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Note

Autophagy Genes *unc-51* and *bec-1* Are Required for Normal Cell Size in *Caenorhabditis elegans*

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ABSTRACT

Here we show that in the nematode *Caenorhabditis elegans* mutational inactivation of two autophagy genes *unc-51/atg1* and *bec-1/atg6/beclin1* results in small body size without affecting cell number. Furthermore, loss-of-function mutations in *unc-51* and *bec-1* suppress the giant phenotype of mutant animals with aberrant insulin-like growth factor-1 (insulin/IGF-1) or transforming growth factor-β (TGF-β) signaling. This function for *unc-51* and *bec-1* in cell size control and their interaction with these two growth modulatory pathways may represent a link between the hormonal and nutritional regulation of cell growth.

In multicellular organisms, the regulation of cell size is intimately linked to nutrient and growth factor availability and requires a well-controlled balance between macromolecule synthesis and degradation (Klionsky and Emr 2001; Saucedo and Edgar 2002; Oldham and Hafen 2003; Danielpour and Song 2005; Leevers and Mcneill 2005). Cells divide only after they reach a critical size. Thus, cell growth is a prerequisite of cell proliferation and may be dysregulated in human malignancies. The insulin-like growth factor-1 (insulin/IGF-1) and transforming growth factor-β (TGF-β) signaling cascades are known as major regulatory systems for cell growth, proliferation, and differentiation (Saltiel and Kahn 2001; Derynck and Zhang 2003; Oldham and Hafen 2003). However, little is known about cellular pathways that mediate these processes.

In *Caenorhabditis elegans*, both insulin/IGF-1 and TGF-β signaling pathways affect body length by controlling cell size (Krishna *et al.* 1999; Suzuki *et al.* 1999; Morita *et al.* 1999, 2002; Mcculloch and Gems 2003). For example, loss-of-function mutations in the gene *daf-2*, which encodes the nematode IGF-1 receptor, extend body length, compared to the wild type (Mcculloch and Gems 2003). The type I TGF-β receptor SMA-6 also influences body size in nematodes (Krishna *et al.* 1999). SMA-6 is activated by the growth factor DBL-1 and represses the expression of the PR-related protein LON-1, which is a novel negative regulator of cell growth (Suzuki *et al.* 1999; Morita *et al.* 1999, 2002). Mutations that eliminate the activity of LON-1 increase body length by 1.5-fold, whereas animals with elevated DBL-1 activity are also longer than the wild type.

The insulin/IGF-1 and TGF-β hormonal systems also control reproductive growth in this organism (Riddle and Albert 1997). Mutant nematodes with decreased DAF-2/IGF-1 receptor activity enter into a state of developmental diapause called dauer, which is an arrested larval form specialized to survive unfavorable conditions. In addition, mutations that inactivate the type I and type II TGF-β receptors, DAF-1 and DAF-4, respectively, result in constitutive dauer development independently of environmental cues (Estevez *et al.* 1993; Gunther *et al.* 2000). It was shown that dauer development in insulin/IGF-1- and TGF-β-signaling mutant nematodes requires the function of autophagy genes, and that normal dauer morphogenesis is associated with increased autophagy (Meléndez *et al.* 2003). Autophagy is a highly regulated cellular pathway used by eukaryotic cells to degrade parts of their contents during development and to survive nutrient deprivation (Klionsky and Emr 2001). Autophagic degradation of cytosolic materials is a major route for turnover of cellular macromolecules and organelles, in particular proteins and mitochondria. In this study, we investigate whether *unc-51* and *bec-1*, which are mutationally characterized *C. elegans* autophagy genes, are required for maintaining normal cell size.

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TABLE 1

Mutational inactivation of *unc-51* or *bec-1* reduces body length in *C. elegans*

