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).

Communicated by: Eisuke Nishida
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DOI: 10.1111/j.1365-2443.2004.00792.x

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 signalling 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 (*MAFb*x-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).

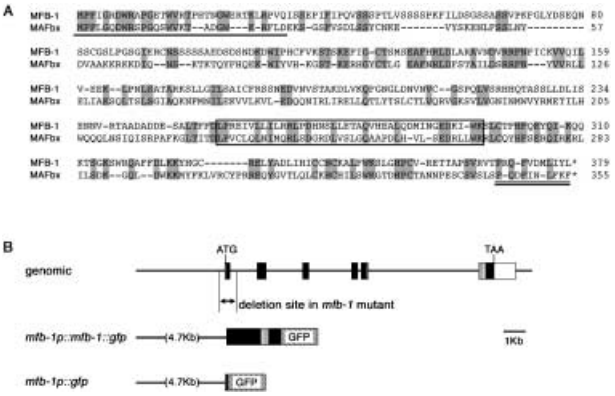

Figure 1 Structural analysis of *mfb-1*. (A) The primary structure of MFB-1 aligned with the human MAFbx/Atrogin-1. Identical residues are shaded. Gaps were introduced with dashes. The asterisk denotes the stop codon. The predicted F-box domain is boxed. The deletion region in the *mfb-1* mutant is indicated 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.

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 ~7.5×10^5 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

