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A *Caenorhabditis elegans* TGF-β, DBL-1, controls the expression of LON-1, a PR-related protein, that regulates polyploidization and body length

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Using cDNA-based array analysis combined with double-stranded RNA interference (dsRNAi), we have identified yk298h6 as a target gene of *Caenorhabditis elegans* TGF-β signaling. Worms overexpressing *dbl-1*, a TGF-β ligand, are 16% longer than wild type. Array analysis shows yk298h6 to be one of several genes suppressed in such worms. Disruption of yk298h6 function by dsRNAi also resulted in long worms, suggesting that it is a negative regulator of body length. yk298h6 was then mapped to, and shown to be identical to, *lon-1*, a known gene that affects body length. *lon-1* encodes a 312 amino acid protein with a motif sequence that is conserved from plants to humans. Expression studies confirm that LON-1 is repressed by DBL-1, suggesting that LON-1 is a novel downstream component of the *C.elegans* TGF-β growth regulation pathway. Consistent with this, LON-1 is expressed mainly in the larval and adult hypodermis and has dose-dependent effects on body length associated with changes in hypodermal ploidy, but not hypodermal cell proliferation.

Keywords: body length/*Caenorhabditis elegans*/hypodermis/polyploidization/TGF-β

Introduction

Some of the most fundamental, but least understood, aspects of animal development are the mechanisms by which body size is determined (Conlon and Raff, 1999; Stern and Emlen, 1999; Day and Lawrence, 2000). Body size is also the most obvious way in which animal species differ from each other (Bonner, 1989). This is particularly true for nematodes, which vary in size between 3.0×10^{-1} and 8.0×10^{4} mm, but are otherwise quite morphologically uniform (Flemming *et al*., 2000). Wild-type strains of the nematode *Caenorhabditis elegans* are 1.2 mm long. Mutations that affect one aspect of body size, length, have long been known in this worm (Brenner, 1974), causing adults to be either long (Lon) or small (Sma). The frequency and viability of these mutants indicate that the worm might be an especially good organism in which to study the molecular regulation of body size.

Recent studies have shown that some *C.elegans* Sma mutants affect genes that encode components of a transforming growth factor-β (TGF-β) signaling pathway (Patterson and Padgett, 2000). These genes include: *sma-6*, a homologue of a vertebrate type I Ser/Thr kinase receptor (Krishna *et al*., 1999), *daf-4*, a type II Ser/Thr kinase receptor (Estevez *et al*., 1993) also used in dauer larva formation (Georgi *et al*., 1990; Ren *et al*., 1996) and *sma-2*, *sma-3* and *sma-4*, cytoplasmic SMADs (Massague, 1996; Heldin *et al*., 1997), that translocate into the nucleus upon signal activation (Savage *et al*., 1996). Since DAF-4 can function as a type II receptor for BMP signaling in mammalian cells (Estevez *et al*., 1993) there appears to be substantial functional conservation between nematode and mammalian TGF-β signaling. More recently, DBL-1/CET-1, belonging to the TGF-β superfamily of proteins, has been identified as the ligand that triggers the *sma* signaling pathway (Morita *et al*., 1999; Suzuki *et al*., 1999). Loss-of-function mutations in *dbl-1* also have a Sma phenotype, while worms that overexpress it are Lon. DBL-1 is presumed to be a secreted growth factor, but how it actually regulates body length remains largely unknown.

One obvious way in which body length might be controlled by the TGF-β pathway is by regulation of cell proliferation. However, Sma and Lon mutants appear to have wild-type cell numbers (Morita *et al*., 1999; Suzuki *et al*., 1999; Flemming *et al*., 2000). This is not surprising, since ~50% of the growth of *C.elegans* occurs during adulthood, when no cell proliferation takes place (Knight *et al*., 2001). This growth, and even part of larval growth, must then be due to increases in cell size (Flemming *et al*., 2000). Increases in cell size of at least some tissues such as the hypodermis and intestine are, in *C.elegans*, associated with increases in somatic ploidy via endoreduplication (Hedgecock and White, 1985). Recently, worms that lack TGF-β activity due to mutations in *daf-4*, *sma-2* and *dbl-1* have been shown to have reduced hypodermal ploidy relative to wild type (Flemming *et al*., 2000; Nyström *et al*., 2002). This observation also suggests a possible mechanism for body size evolution since the degree of somatic polyploidization appears to be, in part, correlated with evolved differences in body size among nematode species related to *C.elegans* (Flemming *et al*., 2000). Identification of further components of this pathway that

affect endoreduplication is, therefore, of interest for an understanding not only of the regulation of body size, but also of nematode morphological diversity.

Downstream target genes of *dbl-1* signaling might be identified by mutational screens that enhance or suppress the Sma phenotype. However, this kind of genetic screen is often time-consuming and laborious. On the other hand, recent DNA macro/microarray technology (Galitski *et al*., 1999; Lockhart and Winzeler, 2000; Young, 2000) has permitted the simultaneous screening of thousands of expressed genes. It is even possible, in principle, to examine the expression of all 19 000 *C.elegans* genes in two different genetic backgrounds or growth conditions (Mochii *et al*., 1999; Reinke *et al*., 2000). Another powerful approach recently developed to address gene function is double-stranded RNA interference (dsRNAi), which permits the sequence-specific inactivation of genes (Fire *et al*., 1998; Bass, 2000; Zamore *et al*., 2000). dsRNAi has accelerated the high through-put analysis of gene function not only for *C.elegans* but also for other model organisms (Kennerdell and Carthew, 1998; Ngo *et al*., 1998; Sanchez Alvarado and Newmark, 1999; Chuang and Meyerowitz, 2000).

Here we report large-scale analyses of gene expression profiles in different genetic backgrounds displaying Sma and Lon phenotypes. We show that one of the genes recovered from this screen has a Lon phenotype when its function is inhibited by dsRNAi. We show that loss-of-function mutations in a known regulator of body length, *lon-1*, interrupt this gene, and that *lon-1* transcription is negatively regulated by *dbl-1* signaling. *lon-1* encodes an evolutionarily conserved protein that defines the 'PR-1 related protein superfamily' from yeast, plant, insect to human, whose biological functions are little known (Szyperski *et al*., 1998). Moreover, we provide evidence that DBL-1 regulation of hypodermal polyploidization is mediated by LON-1.

Results

Identification of DBL-1-regulated genes by differential hybridization analysis using cDNA-based macroarray

As we reported previously, cDNA array of *C.elegans* mutants is an efficient way of screening signal-specific target genes (Mochii *et al*., 1999). To understand the molecular pathway regulating *C.elegans* body length, we used the same DNA macroarray and screened for genes regulated by *dbl-1* signal. In this study, we compared the gene expression profile between *dbl-1* null mutants displaying Sma phenotype, *dbl-1(nk3)* (Morita *et al*., 1999) and worms overexpressing *dbl-1* (which contain a multi-copy of the *dbl-1* genomic fragment) with Lon phenotype, *ctIs40* (Suzuki *et al*., 1999). Out of ~8000 genes arrayed on nylon membranes, 16 genes were found to be up-regulated and seven down-regulated. Gene identification and fold increases of hybridization intensity are summarized in Table I. Altered expression was confirmed for all identified genes by conventional northern blotting. Of the 23 genes identified by cDNA array signals as being regulated by DBL-1, we failed to confirm seven by northern blots.

