 3). Therefore, loss of *mfb-1* function is required for the enhancement of the Daf-c phenotypes observed in the *mfb-1;daf-7* double mutants.

The F-box proteins are a component of the SCF-type E3 ubiquitin ligases. The F-box domain is necessary for
association with the Skp1 protein, and consequent formation of the SCF complex. The F-box domain in MAFbx/Atrogin-1 has been demonstrated to mediate binding to Skp1 (Gomes et al. 2001; Bodine et al. 2001).

To determine whether the F-box domain in MFB-1 is functional in vivo, we constructed \( hsp16-2::mfb-1 \) cDNA or control vector \( hsp16-2p \) vector (pPD49.78) and incubated at 20 °C. Total numbers of worms counted are indicated above each column. Error bars indicate the SE of proportion.

**Figure 3** Rescue of enhancement of Daf-c phenotype in \( mfb-1;daf-7 \) double mutants by \( mfb-1 \). \( mfb-1;daf-7 \) double mutants were transformed with \( hsp16-2p::mfb-1 \) full-length cDNA, \( hsp16-2p::mfb-1\Delta F-box \) cDNA or control vector \( hsp16-2p \) vector (pPD49.78) and incubated at 20 °C. Total numbers of worms counted are indicated above each column. Error bars indicate the SE of proportion.

to rescue the enhancement of the Daf-c phenotypes (Fig. 3). These results suggest that the F-box domain of MFB-1 is required in vivo to regulate dauer formation.

**MFB-1 over-expression suppress the Daf-c phenotype of DAF-7 signalling mutants**

We next examined whether over-expression of the \( mfb-1 \) gene could suppress the Daf-c phenotype caused by defective DAF-7 signalling. We transformed mutants of the DAF-7 pathway, \( daf-4, daf-7 \) and \( daf-14 \), or the DAF-2 pathway, \( daf-2 \), with the \( hsp16-2p::mfb-1 \) gene or the \( hsp16-2p \) vector as a control, along with the GFP transformation marker \( sur-5::gfp \) (Yochem et al. 1998). The embryos were subjected to heat treatment and the progenies were grown at 20 °C or 25 °C, except for the \( daf-2 \) transgenic worms, which were grown at 22.5 °C or 25 °C as \( daf-2 \) single mutants exhibit no Daf-c phenotype at 20 °C (data not shown). Examination of GFP-positive, transgenic worms showed that exogenous MFB-1 expression significantly suppressed the Daf-c defects caused by mutation of \( daf-4 \), \( daf-7 \) or \( daf-14 \) at 20 °C, compared with the vector-transformed controls (Fig. 4). At 25 °C, all of these progeny were arrested at the dauer larval stage (data not shown). However, exogenous MFB-1 expression did not suppress the Daf-c phenotype in \( daf-2 \) worms at either 22.5 °C or 25 °C (Fig. 4 and data not shown). These results support the idea that MFB-1 is involved in the DAF-7 pathway, but not in the DAF-2 pathway.