Genotype	Body length (mm)	*N*	Phenotype
Wild type	1.28 ± 0.07	241	Wild-type
unc-51(e369)	0.87 ± 0.09	266	Sma
unc-51(e1189)	0.9 ± 0.08	304	Sma
bec-1(ok691); Ex[*bec-1(+)*]	0.99 ± 0.08	157	Sma
bec-1(ok700); Ex[*bec-1(+)*]	1.04 ± 0.14	154	Sma
lon-1(e185) [TGF-β][a]	1.72 ± 0.08	228	Lon
lon-2(e678) [TGF-β][a]	1.51 ± 0.09	243	Lon
dbl-1(+++) [TGF-β][a]	1.38 ± 0.09	252	Lon
daf-2(e1370) [insulin/IGF-1][a]	1.36 ± 0.08	185	Lon
unc-51(e1189); lon-1(e185)	1.12 ± 0.1	288	Sma
unc-51(e1189); dbl-1(+++)	1.05 ± 0.06	279	Sma
unc-51(e1189); daf-2(e1370)	0.98 ± 0.06	250	Sma
unc-51(e369); lon-1(e185)	1.07 ± 0.06	225	Sma
unc-51(e369); lon-2(e678)	1.16 ± 0.11	253	Non-Lon
unc-51(e369); dbl-1(+++)	1.05 ± 0.09	265	Sma
bec-1(ok691); Ex[*bec-1(+)*]*; lon-2(e678)*	1.1 ± 0.22	47	Sma
bec-1(ok691); Ex[*bec-1(+)*]*; dbl-1(+++)*	1.04 ± 0.17	58	Sma
bec-1(ok700); Ex[*bec-1(+)*]*; lon-2(e678)*	1.08 ± 0.2	47	Sma
bec-1(ok700); Ex[*bec-1(+)*]*; dbl-1(+++)*	1.12 ± 0.13	42	Sma
bec-1(ok700); Ex[*bec-1(+)*]*; lon-1(e185)*	1.08 ± 0.1	65	Sma
bec-1(ok700); Ex[*bec-1(+)*]*; daf-2(e1370)*	1.02 ± 0.09	89	Sma

Loss-of-functions mutations in *unc-51* or *bec-1* reduce body length and suppress, at least partially, the Lon phenotype of insulin/IGF-1 and TGF-β mutants. *dbl-1(+++)* indicates the DBL-1-overexpressing strain. Body-length measurements were performed as follows. Well-fed, young adult nematodes, which on the day when they become gravid, *i.e.*, start to produce embryos, were anesthetized with 10 mM sodium-azide solution for 5 min. This treatment allows for coiled *unc-51* mutants to be relaxed. Body length of anesthetized worms was measured with a dissecting microscope and micrometer. Note that *unc-51* mutant animals are defective in movement, therefore, only those individuals were selected for body-length measurement that had remained in the bacterium layer and thus appeared well fed. This criterion may help to avoid the potential effect of restricted food intake on cell growth. Data are given as mean ± standard deviation. For single mutant animals, $P < 0.0001$ (unpaired *t*-test, body length of single mutants were compared with that of wild type). For double mutant genotypes, $P < 0.0001$ (unpaired *t*-test, body length of double mutants were compared with that of the corresponding "Lon" single mutants). Sma, small body size; Lon, long body length.

[a] *daf-2* encodes the IGF-1 receptor, whereas *dbl-1*, *lon-1*, and *lon-2* encode components of the TGF-β signaling axis.

The wild-type *C. elegans* strains display a characteristic body length of 1.2 mm (Brenner 1974). We examined *unc-51* loss-of-function mutant nematodes at a well-defined developmental stage (see Table 1) and found that they show a marked shortening in mean body size. For example, body length at the young adult stage was 0.89 ± 0.04 mm in *unc-51(e369)* mutants *vs.* 1.25 ± 0.05 mm in wild-type animals (unpaired *t*-test; $N = 250$, $P < 0.0001$) (Figure 1 and Table 1). *unc-51* encodes a serine/threonine kinase similar to the yeast autophagy protein Atg1 (Ogura *et al.* 1994), which is a key regulator of autophagosome formation (Klionsky 2005). Moreover, the autophagic process appeared to be defective in *unc-51* mutants. According to our electron microscopic observations, autophagic vacuoles were present almost exclusively in lateral hypodermal cells, and their membrane was excessively whorled (Figure 2). This may be indicative of defective autophagic vacuole formation in these animals. We also monitored the effects of mutations in another *C. elegans* autophagy gene, *bec-1*, on body length. *bec-1* is an essential gene that is the nematode ortholog of the human tumor suppressor gene *Beclin1* and yeast *atg6* (Meléndez *et al.* 2003; Takács-Vellai *et al.* 2005). Because *bec-1* loss-of-function mutations arrest development at different stages (Takács-Vellai *et al.* 2005), we rescued the lethality of *bec-1* mutants by an unstable (extrachromosomal) transgene array containing wild-type copies of *bec-1* (*bec-1(−); Ex*[p*bec-1*::BEC-1::GFP + *rol-6(su1006)*]). On average, randomly selected adults of *bec-1(−); Ex*[*bec-1(+)*] genotype were significantly shorter than wild-type nematodes (Figure 1 and Table 1). The reduced body length of certain *bec-1(−); Ex*[*bec-1(+)*] adults may result from incomplete rescue of *bec-1* in somatic cells. The possibility that this is due to an unlinked mutation that is not rescued by the array is unlikely because both *bec-1* alleles *ok691* and *ok700* behaved similarly. In good agreement with the body length data of *bec-1(−); Ex*[*bec-1(+)*] and *unc-51* mutant adults, their body volume was also markedly reduced as compared with