Table I. List of genes identified and dsRNAi phenotype

Clone name	Gene	Fold increase (HDF)[a]	dsRNAi phenotype
yk225d6	K04E7.1	2.3	Sma
yk253f12	F25H8.5	2.2	no phenotype
yk355c4	C17G1.6	3.4	molting defect
yk412e5	Y38H6C.1	1.9	no phenotype
yk608d12	no gene found	1.9	weak Sma
yk174b12	Y57G11C.15	2.0	Let (embryonic lethal)
yk479h1	C07E3.10	2.0	no phenotype
yk532c7	ZK1290.8	2.0	no phenotype
yk570a11	F27E11.3	2.5	no phenotype
yk563h1	C44H4.3	1.9	no phenotype
yk604a4	W04G3.8	2.0	Dpy[b]
yk355e1	F44E2.4	2.8	no phenotype
yk430h5	F23B2.11	3.0	no phenotype
yk479e3	C44H4.2	2.0	no phenotype
yk500g10	VC5.3	6.0	no phenotype
yk44c1	K10C2.1	2.2	no phenotype
yk257f11	T21C9.5	0.3	Unc[c]
yk361c1	K10C2.3	0.5	no phenotype
yk530e1	no gene found	0.4	no phenotype
yk553c5	no gene found	0.5	no phenotype
yk71c6	T21C9.2	0.5	no phenotype
yk298h6	F48E8.1	0.5	Lon
yk605h1	F55A4.2	0.5	no phenotype

[a]The data are the average of four experiments. HDF, high density filter.
[b]Dpy, Dumpy.
[c]Unc, uncoordinated.

yk298h6 encodes the lon-1 gene

In order to examine the biological relevance of the identified genes to the determination of body length in relation to *dbl-1* signaling, we carried out dsRNAi, a method developed recently by which particular genes can be rapidly inactivated (Fire *et al*., 1998). Among the genes regulated both up and down in our microarray screen, seven showed various dsRNAi phenotypes (Table I), some of which resembled the Sma and Lon phenotypes of known body length mutations. We focused on yk298h6 (F48E8.1), one of the genes suppressed by *dbl-1* because its disruption by dsRNAi caused a typical Lon phenotype with a high frequency. Importantly, yk298h6 mapped close to the *lon-1* locus (Figure 1A) by database analysis. Expecting that yk298h6 is the gene responsible for *lon-1* mutation, we performed rescue experiments using a fragment of cosmid F48E8 harboring the entire yk298h6 sequence. The 8.0 kb genomic fragment was sufficient to rescue the *lon-1* body length phenotype, indicating strongly that yk298h6 encodes the *lon-1* product (Table II). This was also confirmed by identifying the mutational lesion in nine *lon-1* alleles (Figure 1B). Sequence analysis of PCR-amplified *lon-1* alleles identified missense and nonsense mutations that cause non-conservative amino acid substitutions in the following alleles: *e1137*, *ct410*, *e185*, *ct411*, *n1130*, *e44* and *sp1* (Figure 1B). Alleles *sp3* and *e43* each have a stop codon caused by a CG-to-TA transition that converts codon TGG (W83) to the opal terminator TGA and TGG (W150) into the amber terminator TAG, respectively. Based on these findings, we conclude that yk298h6 (F48E8.1 for gene) encodes *lon-1*.

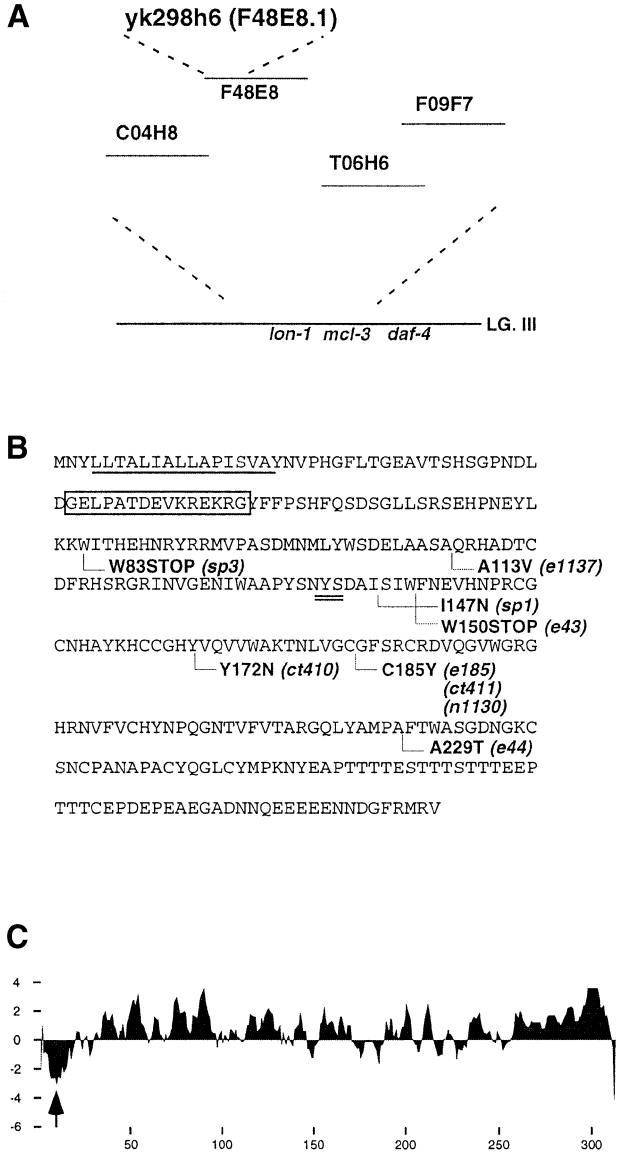

Fig. 1. yk298h6 encodes the *lon-1* gene. (**A**) The yk298h6 (F48E8.1) gene maps to the *lon-1* locus. (**B**) Primary structure of LON-1 and point mutation site. LON-1 is a 312 amino acid protein. The N-terminal hydrophobic region is underlined. A putative *N*-glycosylation site is double underlined. The peptide sequence that generates the LON-1 antibody is boxed. Amino acid substitutions in *lon-1* alleles are indicated below the sequence. *e43* and *sp3* each have a nonsense mutation (W150 to stop and W83 to stop, respectively). *ct410*, *ct411*, *e44*, *e185*, *e1137*, *n1130* and *sp1* have missense mutations. (**C**) The LON-1 Kyte–Doolittle hydrophobicity plot shows a hydrophobic stretch at the N-terminus of the protein (amino acids 4–18, see arrow).

LON-1 belongs to the PR-protein superfamily conserved among yeast, plant, insect and human, and may be a type II transmembrane protein

The *lon-1* gene encodes a 312 amino acid protein (Figure 1B). By a database search, we have found that genes encoding proteins homologous to *lon-1* belong to the PR-protein superfamily and are present in other organisms including yeast, plant, insect and human (Szyperski *et al*., 1998) (see also Discussion). Although overall sequence similarity between them is not outstanding (>30%), 18 amino acid residues in the stretch of ~100 amino acids compared are well aligned (Figure 2). Interestingly, almost all the amino acids identified as mutation sites in *lon-1* alleles include those conserved between species, indicating that these amino acids are essential for LON-1 function (Figure 2, indicated by asterisk). There are no defined functional motifs within this family, but members of the family are predicted to be secreted or glycosylphosphatidylinositol (GPI)-anchored proteins. Two of the members of this family, pathogenesis related protein-1 (PR-1) in plant and glioma-pathogenesis related protein-1 (GliPR-1) in human, are secreted from cells (Szyperski *et al*., 1998). Yeast PR-1 like protein-3 (YPR-3) in yeast and cysteine-rich protease inhibitor are predicted to be GPI-anchored proteins (Hamada *et al*., 1998). However, the analysis of the LON-1 sequence shows that there is a hydrophobic stretch at the N-terminus of the LON-1 protein, indicating that LON-1 might be a transmembrane protein (Figure 1C). To characterize the LON-1 protein, we generated antibodies against a LON-1 peptide corresponding to 42–56 amino acid residues. This antibody (α-LON-1) detected ~35 kDa of protein prepared from whole worm extract by immunoblotting (Figure 3A). The molecular mass of this protein is the same as that of the predicted LON-1 protein.

To address the subcellular localization of LON-1 protein, we fractionated the extract of N2 worms. We detected the LON-1 protein in the membrane fraction, but not the soluble fraction, by western blot analysis (Figure 3B). These results indicate that LON-1 may be a membrane-integrated or -associated protein. Furthermore, the LON-1 protein remains associated with the membrane fraction after treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) or an alkaline wash (Figure 3C). As the sequence following the signal sequence does not closely match with consensus cleavage sequence, we propose that the signal sequence is not cleaved and instead acts as an anchor region. We conclude that LON-1 is likely to be a type II integral membrane protein.

Table II. Effect of *lon-1* gene dosage on body length

Genotype	Body length (mm)[a]	*N*
N2	1.28 ± 0.03	85
lon-1(e185)	1.71 ± 0.01	93
lon-1(e43)	1.43 ± 0.04	87
lon-1(ct410)	1.60 ± 0.01	103
lon-1(sp3)	1.35 ± 0.05	19
lon-1(RNAi)	1.63 ± 0.06	35
lon-1(e43)/Df[b]	1.49 ± 0.11	8
lon-1(sp3)/Df	1.29 ± 0.06	19
nkIs10[c]	1.00 ± 0.03	43
lon-1(e185);Exnk51[lon-1(+)]	1.24 ± 0.11	65
lon-1(e185);Exnk52[lon-1(+)]	1.19 ± 0.09	65
lon-1(e185);Exnk53[lon-1(+)]	0.89 ± 0.10	62

[a]Mean ± SE. *N*, measured animals. Body lengths of various mutants were measured using a micrometer at high magnification at 120 h post-hatching.
[b]Deficiency line.
[c]Lon-1-overexpressed Sma worm.