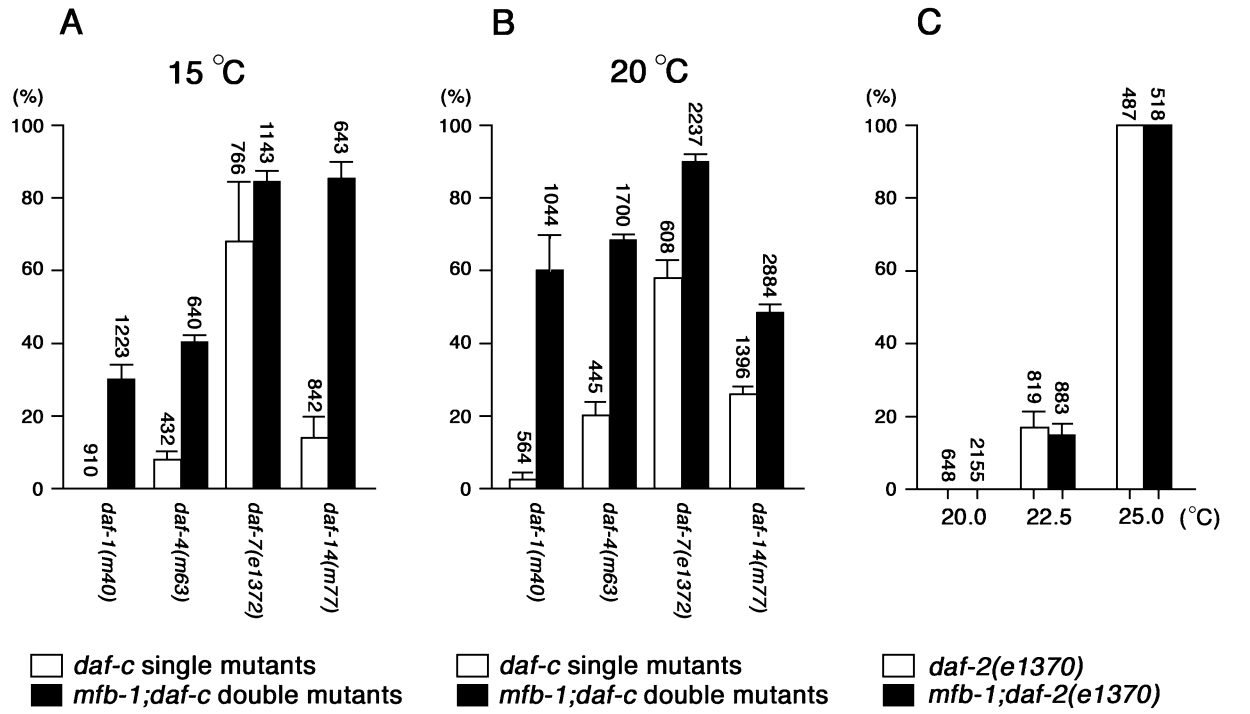

Figure 2 Genetic interactions between *mfb-1* and Daf-c mutations in DAF-7 pathway genes. Percentage of dauer formation in DAF-7 pathway single mutants and double mutants with *mfb-1* at 15 °C (A) and 20 °C (B). (C) Genetic interactions between *mfb-1* and *daf-2* mutations. Percentage of dauer formation in *daf-2 (e1372)* single and double mutants with mfb-1 at 20 °C, 22.5 °C and 25 °C. Total numbers of worms counted are indicated above each column. Error bars indicate the SE of proportion.

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-c* double mutants.

The F-box proteins are a component of the SCF-type E3 ubiquitin ligases. The F-box domain is necessary for

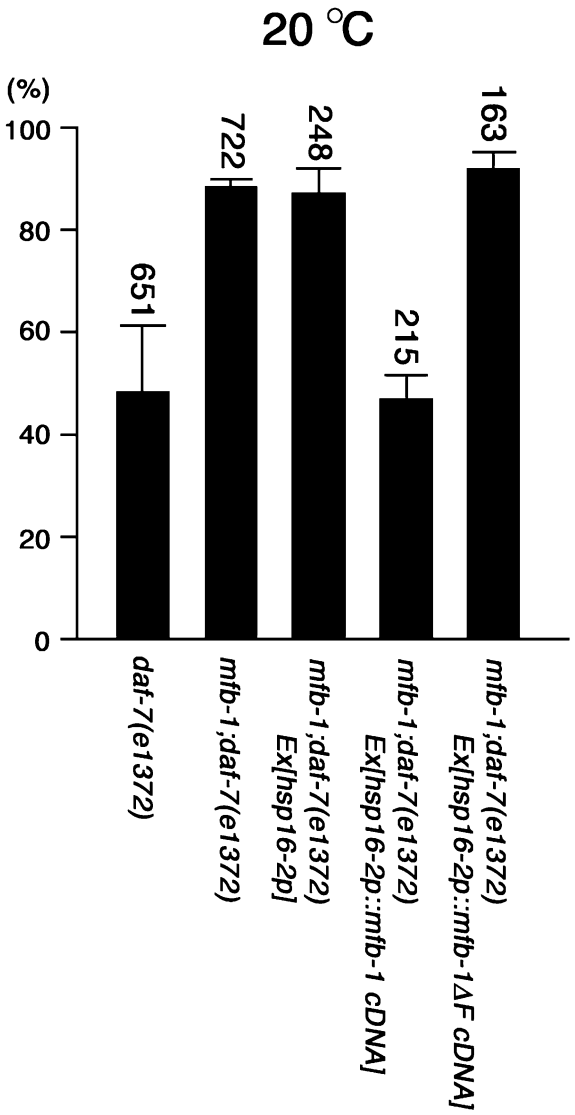

Figure 3 Rescue of enhancement of Daf-c phenotype in *mfb-1;daf-7* double mutants by *mfb-1*. *mfb-1;daf-7* double mutants were transformed with *hsp16-2p::mfb-1* full-length cDNA, *hsp16-2p::mfb-1ΔF-box* cDNA or control vector *hsp16-2p* vector (pPD49.78) and incubated at 20 °C. Total numbers of worms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

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 toSkp1 (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-1ΔF*, which has a deletion in the F-box domain in the *mfb-1* cDNA. Introduction of the *hsp16-2::mfb-1ΔF* transgene into *mfb-1;daf-7* double mutants and heat treatment failed 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