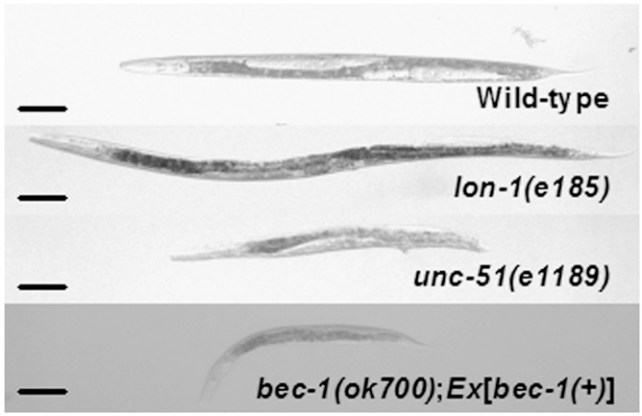

FIGURE 1.—Body size of mutant nematodes with reduced autophagy. The TGF-β genetic pathway component *lon-1* encodes a negative regulator of cell size, whereas *unc-51* and *bec-1* are two autophagy genes. Bars, 0.1 mm.

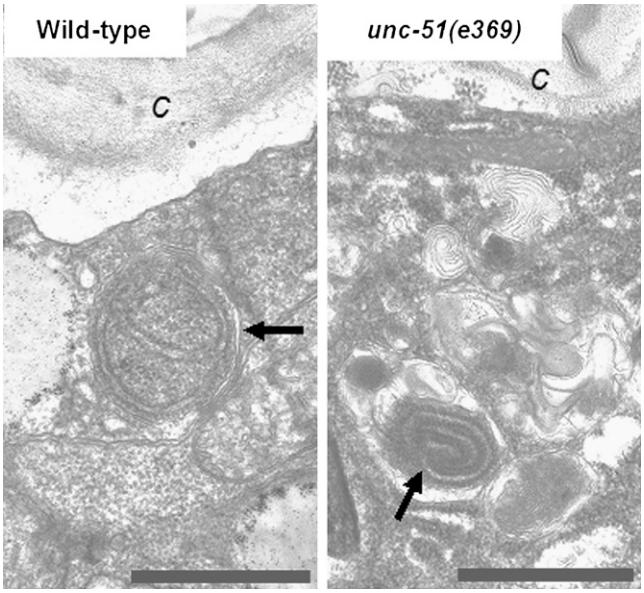

FIGURE 2.—The autophagic process appears to be defective in *unc-51* mutant animals. (Left) Autophagic vacuole with normal double isolation membrane (arrow) in the hypodermis from a wild-type animal. (Right) Abnormal autophagic vacuole (arrow) with strongly myelinated isolation membrane is embedded in a region of the cytoplasm abundant in membrane whorls in a hypodermal seam cell from *unc51(e369)* mutant. c, cuticule; bars, 1 μm. For fixation and embedding of transmission electron microscopic samples, the nematodes were treated individually. They were cut open under a dissecting microscope in a drop of fixative composed of 0.2% glutaraldehyde and 3.2% formaldehyde in 0.15 M cacodylate buffer. After an overnight fixation at 4°, the fixative was changed to washing buffer (0.1 M cacodylate buffer), and the samples were embedded in agar, post-fixed with 0.5% cacodylate-buffered OsO_4, stained with 2% uranyl acetate, dehydrated in ethanol and propylene oxide, and embedded in Durcupan (Fluka Chemical, Buchs, Switzerland). Thereafter the samples were cut along the longitudinal body axis with Reichert-Jung Ultracut-E type ultramicrotome, stained with lead citrate, and examined in a JEM100CX II electron microscope.