Characterization of lon-1 alleles

To analyze the relationship between the mutation site of *lon-1* alleles and their phenotype, we compared the expression level of LON-1 protein and body length in

Fig. 2. Alignment of LON-1 protein with nine related proteins. The sequences are LON-1 (*C.elegans*), CG8483 (*Drosophila*), GliPR-1 (human) (Murphy *et al*., 1995), PR-1 (*Triticum aestivum*), SGP28 (human) (Kjeldsen *et al*., 1996), TPX-1 (testis-specific protein) (pig) (Kasahara *et al*., 1989), venom allergen 5 (*Vespa mandarinia*) (Lu *et al*., 1993), cystein-rich secretory protein (horse), cystein-rich protease inhibitor (mouse), YJH8 (yeast). Asterisks indicate the mutation sites identified in *lon-1* alleles. Conserved amino acid residues (at least seven residues) are by bold type.

mutants. *lon-1(e185)* is thought to be the strongest allele because they are the longest worms (Table II). By western blot analysis, LON-1 protein was found to be almost completely undetectable as a result of the point mutation (Figure 3A). Cysteine residues, including C185, are conserved in the PR-protein family and might form disulfide bonds, essential for the structural integrity of the protein. The missense mutation might cause misfolding of LON-1 by creating unpaired cysteine residues and may even prevent LON-1 from being exported from the endoplasmic reticulum, thereby resulting in a severe loss of LON-1 function. Compared with *lon-1(e185)*, nonsense mutation alleles *e43* and *sp3* display mild phenotypes in terms of body length. LON-1 protein in *e43* was weakly detected by western blot analysis (Figure 3A). The weak Lon phenotype of *e43* and *sp3* may be due to low levels of LON-1 protein produced by translational readthrough. Like other animals, *C.elegans* has $tRNA^{[Ser]Sec}$ that can insert selenocysteine at UGA codons (Lee *et al*., 1990). In prokaryotes, substitution of tryptophan at opal stops occurs due to third-position wobble in codon–anticodon recognition. A similar effect in *daf-1* alleles is reported by Gunther *et al*. (2000). Next, we made *e43/Df* and *sp3/Df* animals. These animals have a longer phenotype than *e43* and *sp3* homozygotes. *lon-1(e43)* and some *lon-1* alleles often produce stunted worms and show incomplete penetrance of the Lon phenotype. We cannot rule out that this is due to the secondary induced constriction behind the head that leads to auto-decapitation; nevertheless, dsRNAi of *lon-1* seems to cause only the Lon phenotype (data not shown). We speculate that the *lon-1* allele with the null or most severe phenotype is *lon-1(e185)*.

lon-1 expression is negatively regulated by dbl-1 signaling

lon-1 was initially identified as being down-regulated in worms overexpressing *dbl-1* in our cDNA macroarray (Table I). Northern blot analysis confirmed this, showing that *lon-1* transcript levels seem to be regulated in a stepwise manner depending on the gene dosage of *dbl-1* (Figure 3D). The change in expression level of *lon-1* transcript between *ctIs40;dbl-1*-overexpressing worms and the *dbl-1(–)* mutation was estimated as ~5-fold.

As expected from the *dbl-1*-dependent repression profiled by cDNA array analysis and northern blotting, green fluorescent protein (GFP) fluorescence confirmed that the *lon-1* promoter is negatively regulated by the *dbl-1* signal. The fluorescence of *lon-1::gfp* appeared to be significantly down-regulated in worms overexpressing *dbl-1;dbl-1(++);ctIs40*, compared with *dbl-1(–)* (Figure 3E). These results indicate that *lon-1* expression is negatively regulated by *dbl-1* signaling at the transcriptional level.

To analyze the genetic relationship of *dbl-1* and *lon-1*, we made double mutants of *lon-1* alleles with *dbl-1(–)* and measured body length (Table IV). The *lon-1(ct410);dbl-1(nk3)* double mutant was almost the same length as the *lon-1(ct410)* single mutant. This result indicates that the *lon-1(ct410)* mutation is completely epistatic to *dbl-1*. However, the double mutants of *lon-1(e185)* and *lon-1(e43)* with *dbl-1(nk3)* had intermediate body lengths. The cause of this incomplete epistacy is unclear.

Worms overexpressing LON-1 have a Sma phenotype

Loss-of-function mutations in *dbl-1* cause a Sma phenotype, while worms that overexpress *dbl-1* are Lon (Morita *et al*., 1999; Suzuki *et al*., 1999). DBL-1 negatively regulates LON-1, and loss-of-function mutations in *lon-1* give a Lon worm. If LON-1 is also a dose-dependent regulator of body length, overexpressing *lon-1* should give a Sma worm. To address this possibility, we injected high concentrations of *lon-1* DNA into *lon-1* mutant or N2 worms (see Materials and methods). As predicted, higher dosages of *lon-1* caused the Sma phenotype against both *lon-1* and N2 backgrounds (Table II; data not shown). The body length of worms overexpressing *lon-1;nkIs10* was close to *sma* mutants such as *dbl-1*, *sma-2*, *sma-3* and *sma-4* but, as discussed below, do not show the male tail phenotype (data not shown).

To determine when mutants become longer than wild type, we measured the length of N2 wild-type, *lon-1-*