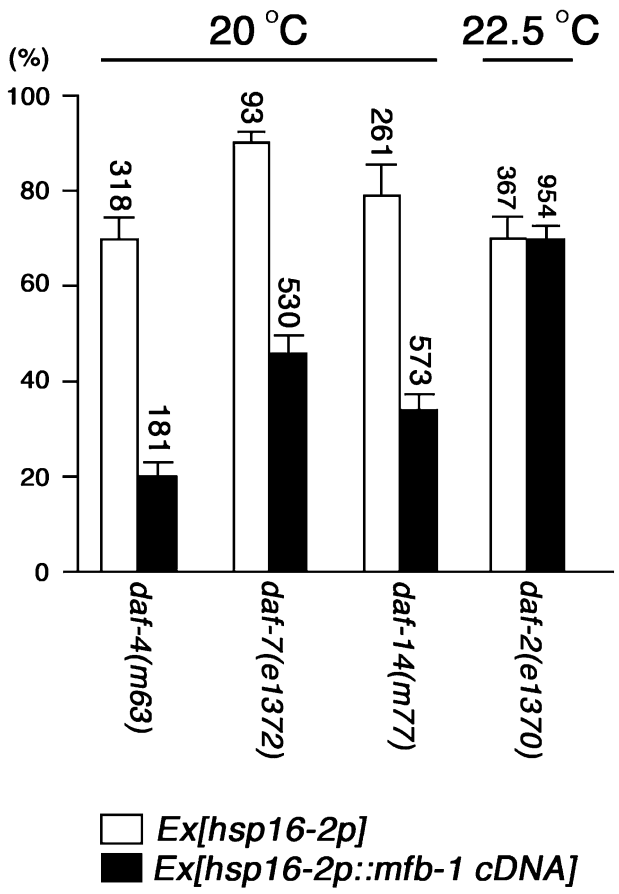

Figure 4 Suppression of Daf-c phenotype in DAF-7 pathway single mutants and *daf-2* mutant by over-expression of *mfb-1* cDNA. Control is *hsp16-2p* vector (pPD49.78) vector. Following heat treatment, daf-4, daf-7 and daf-14 mutants were grown at 20 °C, and daf-2 mutants at 22.5 °C. Total numbers of worms counted are indicated above each column. Error bars indicate the SE of proportion.

enhancement of the Daf-c phenotype of the *mfb-1;daf-7* mutant, and was prominently detected in some head and tail ganglia, weakly in the ventral nerve cord, but little or not at all in the hypodermis, intestine, seam cells and muscles (Fig. 5D). Moreover, in the head and tail ganglia, MFB-1::GFP was preferentially localized to the nuclei (Fig. 5E,F). These expression patterns were extremely similar to those of molecules in the DAF-7 signalling pathway. These results are consistent with a role for MFB-1 in the regulation of the DAF-7 pathway.

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. 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 penotypes 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 neurones 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