**mfb-1::GFP is expressed in many cells in a pattern similar to components of the DAF7 pathway**

The DAF-7 pathway-related genes, \( daf-1 \), \( daf-3 \), \( daf-4 \), \( daf-5 \) and \( daf-14 \), are expressed in the nervous systems, intestine, muscles and distal tip cells (Patterson et al. 1997; Gunther et al. 2000; Inoue & Thomas 2000; da Graca et al. 2004). To examine the expression pattern of MFB-1, we constructed \( mfb-1p::gfp \), consisting of \( mfb-1 \) regulatory sequences starting 4.7 kb upstream of the \( mfb-1 \) start site, the first nine codons of the \( mfb-1 \) exon 1, and an in-frame fusion to the \( gfp \) cDNA (Fig. 1B). We also constructed MFB-1::GFP, which is the same as \( mfb-1p::gfp \) except that it contains the entire \( mfb-1 \) cDNA followed by the \( gfp \) cDNA (Fig. 1C). Animals transformed with \( mfb-1p::gfp \) exhibit GFP fluorescence in the late embryo and through the larval and adult stages, strong expression in the head and tail ganglia, the ventral nerve cord, the tail hypodermal cells and the intestine, weak expression in some lateral neurones, seam cells and body wall muscles in some lines, and no expression in the pharynx and distal tip cells (Fig. 5A–C). MFB-1::GFP partially rescued the
Deletion of the mfb-1 gene significantly enhances the Daf-c phenotype caused by genetic disruption of DAF-7/TGF-β-like signalling, but not of DAF-2 insulin receptor-like signalling. Furthermore, over-expression of mfb-1 partially suppresses the Daf-c phenotypes of daf-7, daf-4 and daf-14 mutants. In addition, we found that the enhancement of Daf-c phenotype of mfb-1 mutant also caused by the daf-11/guanylyl cyclase signal mutant (data not shown). As recent reports indicated that daf-11 regulates daf-7 gene expression, this enhancement of Daf-c phenotype may be caused by the reduction of DAF-7 signalling. These genetic analyses in C. elegans indicate that MFB-1 functions genetically downstream in the DAF-7 pathway to negatively regulate dauer formation. Moreover, daf-7, daf-14 mutations cause three other phenotypes, Cpy, Din and Egl, in addition to their Daf-c phenotype (Thomas et al. 1993). However, the over-expression of MFB-1 could not suppress these phenotypes in daf-7, daf-14 mutations (data not shown). Interestingly, over-expression of mfb-1 did not suppress the Sma phenotype in daf-4 mutants (data not shown). The daf-4 gene encodes a unique type II receptor in C. elegans (C. elegans Sequencing Consortium 1998) and regulates both the DAF-7 and DBL-1 pathways (Padgett et al. 1998). Accordingly, daf-4 mutants exhibit rather pleiotropic phenotypes, such as Daf-c, Egl, Sma (Trent et al. 1983). Taken together, these genetic analyses suggest that MFB-1 is epistatic to daf-4, daf-7 and daf-14, but is not involved in the DBL-1 pathway.

MAFbx/Atrogin-1 has been identified as a ubiquitin ligase required for skeletal muscle atrophy. We have also isolated MAFbx/Atrogin-1 as a TGF-β-inducible gene (H. Ide, S. Higuchi and T. Akiyama, unpublished results), and therefore expected that the mfb-1 gene would be induced by DAF-7 signalling in C. elegans. However, we could not detect significant induction of the mfb-1 mRNA in transgenic lines over-expressing the daf-7 gene (data not shown). Moreover, while MAFbx/Atrogin-1 was expressed specifically in cardiac and skeletal muscle (Bodine et al. 2001; Gomes et al. 2001), MFB-1 was expressed in many tissues, including neurons and intestine (Fig. 5). Only one homologue of MAFbx/atrogin-1 exists in C. elegans and Drosophila (Gomes et al. 2001), although there is another close relative in mammals; Fbxo25 (Cenciarelli et al. 1999; Bodine et al. 2001; Gomes et al. 2001). Fbxo25 shows 60% amino acid identity to MAFbx/Atrogin-1, and is ubiquitously expressed in mouse embryos (H. Ide, S. Higuchi and T. Akiyama, unpublished results). Therefore, it is likely that MAFbx/atrogin-1 and Fbxo25 may be evolutionally diverged in mammals.

While MAFbx/Atrogin-1 is well understood to be involved in the ubiquitin-proteasome pathway in skeletal muscles, the role of the C. elegans MFB-1 homologue in the DAF-7 signalling pathway is not well understood. The results presented here suggest that MFB-1 may have a role in the regulation of DAF-7 signalling, and that it is a potential target for further study in understanding the molecular mechanisms underlying dauer formation in C. elegans.