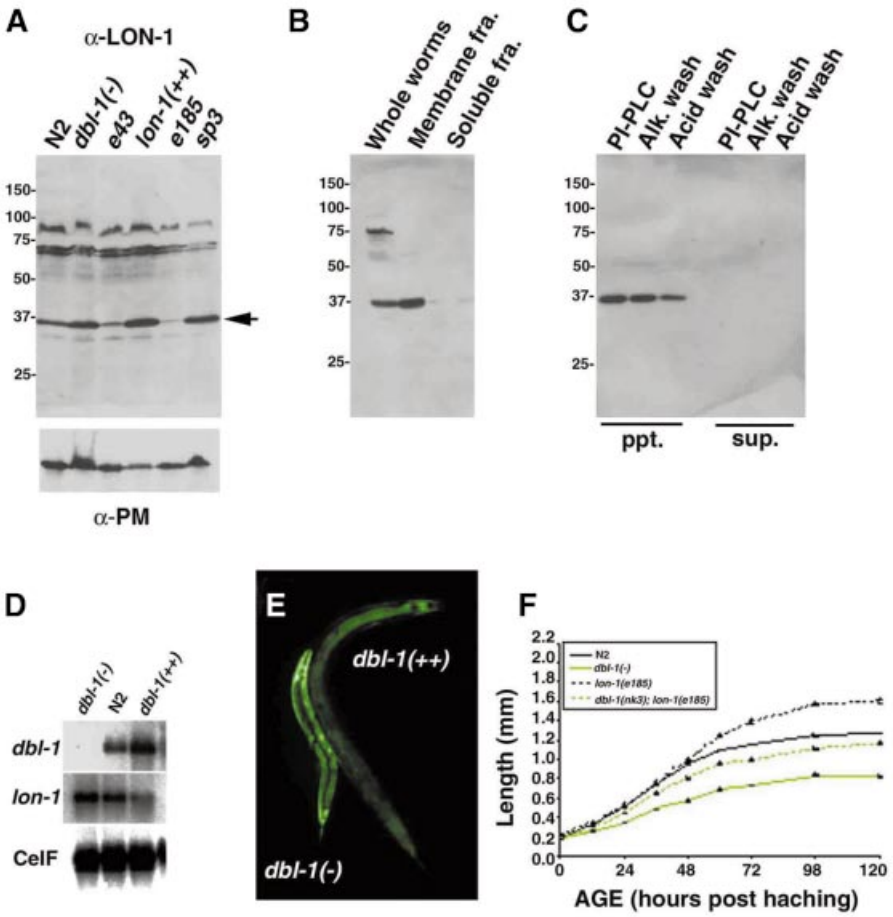

Fig. 3. LON-1 expression is negatively regulated by *dbl-1* signaling. (A–C) Western blot analysis using α-LON-1 antibody. (**A**) Whole worm extracts of *dbl-1(–)*, N2 wild-type, *lon-1(++)* and *lon-1(–)* alleles *sp3*, *e185* and *e43*. The arrowhead indicates the 35 kDa LON-1 protein. Lower panel is the loading control staining with α-paramyosin antibody. (**B**) Cryostat sections of N2 worms were homogenized and extracted by Triton X-100 and fractionated by centrifugation (see also Materials and methods). LON-1 protein is detected in the membrane fraction but not in the soluble fraction. (**C**) The membrane fractions were treated with PI-PLC or alkaline wash (pH 11.5), or acid wash (pH 2.0). LON-1 protein remains associated with the membrane fraction. (**D**) Northern blot analysis. Poly(A)$^+$ RNA (1.0 μg) prepared from mixed stages of *dbl-1(–);dbl-1(nk3)*, N2, *dbl-1(++);ctIs40* worms was blotted and hybridized with *dbl-1*, *lon-1*, CeIF (*C.elegans* initiation factor; for loading control) cDNA. The *dbl-1* signal in *dbl-1*-overexpressing animals increased 3-fold compared with N2, and *lon-1* in *dbl*-1-overexpressing animals decreased 5-fold compared with *dbl-1* null animals. (**E**) *lon-1::gfp* expression is up- or down-regulated by *dbl-1* dosage. The fluorescence indicating *lon-1::gfp* expression was increased with a *dbl-1(–);dbl-1(nk3)* background and decreased with a *dbl-1(++);ctIs40* background. (**F**) Growth curve of N2 wild type, *dbl-1(nk3)*, *dbl-1(nk3);lon-1(e185)* and *lon-1(e185)*. The body length of mutants was measured at various times after hatching.

(e185), *dbl-1(nk3)* and *dbl-1(nk3);lon-1(e185)* hermaphrodites at different times after hatching (Figure 3F). All mutants hatch as L1 larvae indistinguishable in size from wild-type larvae, but the Lon and Sma phenotypes are seen at late L1 stage, and these phenotypes are sustained through to adult stages. These results demonstrate that *dbl-1* signaling is effective from all larval stages to adult.

LON-1 is expressed in hypodermal and intestinal cells

To examine the expression pattern of *lon-1 in vivo* at the cellular level, we stained the worms with LON-1 antibody (α-LON-1). Fluorescence was detected in almost all hypodermal and intestinal cells (Figure 4A). The signal of hypodermal cells is colocalized with the staining of MH27, which stains the adherens junction of the hypodermal cells (Figure 4A–C). Colocalization of LON-1 and MH27 antigen are also observed around hypodermal seam cells (Figure 4D–F). These expression patterns persisted through the subsequent larval stages to the adult stage. All signals were observed at the surface of the cell membrane. These observations are consistent with the result that LON-1 is a type II transmembrane protein. The signal disappeared completely in the presence of the antigen peptide (Figure 4G). In the *lon-1(e43)* nonsense mutation allele, LON-1 staining was substantially less (Figure 4F).

Expression of lon-1 cDNA in hypodermal but not in intestinal cells rescued Lon phenotype

We show that LON-1 protein is expressed in the intestinal inner membrane and hypodermal adherens junction where MH27 antigen is also expressed (Figure 4). To address the functional sites of LON-1 protein in the regulation of body length, we expressed a *lon-1* cDNA under region-specific promoters. We tested four cell type-specific promoters: $P_{dpy\text{-}7}$, P_{yk92e8}, $P_{unc\text{-}54}$ and $P_{dbl\text{-}1}$. $P_{dpy\text{-}7}$, the promoter for the collagen gene *dpy-7*, is specific for hypodermal cells. P_{yk92e8} is expressed only in intestinal cells (Mochii *et al.*,

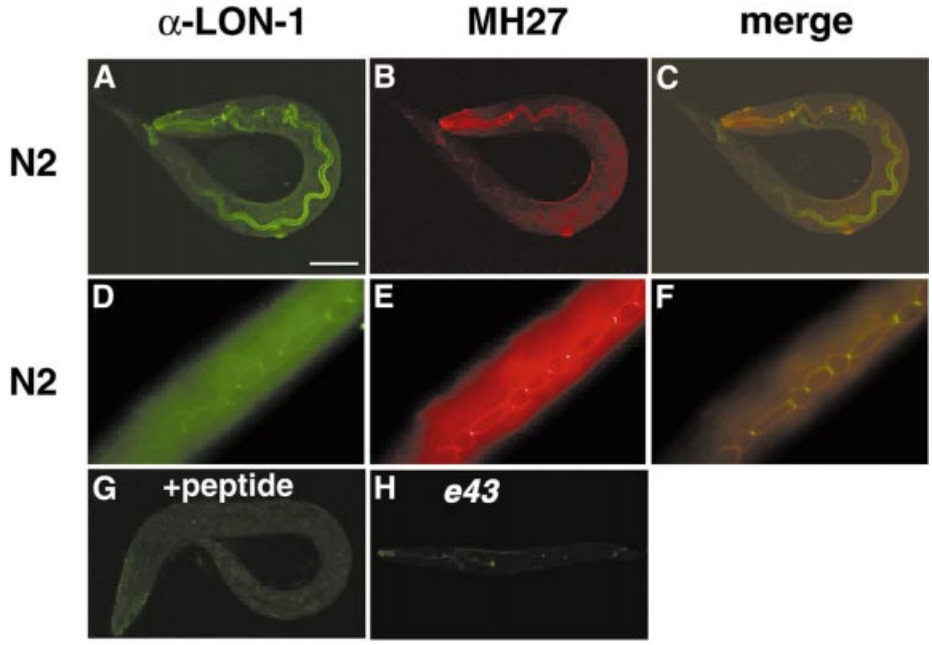

Fig. 4. LON-1 proteins are expressed in hypodermal and intestinal cells. All images are stained with purified anti-rabbit LON-1 peptide antibody (1:300 dilution) (green) and MH27 monoclonal antibody (1:100 dilution) (red). (A–C) N2 wild-type L1 stage animal. (**A**) Staining image of α-LON-1. The fluorescence is observed around the hypodermal cells and in the inner membrane of intestinal cells. (**B**) MH27. Signal is seen in the hypodermal adherens junction. (**C**) Merged image of (A) and (B). (D–F) Hypodermal region of N2 wild-type L3 stage animal. (**D**) α-LON-1, (**E**) MH27, (**F**) merged image of (D) and (E), the signals are observed around the seam cells. (**G**) Staining image of α-LON-1 in the presence of antigen peptide. Staining of α-LON-1 has disappeared completely. N2 wild-type L1 stage animal. (**H**) Staining image of α-LON-1 to *lon-1(e43)* mutant L1 stage animal. Weak expression is detected.

1999; Yoshida *et al*., 2001). P_{unc-54}, the promoter for the myosin heavy chain, is expressed in major body wall muscle. P_{dbl-1}, the promoter for *dbl-1*, the ligand of the Sma pathway, is expressed almost exclusively in neuronal cells (Morita *et al*., 1999; Suzuki *et al*., 1999). Using these promoters, we expressed the *lon-1* cDNA. The cell type specificity of these promoters was confirmed by GFP fluorescence by the injection of a GFP cDNA with each of above tissue-specific promoters (data not shown). Hypodermal cell-specific expression of LON-1 (P_{dpy-7} *lon-1*) rescued the Lon phenotype, but the intestinal cell-specific expression (P_{yk92e8} *lon-1*) did not (Table III). Two other promoters, P_{unc-54} and P_{dbl-1} also could not rescue the Lon phenotype. These results indicate that expression of LON-1 protein in the hypodermal cells is sufficient to regulate body length.