the wild type (Table 2). Together, these results suggest that *bec-1(−); Ex*[*bec-1(+)*] and *unc-51* mutant animals display a characteristic small body size (Sma) phenotype.

unc-51 and *bec-1* mutant animals also exhibited delay in development (data not shown), but had wild-type cell numbers as revealed by scoring different cell types expressing reporters labeled with green fluorescent protein. For example, we used the *tra-1::gfp* and *prk-1::gfp* markers to visualize intestinal cells; *mec-7::gfp*, which is expressed in certain Q-cell descendants; and *cdh-3::gfp* and *ajm-1::gfp* to label lateral seam cells. We found twenty GFP-positive intestinal cells in both wild type as well as in *unc-51(e369)* and *bec-1(ok691); Ex*[*bec-1(+)*] mutant animals (Figure 3 and results not shown). Furthermore, mutations in *unc-51* and *bec-1* also did not affect the number of Q-cell progeny and hypodermal seam cells. We next monitored the mean longitudinal diameter of gut cells by Nomarski microscopic analysis of strains expressing *prk-1::gfp* (Figure 3D). For example, mean gut cell diameter was only 41.9 ± 1.9 μm in *unc-51(e369)* mutants ($N = 35$ animals), as compared with 56.1 ± 3.2 μm in wild-type animals ($N = 30$). We also measured the volume of the intestine and the length and area of seam cells, as described previously (WANG *et al.* 2002; HIROSE *et al.* 2003). Our data shown in Tables 3 and 4 indicate that the reduced body size of *unc-51* mutants was due to a decrease in cell size (mutants deficient for BEC-1 were not examined for this trait). Thus, autophagy genes, or at least some of them, are required for normal cell growth.

Next, we evaluated the effects of inhibiting the function of UNC-51 and BEC-1 on the long body size (Lon) phenotype of mutant strains defective in insulin/IGF-1 or TGF-β signaling. We found that *daf-2(e1370)*,

TABLE 2

***unc-51* and *bec-1* mutant adults have decreased body volume**

Genotype	Body length (mm)	Diameter (mm)	Volume (mm^3)	% body volume	*N*
Wild type	1.2 ± 0.02	0.083 ± 0.0015	0.00619 ± 0.0002	100	8
unc-51(e1189)	0.86 ± 0.07	0.076 ± 0.0013	0.0036 ± 0.00019	58.17	11
bec-1(ok700); Ex[*bec-1(+)*]	1.02 ± 0.15	0.083 ± 0.001	0.0041 ± 0.0003	66.88	11

Body diameter and volumes were determined as described (KAMMENGA *et al.* 2007). For mutant body volumes, $P < 0.001$ (unpaired *t*-test). Data are given as mean ± standard deviation.

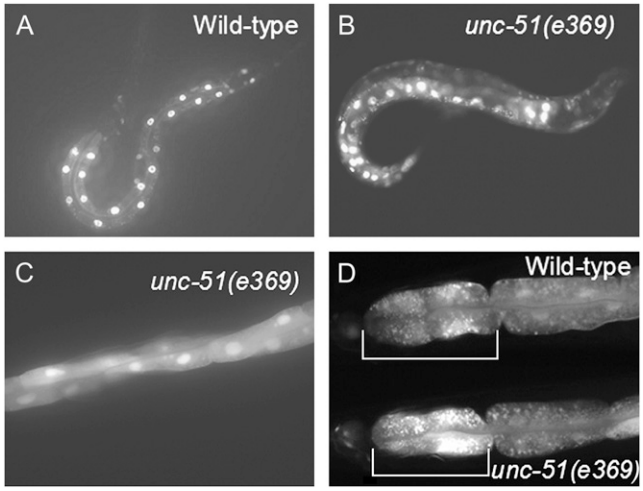

FIGURE 3.—Visualization of intestinal cells in adult nematodes. (A) TRA-1::GFP is expressed in the nucleus of gut cells from wild-type adult. (B) TRA-1::GFP expression in *unc-51(e369)* mutant adult. (C) Expression of PRK-1::GFP in the intestine of *unc-51(e369)* animal. (D) Expression of PRK-1::GFP in individual intestinal cells of wild-type (top) and *unc-51(e369)* mutant (bottom) animals. Brackets indicate the longitudinal border of the first four individual intestinal cells. Both images were made with the same magnification.