Discussion

In the present study, we identified mfb-1 as a C. elegans homologue of the human MAFbx/atrogin-1 gene, and characterized its in vivo functions using deletion mutants.
muscle atrophy, the molecular mechanisms underlying this involvement and the precise in vivo functions of MAFbx/Atrogin-1 have been unclear. Our results suggest that MAFbx/Atrogin-1 is involved in the TGF-β family signalling pathway. Furthermore, we demonstrate that the F-box region of MFB-1 is indispensable for its function in DAF-7/TGF-β signalling. These findings provide the first evidence that MAFbx/atrogin-1 functions in a specific cellular signalling pathway in vivo, and demonstrate the significance of the F-box of MAFbx/atrogin-1 in dauer formation in C. elegans. The F-box facilitates binding to substrates and mediates association with the SCF complex and E2 enzyme involved in ubiquitination (Ilyin et al. 2000; Kipreos & Pagano 2000). MAFbx/atrogin-1 has been characterized as an E3 ligase (Bodine et al. 2001; Gomes et al. 2001). This raises the question of whether the E3 ligase may also be a substrate of MFB-1. MFB-1, along with DAF-7 signalling, negatively regulates dauer formation. Conversely, genetic analyses have shown that DAF-3 and DAF-5 negatively regulate the DAF-7 signalling pathway and are required for initiation of dauer development. Amino acid sequence homology indicates that DAF-3 is a co-SMAD (Patterson et al. 1997), but it functions as a transcriptional co-repressor, such as the mammalian SnoN (Stroschein et al. 1999; Sun et al. 1999). DAF-5 is a homologue of Sno/Ski and binds to and functions as a cofactor for DAF-3 (da Graca et al. 2004). These results raise the possibility that DAF-3 or/and DAF-5 may be a target of MFB-1 E3 ligase activity. We observed that a MFB-1::GFP construct was expressed predominantly in the head and tail ganglia. In these cells, GFP mainly localized to nuclei. This expression pattern is extremely similar to that of DAF-5::GFP (da Graca et al. 2004). Although the exact identity of the specific substrate(s) of MAFbx/atrogin-1 is unclear at present, it is clear that MAFbx/atrogin-1 must be involved in the degradation of a yet-to-be-identified substrate that functions in the TGF-β family signalling pathway. Further studies will be needed to identify the precise target substrate of the MFB-1 E3 ligase in the DAF-7 signalling pathway.

**Experimental procedures**

**Strains and genetics**

The basic methods of maintenance of worms are as described by Brenner (1974). All mutants were maintained on NGM plates at 15 °C unless otherwise noted. The following strains were used in this work: wild-type C. elegans Bristol strain N2, daf-1(m40) IV, daf-1(e1287) IV, daf-2(e1370) III, daf-3 (mgDf90) X, daf-4(m63) III, daf-7(e1372) III, daf-14(m77) IV.

**Cloning and sequencing of cDNAs**

Database searches for sequence homologies to the human MAFbx/Atrogin-1 gene in the C. elegans genome were performed.
The isolation of mfb-1 deletion allele

A library consisting of 7.5 × 10^5 mutagenized animals was screened for deletion in the mfb-1 genomic locus by nested PCR assay, using the method of Gengyo-Ando & Mitani (2000). The deletion library, which was a kind gift of the Mori lab of Nagoya University, was prepared by the TMP–UV method as described (Gengyo-Ando & Mitani 2000). First primers used for this nested PCR assay for mfb-1 gene were DY3.6F1 (5′-CAGTGAGGAGGAAAGAGCAGTCTTCCCA-3′) and DY3.6R1 (5′-TTTGGATCCATATACATGACACACCAGTC-3′). Second primers were DY3.6R2 (5′-AGATCGCTTTCCCTGATGAGCAATCTTTC-3′) and DY3.6R2 (5′-AGATCGCCGAGGAGCATGTATATATAGTGCACACGTC-3′). These primer sets are about 1.4 kb apart from each other in the intact genome across the first exon of mfb-1. The deletion site was determined by sequencing of the PCR product. Prior to phenotypic analysis, the mfb-1 deletion allele was backcrossed nine times against an N2 background.