LON-1 regulates the polyploidization of hypodermal nuclei

Recently, loss-of-function mutations in *daf-4* and *sma-2*, both of which encode signaling components downstream of *dbl-1*, have been reported to decrease polyploidy of the hypodermal nuclei (Flemming *et al*., 2000). The *C.elegans* TGF-β growth pathway is thus implicated in the regulation of endoreduplication. If *lon-1* is a downstream target of TGF-β signaling, it may also be a dose-dependent regulator of hypodermal ploidy. To address this possibility, we estimated the hypodermal ploidy of loss-of-function mutations of *lon-1* and worms that overexpress *lon-1*. We found that endoreduplication of hypodermal nuclei was indeed increased in *lon-1(e185)* and *lon-1(RNAi)* worms by 19 and 13%, respectively, compared with wild type (Figure 5 and Table IV). Importantly, *lon-1*-overexpressing Sma worms (*nkIs10*) have reduced hypodermal ploidy, like *dbl-1*, *daf-4* and *sma-2* mutants (Table IV; Flemming *et al*., 2000; Nyström *et al*., 2002). It is intriguing to note that Nyström *et al*. (2002) failed to detect increased hypodermal ploidy in worms that overexpress *dbl-1*, despite the general phenotypic similarity of these worms to *lon-1(–)*. Furthermore, *dpy-2(–)* and *lon-3(–)* mutants both have altered body size but wild-type ploidy (Flemming *et al*., 2000; Nyström *et al*., 2002). Indeed, among the many mutations that affect body size in *C.elegans*, so far only the TGF-β signal mutants and *lon-1* loss-of-function and overexpression mutants have increased or decreased ploidy in hypodermal cells.

Discussion

lon-1 regulates body length of C.elegans and is negatively regulated by TGF-β signaling

In this study, we first show that DNA array technology combined with dsRNAi is capable of identifying a biologically important gene. Although not all genes identified displayed a phenotype when inactivated by dsRNAi, one of the genes that did was found to encode *lon-1* whose inactivation causes a Lon worm. Our finding that *lon-1* expression is negatively regulated by *dbl-1* provides further insight into the important pathway regulating nematode body size yet identified. The *lon-1* expression profile suggests that it most likely acts as a downstream component of the *C.elegans* TGF-β growth pathway.

Table III. Expression of *lon-1* cDNA in hypodermal, but not intestinal, cells rescued a *lon-1* phenotype

Genotype	Transgene	Expression sites	Body length (mm)[a]	*N*
lon-1(e185)	–	–	1.71 ± 0.01	93
lon-1(e185)	P_{dpy-7} *lon-1*	hypodermal cells	1.12 ± 0.12	108
lon-1(e185)	P_{yk92e8} *lon-1*	intestinal cells	1.59 ± 0.01	60
lon-1(e185)	P_{unc-54} *lon-1*	body wall muscle	1.60 ± 0.01	47
lon-1(e185)	P_{dbl-1} *lon-1*	neural cells	1.57 ± 0.01	37
lon-1(e185)	P_{lon-1} *lon-1*	hypodermal and intestinal cells	1.30 ± 0.02	68

[a]Mean ± SE.

Table IV. Hypodermal ploidy of mutants and double mutants

Mutant	Hypodermal ploidy[a]	Body length (mm)
N2	12.47 ± 0.22	1.50 ± 0.02
dbl-1(nk3)	7.81 ± 0.25 ($p < 0.0001$)	1.01 ± 0.02 ($p < 0.0001$)
nkIs10	10.73 ± 0.33 ($p < 0.003$)	1.00 ± 0.03 ($p < 0.0001$)
lon-1(e185)	14.36 ± 0.40 ($p < 0.0001$)	1.86 ± 0.03 ($p < 0.0001$)
lon-1(e43)	11.78 ± 0.43 NS[b]	1.36 ± 0.06 ($p < 0.01$)
lon-1(ct410)	10.47 ± 0.40 NS	1.60 ± 0.02 ($p < 0.01$)
lon-1(sp3)	11.98 ± 0.78	1.35 ± 0.05
lon-1(RNAi)	13.70 ± 1.34	1.63 ± 0.06
lon-1(e43)/Df	11.63 ± 1.18	1.49 ± 0.11
lon-1(sp3)/Df	13.86 ± 0.72	1.29 ± 0.06
dbl-1(nk3);lon-1(e185)	8.81 ± 0.29	1.34 ± 0.02
dbl-1(nk3);lon-1(e43)	9.64 ± 0.37	1.21 ± 0.03
dbl-1(nk3);lon-1(ct410)	11.48 ± 0.27	1.60 ± 0.02

[a]Mean ± SE. *p* values represent the probability of rejecting the null hypothesis of no difference between a given genotype and wild type, as estimated from an *F* distribution. Preliminary experiments indicate that the Lon phenotype was variably penetrant in some genotypes. In order to circumvent this, we obtained an unbiased estimate of the 80th percentile for length for each genotype. We raised 50 worms (five per plate, 20–30 replicate plates), measured all of them at 120 h of age and then compared only the longest worm of each plate, thus ensuring independence of replicates.
[b]Not significant.

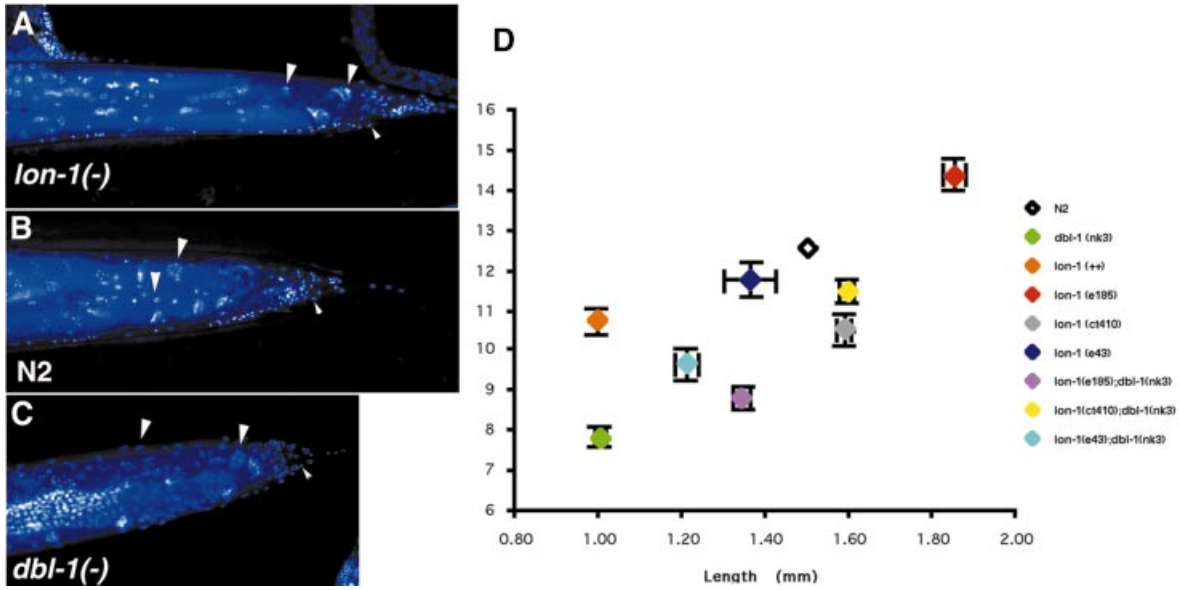

Fig. 5. LON-1 regulates the polyploidization of hypodermal nuclei in a dose-dependent manner. (A–C) DAPI staining of the tail region of an adult hermaphrodite. (**A**) *lon-1(e185)*, (**B**) N2 wild type, (**C**) *dbl-1(nk3)*. Large arrowheads indicate the nuclei of hypodermal cells (polyploid nuclei) and small arrowheads diploid nuclei. Anterior to the left and posterior to the right. (**D**) The relationship between hypodermal ploidy and body length in *lon-1(–);dbl-1(0)* double mutant worms.

Although *lon-1* controls body length acting downstream of *dbl-1*, it is not likely be involved in all processes of *dbl-1* function. Another phenotype commonly found in *dbl-1*, *daf-4*, *sma-6* and *sma-2*, *sma-3*, *sma-4* mutants is a change in male tail ray identity (Estevez *et al*., 1993; Savage *et al*., 1996; Krishna *et al*., 1999; Morita *et al*., 1999; Suzuki *et al*., 1999). *mab-21*, a gene that also regulates male tail identity, has been shown to be downstream of *dbl-1* signaling (Morita *et al*., 1999). Since *mab-21* is thought not to regulate body length (Chow

et al., 1995; Morita *et al*., 1999), the TGF-β signaling pathway must bifurcate upstream of *mab-21*, one branch regulating body length and the other regulating tail ray patterning. Consistent with this, we find no evidence of altered ray identities in *lon-1(–)* worms, although they do have an elongated male tail. Similarly, worms overexpressing LON-1 (*nkIs10*) have wild-type tails (data not shown). This suggests that *lon-1* functions downstream of the bifurcation point and is involved exclusively in body length regulation.