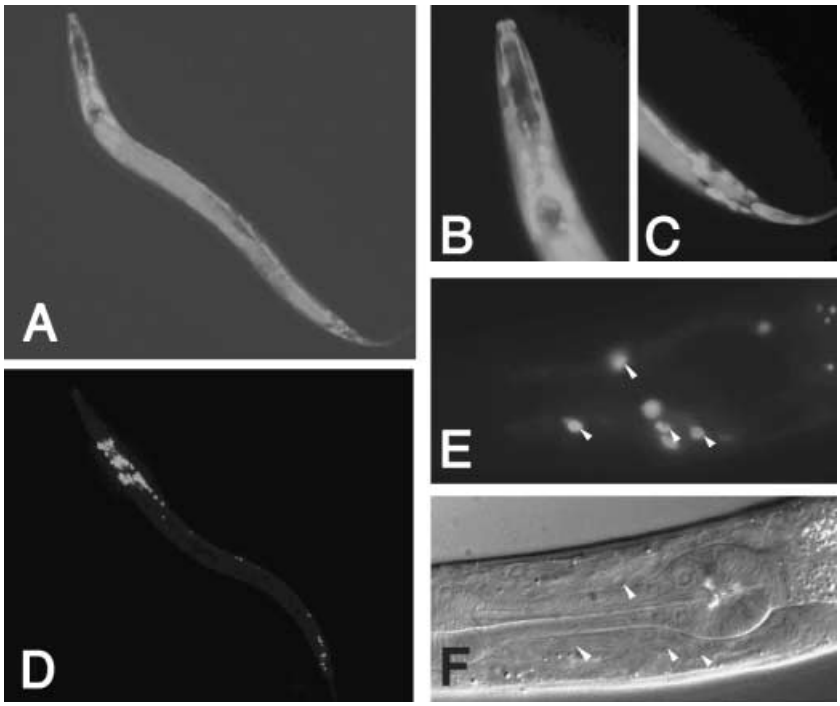

Figure 5 Expression pattern of MFB-1. (A) *mfb-1p::GFP* expression in L3 larva. Bright GFP expression was observed in neuronal cells, including head and tail ganglia, ventral nerve code and non-neuronal cells, including intestines and tail hypodermis. (B) GFP in head neurone. (C) GFP in tail ganglia and hypodermis. Fluorescence images of MFB-1::GFP in L3 larva (D, E). (D) MFB-1::GFP is predominantly expressed in head and tail ganglia. (E) MFB-1::GFP is mainly localized to the nucleus in head ganglia. (F) Nomarski interference image contrasts with images shown in panel E. White triangles indicate some neurones in head ganglia.

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

by BLAST, using the *C. elegans* BLAST server Web site of the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/C_elegans/blast_server.shtml). The cDNA clone of *mfb-1* was isolated by PCR from the pNVLeu cDNA library of *C. elegans* (Kawasaki *et al*. 1999) using the SL1 or SL2 sequences as the 5′ primers and DY3.6–3′Rv1 (5′-TGTGGCGAAAATATTTGAGCTTTCACAAAG-3′) as the outer 3′ primer and DY3.6–3′Rv2 (5′-TCAGTAAAAAAAGGGGATCAAAAATTTAC-3′) as the inner primer. Both 3′ primers were designed from the sequence of DY3.6 based on the Sequence Report Web site of WormBase (http://www.wormbase.org/db/seq/sequence). PCR products of approximately 1.1 kb were subcloned into pCR2.1-TOPO vector (Invitrogen) and sequenced.

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′-CACGTAACTTCCAGTTTTCTTCCC TCA-3′) and DY3.6R1 (5′-TTTGACATATACAATAGTGCACACGTC-3′). Second primers were DY3.6F2 (5′-TCTCTTCCATTCCACCAATTTGTTTTT-3′) and DY3.6R2 (5′-AGATGCGCGAGGAGCATGTATATGCGG-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′-CAGTCGACCGTTTGATGAAGAGGAAACCGGCGG-3′) and DY3.6p-R (5′-TAGGATCCCGCCAATCACGTCCAATGAATGGCAT-3′). A *Sal*I site and a *Bam*HI 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 phsp-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 *Bam*HI 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; mfb1ΔFMF (5′-AGTGCTCTTTCCCTTTGCACATTTCACTTC-3′) and mfb1ΔFMR (5′-GCAAAGGGAAAGAGCACTTTCATCATCGG-3′). This product was inserted downstream of the *hsp16-2* promoter at the *Bam*HI site of pPD49.78 to construct the plasmid phsp-mfb1ΔF, containing *hsp16-2p:: mfb-1ΔF-box*.

Transgenic strains

Germ-line transformation was performed as described (Jin 1999). phsp-mfb1 (50 ng/μL) was injected together with 50 ng/μL *sur-5::gfp* plasmid pTG96 (Yochem *et al*. 1998) as an injected 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(m77)* were obtained by crossing with N2 array lines, as well as control lines. phsp-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-1p::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 ptTopo-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.

Acknowledgements

We thank I. Mori and H. Inada for their deletion library and their technical advice on screening for knock-out worms. We also thank A. Coulson for YAC DY3, N. Ueno for some nematode strains, K. Matsumoto, N. Hisamoto for their cDNA library pNVLeu and special advice, Y. Kimura for Fire lab's vector, K. Shirakabe for excellent advice and M. Lamphier for critical reading of the manuscript. Most of the *C. elegans* strains used in this work were provided by the *Caenorhabditis elegans* Genetic Center, which is founded by the National Institute of Health centre for Research Resources. This work was supported by the Center of Excellence Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone, Grants-in-Aid for

scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Yamanouchi Foundation for Research on Metabolic Disorders and Center of Excellence Program for Frontier Research.

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Received: 28 July 2004
Accepted: 17 August 2004