Dauer formation assay

Some gravid adult hermaphrodites were allowed to lay eggs on fresh NGM agar plates for 12 h at 25 °C, 24 h at 20 °C and 15 °C. After parent animals were removed, F1 progeny were returned at each assay temperature and incubated for 2 days at 25 °C, 3 days at 20 °C and 5 days at 15 °C.

Plasmid construction

The mfb-1::gfp transcriptional fusion was prepared using PCR to amplify the region 4.7 kb upstream of the predicted start site of mfb-1 and the first nine amino acids of its coding region from DY3, using the primers DY3.6p-F (5′-CAGTGAGGAGGAAAGAGCAGTCTTCCCA-3′) and DY3.6p-R (5′-TTTGGATCCATATACATGACACACCAGTC-3′). A SalI site and a BamHI site were designed into the PCR primers and used to insert the PCR product into pPD95.75 (A. Fire, personal communication), generating plasmid pmfb1p::gfp. The plasmid pmsp-mfb1, which contains hsp16-2p::mfb-1 was constructed by inserting the full-length mfb-1 cDNA downstream of the hsp16-2 promoter at the BamHI site in pPD49.78 (A. Fire, personal communication). The mfb-1ΔF-box cDNA deletes the F-box region of MFB-1 using the following primers; mfb1AFMF (5′-AGTGCTCTTTCTCCTTTGACAATTCTCCTG-3′) and mfb1AFMR (5′-GCAAAGGAAAAAGCAGTCTTTCATCATC-3′). This product was inserted downstream of the hsp16-2 promoter at the BamHI site of pPD49.78 to construct the plasmid pmsp-mfb1ΔF, containing hsp16-2p::mfb-1ΔF-box.

Transgenic strains

Germ-line transformation was performed as described (Jin 1999). pssp-mfb1 (50 ng/µL) was injected together with 50 ng/µL sur-5::gfp plasmid pTG96 (Yochim et al. 1998) as an injection marker into N2 and mfb-1;daf-7 double mutants, and at least two independent transgenic lines from N2 Ex[hsp16-2::mfb-1cDNA, sur-5::gfp] and mfb-1;daf-7 Ex[hsp16-2::mfb-1cDNA, sur-5::gfp] were obtained. As a control, pPD49.78 was injected under the same conditions into N2 and mfb-1;daf-7 mutants to obtain N2 Ex[hsp16-2, sur-5::gfp] and mfb-1;daf-7 Ex[hsp16-2, sur-5::gfp]. Ex[hsp16-2::mfb-1cDNA, sur-5::gfp] line in daf-1(m40), daf-2(e1370), daf-4(m63), daf-7(e1372) and daf-14(n77) were obtained by crossing with N2 array lines, as well as control lines. pssp-mfb1ΔF (50 ng/µL) was injected together with pTG96 (50 ng/µL) into the mfb-1;daf-7 mutant to generate mfb-1;daf-7 Ex[hsp16-2::mfb-1ΔF-box, sur-5::gfp]. pmfb1p::gfp (100 ng/µL) was injected into N2 animals together with the rol-6 (su1006) plasmid pRF4 (50 ng/µL) (Mello et al. 1991) as an injection marker to obtain the mfb-1::GFP transgenic line. Expression patterns were observed in two independent transgenic lines. Heat shock treatments were carried out for 30 min at 33 °C at the embryonic stage within 3 h after egg-laying.

RNAi

The mfb-1 coding region was amplified with both T7 promoter-tagged primers from pTopo–mfb1. RNA was prepared with Riboprobe Systems–T7 (Promega), using the PCR product as template. One microgram per microlitre of mfb-1 dsRNA or DH20 as a control were injected into worms. After injection, the animals were allowed to recover at 15 °C for 24 h, then were transferred to fresh plates and allowed to lay eggs at the assay temperature for 3 h.

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References


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