Our results also permit insight into an unsolved problem of *C.elegans* growth regulation, namely, which tissue is most critical in determining the size of a worm. Obvious candidates include the hypodermis which covers the entire worm and synthesizes the cuticle, and the body wall muscle that underlies the hypodermis. The TGF-β ligand DBL-1 is expressed mainly in neuronal cells such as ventral nerve cord and AFD (Morita *et al*., 1999; Suzuki *et al*., 1999), but although the ventral cord may the source of important growth factors, it is not likely to be a critical structural component of worm length. Expression of another TGF-β component, *daf-4*, the type II receptor, has shed little light on this question since it is almost ubiquitously expressed (Gunther *et al*., 2000). More interestingly, we have found recently that the type I receptor *sma-6* is highly expressed in hypodermis (Yoshida *et al*., 2001) and that expressing *sma-6* exclusively in the hypodermis by a hypodermal-specific promoter is sufficient to rescue the *sma-6(–)* Sma phenotype (Yoshida *et al*., 2001). We also show that LON-1 expression in hypodermal cells is essential for body length regulation (Table III). Taken together with these results, we suggest that the hypodermis is the target tissue of *dbl-1* in the determination of body length.

LON-1 may control polyploidization of hypodermal nuclei

In the development of almost all plants and animals, specialized polyploid and polytene cell types arise through endocycles, cell cycles lacking cell division (Brodsky and Uryvaeva, 1985; Royzman and Orr-Weaver, 1998). Endoreduplication is, therefore, a normal and important component of cell proliferation and growth in many organisms. However, endoreduplication also occurs in pathological conditions such as tumorigenesis (Holland *et al*., 1998; Lengauer *et al*., 1998). Nevertheless, the molecular mechanism of the regulation of polyploidy has remained largely unknown (Reed and Orr-Weaver, 1997; Royzman and Orr-Weaver, 1998; Galitski *et al*., 1999), although Britton and Edgar (1998) have recently shown that the *Drosophila* larval endoreduplication cycle is dependent on nutrition as well as cyclin E and E2F transcription factor expression. Here, we show that LON-1 regulates the polyploidy of hypodermal nuclei from almost 8C to 16C depending on its gene dosage [*dbl-1(0)*, *lon-1*-overexpressing worms and loss-of-function mutant *lon-1(e185)*] (Figure 5 and Table IV). How LON-1 does this is not known, but it could function as an inhibitor of an as yet unknown factor that promotes endoreduplication. Interestingly, *lon-1* is also expressed at all stages in the intestine, another tissue that undergoes endoreduplication in *C.elegans* (Hedgecock and White, 1985), therefore, we are currently examining the ploidy of intestinal cells of *lon-1* mutants.

Regulation of endoreduplication and body length by DBL-1

The observation that polyploid cells (be they due to somatic polyploidization or germ-line polyploidy) are typically larger than diploid cells has often led to the suggestion that genome content directly regulates cell size (Brodsky and Uryvaeva, 1985; Day and Lawrence, 2000). In the absence of other levels of body size regulation, changes in ploidy might, then, have a direct effect upon body size. Flemming *et al*. (2000) proposed that TGF-β mutants might be Sma because they were endoreduplication deficient. They noted, however, that there was no evidence in *C.elegans* for a direct causal connection between ploidy and body length. Our findings that *lon-1* is simultaneously a dose-dependent regulator of body length and of endoreduplication adds strength to the idea that ploidy regulates body length in worms. However, not all evidence from the TGF-β pathway in *C.elegans* tends to confirm this idea. While worms that are DBL-1 defective are Sma and have reduced hypodermal ploidy, worms that overexpress DBL-1 have a Lon phenotype but appear to have a wild-type ploidy (Nyström *et al*., 2002). One possible explanation for this discrepancy may be that DBL-1 does not suppress *lon-1* activity to the same extent as the *lon-1* null mutant; indeed, our northern blot analysis shows that DBL-1 overexpression only reduces the level of *lon-1* transcript by ~50%. This explanation also suggests that relative to the regulation of ploidy, body length determination is more sensitive to a change in DBL-1 activity, since partial suppression of *lon-1* is sufficient to promote the increase in body size seen in *lon-1(–)* mutants. Alternatively, *lon-1* may be able to overcome exogenous DBL-1 activity to block hyper-endoreduplication to some extent. It is also noted that hypodermal polyploidy is unchanged in some alleles such as *lon-1(e43)* and *lon-1(ct410)* in which some residual LON-1 protein exists. It is possible that an interaction of LON-1 protein with an as yet unknown protein(s) may contribute to the suppression of hypodermal ploidy.

We propose here a model for the regulation of body length and hypodermal ploidy by DBL-1. *dbl-1* is expressed in the neuronal cells including those of the AFD amphid neuron and ventral nerve cord (Morita *et al*., 1999; Suzuki *et al*., 1999). DBL-1 might be secreted by nervous cells and delivered to hypodermal cells that express the type I receptor *sma-6* (Yoshida *et al*., 2001). At the hypodermal syncytium, *dbl-1* signaling is activated and negatively regulates the expression of LON-1 through SMA-2, SMA-3 and SMA-4. LON-1 inhibits the endo cell cycle (Royzman and Orr-Weaver, 1998) and regulates the hypodermal ploidy.

It remains possible that the TGF-β signaling pathway and *lon-1* regulate endoreduplication as a result of body size change. LON-1 might, for example, regulate one or more of the many collagens of which the *C.elegans* cuticle is composed. Loss-of-function mutations in many of these collagens give short worms with a characteristic Dumpy (Dpy) phenotype: short and fat worms as distinct from the short and thin Sma phenotype. Indeed, another Lon mutant, *lon-3*, has recently been found to encode a

collagen and, like *lon-1*, is a dose-dependent regulator of body length (Nyström *et al*., 2002). *lon-3(–)* worms, as well as worms overexpressing LON-3, have, however, a wild-type ploidy and are thought to affect body length via changes in cuticle elasticity (Nyström *et al*., 2002; Y.Suzuki and W.B.Wood, manuscript in preparation). Therefore, it is unlikely that body size change is the cause for change in hypodermal ploidy. Also, if *lon-1* regulates collagens, it probably does not regulate *lon-3*, since analysis of double mutants suggests that these genes act largely additively (Nyström *et al*., 2002).

LON-1 belongs to the PR-protein superfamily, which is conserved from yeast, plant, insect to human

Searches of databases revealed that LON-1 has conserved amino acid motifs found in proteins in yeast, plants, insects and humans. One of these proteins, the human GliPR protein, was found to be highly expressed in brain tumors, suggesting that GliPR plays an important role in tumor growth (Murphy *et al*., 1995). LON-1 also has sequence homology with the plant PR-1 protein, which plays a central role in the defense system of plants, for example, during the manifestation of systematic acquired resistance (SAR) (Uknes *et al*., 1992, 1993). PR-1-related proteins from tobacco and tomato have *in vitro* activity against *Phytophthora infestans*, but the underlying molecular mechanism for the action of these proteins is not known (Szyperski *et al*., 1998). The characterization of the three-dimensional structure of GliPR (human) and PR-1 (plant) revealed that the putative active site residues of these proteins is strictly conserved. This suggests strongly that human GliPR and plant PR-1 proteins operate according to the same molecular mechanism, which establishes a possible functional link between the human immune system and a plant defense system (Szyperski *et al*., 1998).

In *C.elegans*, we show that LON-1 regulates the endoreduplication of hypodermal nuclei by demonstrating that loss of function of *lon-1* causes hyperendoreduplication of hypodermal nuclei, and overexpression of *lon-1* causes hypoendoreduplication (Figure 5 and Table IV). Members of the PR-protein superfamily are secreted, GPI-anchored and transmembrane proteins, raising the possibility that LON-1 might function as an extracellular ligand. Alternatively, the PR-protein superfamily, including LON-1, might function as enzymes because putative enzyme active sites are located in the largest surface cleft of the three-dimensional structure of GliPR, PR-1 and LON-1 (Szyperski *et al*., 1998). LON-1 might also function as an inhibitor of enzyme or protease that regulates the endocycle. We are tempted to speculate further that other members of the PR-protein superfamily might also regulate the endo cell cycle under various pathological conditions of plants and animals. Intriguingly, endoreduplication is a common response of plants to the saliva of endoparasitic nematodes (Goverse *et al*., 2000). Future analysis of the molecular mechanism of LON-1 in regulating the polyploidization of hypodermal nuclei may, then, shed light on a diversity of biological problems as well as the functional sites of the PR-protein superfamily.

Materials and methods

General method and strains

Caenorhabditis elegans strains were cultured as described by Brenner (1974). Genetic crosses were performed as described by Wood (1988). The following strains were used: wild-type *C.elegans* variety Bristol strain N2, *lon-1(e43)III*, *lon-1(e44)III*, *lon-1(e185)III*, *lon-1(e1137)III*, *lon-1(ct410)III*, *lon-1(ct411)III*, *lon-1(n1130)III*, *lon-1(sp1)III*, *lon-1(sp3)III*, *dbl-1(–);dbl-1(nk3)V*, *dbl-1(++);dbl-1(ctIS40)*, *him-5(e1492)V*.

cDNA array and differential hybridization

Caenorhabditis elegans cDNA array was prepared as described by Mochii *et al*. (1999). Total RNA was extracted from synchronously cultured *dbl-1(nk3);dbl-1(–)* or *ctIS40;dbl-1(++)* L3 stage with Trizol reagent (Gibco-BRL), and poly(A)$^+$ RNA was purified by using Oligotex-dT 30 (Roche Molecular Biochemicals). For probe labeling, the oligo(dT)-depleted RNA was incubated with random hexamer, Superscript reverse transcriptase (Gibco-BRL) and [^{33}P]dCTP at 37°C for 2 h. Hybridization was performed as described. Hybridized signals were detected using the Fuji BAS system and quantified with HDG Analyzer software (Genomic Solutions). Experiments were carried out four times with independently prepared RNAs. Northern analyses were performed using poly(A)$^+$ RNA and results were quantified using the Fuji BAS system.

dsRNAi analysis

Each template was amplified by PCR from YK clones, using the primers: CMO24: 5′-TTGTAAAACGACGGCCAG-3′ and CMO422: 5′-GCG-TAATACGACTCACTATAGGGAACAAAAGCTGGAGCT-3′. dsRNAs were synthesized from these templates using MEGAscript T7 kit (Ambion). dsRNAs were injected into the gonadal syncytia of adult hermaphrodites, and the phenotypes of F_1 progeny were analyzed. For RNAi feeding, full-length *lon-1* cDNA was inserted in a pPD129.36 vector.

Generation of antibody against LON-1, and western analysis

Antisera LP1-1 was raised against a synthetic peptide with the sequence CGELPATDEVKREKRG (corresponding to amino acids 42–56 in LON-1). The peptide was coupled to keyhole limpet hemocyanin (KLH). Two rabbits were injected with the conjugated peptide. The antiserum was affinity purified on a Sepharose peptide affinity column. Whole worm extracts were prepared by suspending washed worms in 10 vol of SDS sample buffer and immediately boiling for 3 min before loading onto a 10% SDS–polyacrylamide gel. Extracts resolved by SDS–PAGE were transferred to nitrocellulose and the filter was blocked in 5% non-fat dried milk before incubation in a 1:300 dilution of affinity-purified LON-1 peptide antibodies. Anti-paramyosin antibody (5-23) was used in a 1:1000 dilution.

Membrane extracts were prepared as described previously (Epstein *et al*., 1988). One gram of packed worms mixed with 2.0 ml of OTC compound was frozen in liquid N_2. Cryostat sections were dissolved in 10 ml relaxing buffer and washed twice for centrifugation with relaxing buffer. The pellet was resuspended in 1% Triton X-100 in relaxing buffer and homogenized with Teflon homogenizer. Homogenized solutions were centrifuged at 5000 *g* for 10 min and the resulting supernatant was centrifuged at 15 000 *g* for 30 min. The resulting pellet was used as membrane fraction and the supernatant as soluble fraction.

Immunofluorescent staining

Mixed stage populations of each strain were fixed and stained according to the protocol of Finney and Ruvkun (1990). Affinity-purified LON-1 anti-rabbit antibody was used at a concentration of 1:200 and MH27 anti-mouse monoclonal antibody at 1:300. The secondary antibody was FITC-conjugated anti-rabbit antibody (Jackson) and Texas-Red-conjugated anti-mouse antibody (Amersham) diluted 1:100.

Plasmids and germ-line transformation

A *lon-1 Pst*I–*Sca*I genomic fragment (8.2 kb) from cosmid F48E8 was subcloned into the *Pst*I–*Sca*I site of pBluescript KS– to generate pYS1. In rescue and overexpression experiments, pYS1 was injected at 50 or 200 ng/μl as described (Mello *et al*., 1991) with *myo-3::gfp* as the co-injection marker. *lon-1::gfp* was generated by PCR amplification of 3.0 kb upstream of the *lon-1* ORF from cosmid F48E8, and subcloned into pPD95,69 (a gift from A.Fire) in-frame. Extrachromosomal arrays were integrated into chromosomes using UV irradiation, followed by at least two backcrosses with N2 or *him-5*.

Analysis of polyploidization of hypodermal nuclei

Hypodermal DNA content was determined using videomicrodensitometry as described by Flemming *et al*. (2000). On average, 20 nuclei were scored per worm at late adulthood (>120 h after hatching).

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References

Bass,B.L. (2000) Double-stranded RNA as a template for gene silencing. *Cell*, **101**, 235–238.

Bonner,J.T. (1989) *The Evolution of Complexity by Means of Natural Selection*. Princeton University Press, Princeton, NJ.

Brenner,S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics*, **77**, 71–94.

Britton,J.S. and Edgar,B.A. (1998) Environmental control of the cell cycle in *Drosophila*: nutrition activates mitotic and endoreplicative cells by distinct mechanisms. *Development*, **125**, 2149–2158.

Brodsky,V.Y. and Uryvaeva,I.V. (1985) *Genome Multiplication in Growth and Development*. Cambridge University Press, Cambridge, UK.

Chow,K.L., Hall,D.H. and Emmons,S.W. (1995) The *mab-21* gene of *Caenorhabditis elegans* encodes a novel protein required for choice of alternate cell fates. *Development*, **121**, 3615–3626.

Chuang,C.F. and Meyerowitz,E.M. (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA*, **97**, 4985–4990.

Conlon,I. and Raff,M. (1999) Size control in animal development. *Cell*, **96**, 235–244.

Day,S.J. and Lawrence,P.A. (2000) Measuring dimensions: the regulation of size and shape. *Development*, **127**, 2977–2987.

Epstein,H.F., Berliner,G.C., Casey,D.L. and Ortiz,I. (1988) Purified thick filaments from the nematode *Caenorhabditis elegans*: evidence for multiple proteins associated with core structures. *J. Cell Biol.*, **106**, 1985–1995.

Estevez,M., Attisano,L., Wrana,J.L., Albert,P.S., Massague,J. and Riddle,D.L. (1993) The *daf-4* gene encodes a bone morphogenetic protein receptor controlling *C.elegans* dauer larva development. *Nature*, **365**, 644–649.

Finney,M. and Ruvkun,G. (1990) The *unc-86* gene product couples cell lineage and cell identity in *C.elegans*. *Cell*, **63**, 895–905.

Fire,A., Xu,S., Montgomery,M.K., Kostas,S.A., Driver,S.E. and Mello,C.C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, **391**, 806–811.

Flemming,A.J., Shen,Z.Z., Cunha,A., Emmons,S.W. and Leroi,A.M. (2000) Somatic polyploidization and cellular proliferation drive body size evolution in nematodes. *Proc. Natl Acad. Sci. USA*, **97**, 5285–5290.

Fujiki,Y., Hubbard,A.L., Fowler,S. and Lazarow,P.B. (1982) Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.*, **93**, 97–102.

Galitski,T., Saldanha,A.J., Styles,C.A., Lander,E.S. and Fink,G.R. (1999) Ploidy regulation of gene expression. *Science*, **285**, 251–254.

Georgi,L.L., Albert,P.S. and Riddle,D.L. (1990) *daf-1*, a *C.elegans* gene controlling dauer larva development, encodes a novel receptor protein kinase. *Cell*, **61**, 635–645.

Goverse,A.J., de Engler,A.J., Verhees,J., van der Krol,S., Helder,H.H. and Gheysen,G. (2000) Cell cycle activation by plant parasitic nematodes. *Plant Mol. Biol.*, **43**, 747–761.

Gunther,C.V., Georgi,L.L. and Riddle,D.L. (2000) A *Caenorhabditis elegans* type I TGFβ receptor can function in the absence of type II kinase to promote larval development. *Development*, **127**, 3337–3347.

Hamada,K., Fukuchi,S., Arisawa,M., Baba,M. and Kitada,K. (1998) Screening for glycosylphosphatidylinositol (GPI)-dependent cell wall proteins in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, **258**, 53–59.

Hedgecock,E.M. and White,J.G. (1985) Polyploid tissues in the nematode *Caenorhabditis elegans*. *Dev. Biol.*, **107**, 128–133.

Heldin,C.H., Miyazono,K. and ten Dijke,P. (1997) TGF-β signalling from cell membrane to nucleus through SMAD proteins. *Nature*, **390**, 465–471.

Hogan,B.L. (1996) Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev.*, **10**, 1580–1594.

Holland,E.C., Hively,W.P., Gallo,V. and Varmus,H.E. (1998) Modeling mutations in the G1 arrest pathway in human gliomas: overexpression of CDK4 but not loss of INK4a-ARF induces hyperploidy in cultured mouse astrocytes. *Genes Dev.*, **12**, 3644–3649.

Kasahara,M., Gutknecht,J., Brew,K., Spurr,N. and Goodfellow,P.N. (1989) Cloning and mapping of a testis-specific gene with sequence similarity to a sperm-coating glycoprotein gene. *Genomics*, **5**, 527–534.

Kennerdell,J.R. and Carthew,R.W. (1998) Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell*, **95**, 1017–1026.

Kjeldsen,L., Cowland,J.B., Johnsen,A.H. and Borregaard,N. (1996) SGP28, a novel matrix glycoprotein in specific granules of human neutrophils with similarity to a human testis-specific gene product and a rodent sperm-coating glycoprotein. *FEBS Lett.*, **380**, 246–250.

Knight,C., Patel,M.N., Azevedo,R.B.R., Flemming,A. and Leroi,A.M. (2001) A novel mode of ecdysozoan growth in *Caenorhabditis elegans*. *Evol. Dev.*, in press.

Krishna,S., Maduzia,L.L. and Padgett,R.W. (1999) Specificity of TGFβ signaling is conferred by distinct type I receptors and their associated SMAD proteins in *Caenorhabditis elegans*. *Development*, **126**, 251–260.

Lee,B.J., Rajagopalan,M., Kim,Y.S., You,K.H., Jacobson,K.B. and Hatfield,D. (1990) Selenocysteine $tRNA^{[Ser]Sec}$ gene is ubiquitous within the animal kingdom. *Mol. Cell. Biol.*, **10**, 1940–1949.

Lengauer,C., Kinzler,K.W. and Vogelstein,B. (1998) Genetic instabilities in human cancers. *Nature*, **396**, 643–649.

Lockhart,D.J. and Winzeler,E.A. (2000) Genomics, gene expression and DNA arrays. *Nature*, **405**, 827–836.

Lu,G., Villalba,M., Coscia,M.R., Hoffman,D.R. and King,T.P. (1993) Sequence analysis and antigenic cross-reactivity of a venom allergen, antigen 5, from hornets, wasps and yellow jackets. *J. Immunol.*, **150**, 2823–2830.

Massague,J. (1996) TGFβ signaling: receptors, transducers and Mad proteins. *Cell*, **85**, 947–950.

Mello,C.C., Kramer,J.M., Stinchcomb,D. and Ambros,V. (1991) Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.*, **10**, 3959–3970.

Mochii,M., Yoshida,S., Morita,K., Kohara,Y. and Ueno,N. (1999) Identification of transforming growth factor-β-regulated genes in *Caenorhabditis elegans* by differential hybridization of arrayed cDNAs. *Proc. Natl Acad. Sci. USA*, **96**, 15020–15025.

Morita,K., Chow,K.L. and Ueno,N. (1999) Regulation of body length and male tail ray pattern formation of *Caenorhabditis elegans* by a member of TGF-β family. *Development*, **126**, 1337–1347.

Murphy,E.V., Zhang,Y., Zhu,W. and Biggs,J. (1995) The human glioma pathogenesis-related protein is structurally related to plant pathogenesis-related proteins and its gene is expressed specifically in brain tumors. *Gene*, **159**, 131–135.

Ngo,H., Tschudi,C., Gull,K. and Ullu,E. (1998) Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proc. Natl Acad. Sci. USA*, **95**, 14687–14692.

Nyström,J., Aili,M., Shen,Z.Z., Flemming,A., Leroi,A.M. and Tuck,S. (2002) *lon-3* is a dose-dependent regulator of body size in *C.elegans*. *Genetics*, in press.

Patterson,G.I. and Padgett,R.W. (2000) TGF β-related pathways. Roles in *Caenorhabditis elegans* development. *Trends Genet.*, **16**, 27–33.

Patterson,G.I., Koweek,A., Wong,A., Liu,Y. and Ruvkun,G. (1997) The DAF-3 smad protein antagonizes TGF-β-related receptor signaling in the *Caenorhabditis elegans* dauer pathway. *Genes Dev.*, **11**, 2679–2690.

Reed,B.H. and Orr-Weaver,T.L. (1997) The *Drosophila* gene *morula* inhibits mitotic functions in the endo cell cycle and the mitotic cell cycle. *Development*, **124**, 3543–3553.

Reinke,V. *et al.* (2000) A global profile of germline gene expression in *C.elegans*. *Mol. Cell*, **6**, 605–616.

Ren,P., Lim,C.S., Johnsen,R., Albert,P.S., Pilgrim,D. and Riddle,D.L. (1996) Control of *C.elegans* larval development by neuronal expression of a TGF-β homolog. *Science*, **274**, 1389–1391.

Royzman,I. and Orr-Weaver,T.L. (1998) S phase and differential DNA replication during *Drosophila* oogenesis. *Genes Cells*, **3**, 767–776.
Sanchez Alvarado,A. and Newmark,P.A. (1999) Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc. Natl Acad. Sci. USA*, **96**, 5049–5054.
Savage,C., Das,P., Finelli,A.L., Townsend,S.R., Sun,C.Y., Baird,S.E. and Padgett,R.W. (1996) *Caenorhabditis elegans* genes *sma-2*, *sma-3* and *sma-4* define a conserved family of transforming growth factor β pathway components. *Proc. Natl Acad. Sci. USA*, **93**, 790–794.
Stern,D.L. and Emlen,D.J. (1999) The developmental basis for allometry in insects. *Development*, **126**, 1091–1101.
Suzuki,Y., Yandell,M.D., Roy,P.J., Krishna,S., Savage-Dunn,C., Ross,R.M., Padgett,R.W. and Wood,W.B. (1999) A BMP homolog acts as a dose-dependent regulator of body size and male tail patterning in *Caenorhabditis elegans*. *Development*, **126**, 241–250.
Szyperski,T., Fernandez,C., Mumenthaler,C. and Wuthrich,K. (1998) Structure comparison of human glioma pathogenesis-related protein GliPR and the plant pathogenesis-related protein P14a indicates a functional link between the human immune system and a plant defense system. *Proc. Natl Acad. Sci. USA*, **95**, 2262–2266.
Uknes,S., Mauch-Mani,B., Moyer,M., Potter,S., Williams,S., Dincher,S., Chandler,D., Slusarenko,A., Ward,E. and Ryals,J. (1992) Acquired resistance in *Arabidopsis*. *Plant Cell*, **4**, 645–656.
Uknes,S., Dincher,S., Friedrich,L., Negrotto,D., Williams,S., Thompson-Taylor,H., Potter,S., Ward,E. and Ryals,J. (1993) Regulation of pathogenesis-related protein-1a gene expression in tobacco. *Plant Cell*, **5**, 159–169.
Wood,W.B. (1988) *The Nematode Caenorhabditis elegans*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Yamakawa,T., Miyata,S., Ogawa,N., Koshikawa,N., Yasumitsu,H., Kanamori,T. and Miyazaki,K. (1998) cDNA cloning of a novel trypsin inhibitor with similarity to pathogenesis-related proteins and its frequent expression in human brain cancer cells. *Biochim. Biophys. Acta*, **1395**, 202–208.
Yoshida,S., Morita,K., Mochii,M. and Ueno,N. (2001) Hypodermal expression of a *C.elegans* TGF-β type I receptor SMA-6 for the growth and maintenance of body length. *Dev. Biol.*, **240**, 32–45.
Young,R.A. (2000) Biomedical discovery with DNA arrays. *Cell*, **102**, 9–15.
Zamore,P.D., Tuschl,T., Sharp,P.A. and Bartel,D.P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell*, **101**, 25–33.

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