lon-1(e185), and *lon-2(e678)* mutants as well as DBL-1-overexpressing nematodes, which as single mutant animals are each long (KRISHNA *et al.* 1999; SUZUKI *et al.* 1999; MORITA *et al.* 1999, 2002; MCCULLOCH and GEMS 2003), displayed small or wild-type body size when they also carried a loss-of-function mutation in *unc-51* or *bec-1* (Table 1). Note that double mutants carrying the *daf-2* mutation *e1370* were grown at 15° until they reached the L2 larval stage and then transferred at 25° to develop further. This temperature shift prevented these worms from arrest development as abnormal dauer larvae (MELÉNDEZ *et al.* 2003). Suppression of the Lon phenotype in insulin/IGF-1 and TGF-β pathway mutant animals by mutations in *unc-51* and *bec-1* suggests that autophagy genes interact with and, possibly, act downstream of these hormonal systems, as well as suggesting that autophagy may be implicated in cell growth control. In other words, *unc-51* and *bec-1* are epistatic to *daf-2*, *dbl-1*, and *lon-1* to influence body size.

TABLE 3

Cell size measurements in *unc-51* mutant animals

Genotype	Seam cell length (μm)	Seam cell area (μm²)	*N*
ajm-1::gfp	33.2 ± 5.1	1.25 ± 0.12	55
unc-51(e369); ajm-1::gfp	27.4 ± 4.0	0.95 ± 0.2	39

The *ajm-1::gfp* marker localizes to the adherens junctions surrounding the seam cells (MOHLER *et al.* 1998). Seam cell measurements were carried out at the L3 stage. $P < 0.001$ (unpaired *t*-test). Data are given as mean ± standard deviation.

TABLE 4

Organ volume measurements in *unc-51* mutant animals

Genotype	Volume of the intestine (nl)	% relative volume	*N*
prk-1::gfp	0.95 ± 0.27	100	20
unc-51(e369), prk-1::gfp	0.76 ± 0.33	78	20

For intestinal volume measurements, animals were selected at young adulthood. $P < 0.001$ (unpaired *t*-test). Data are given as mean ± standard deviation.

However, in certain double mutant combinations *unc-51* and *bec-1* mutant alleles did not completely suppress body lengths of *lon-1*, *lon-2*, and *daf-2* mutants, as well as DBL-1-overexpressing animals, *i.e.*, the size of these double mutants was intermediate between the corresponding single mutants (Table 1). Alternatively, this implies that parallel pathways might exist in which *unc-51* and *bec-1* control body size independently from insulin/IGF-1 and/or TGF-β signaling. If autophagy genes mediate the effects of both signal transduction axes in the control of cell growth, then *unc-51* and *bec-1* should function both in parallel and downstream of either of these growth modulatory pathways, explaining intermediate body sizes observed (see below).

In *C. elegans*, several components of the TGF-β signaling pathway are involved in male tail ray pattern formation (SUZUKI *et al.* 1999; MORITA *et al.* 1999, 2002). To demonstrate whether UNC-51 and BEC-1 are also interacting with TGF-β signaling to affect male tail development, we assayed patterning of male tail structures in *unc-51(−)* mutants and *bec-1(−); Ex*[*bec-1(+)*] animals. *unc-51* mutant males showed severe tail abnormalities, including the complete loss of the sensory rays and fan (Figure 4B). Moreover, BEC-1 was expressed in all structures of the adult male tail, and in *bec-1* mosaic males the neighboring rays are often fused with each other (Figure 4). Together, our results indicate that *unc-51* and *bec-1* influence male tail patterning, possibly by interacting with the TGF-β system. To determine where these genes may act in the TGF pathway, we analyzed the expression of an integrated p*lgg-1*::GFP::LGG-1 reporter (TÓTH *et al.* 2007), which is supposed to label autophagosomal structures in hypodermal seam cells (MELÉNDEZ *et al.* 2003). Wild-type animals and *sma-6(e1482)* mutants carrying a transgene that expressed GFP::LGG-1 had mainly a diffuse cytoplasmic staining pattern (Figure 5), whereas the number of GFP::LGG-1-positive foci increased markedly in the *lon-1(e185)* background. This suggests that autophagy genes, at least some of them, may act downstream of and are inhibited by LON-1 in body size regulation.

In summary, our data indicate that the insulin/IGF-1 and TGF-β signaling pathways may interact with the UNC-51 and BEC-1 autophagy genes to control cell size in *C. elegans*. The autophagy protein Atg5 has also been

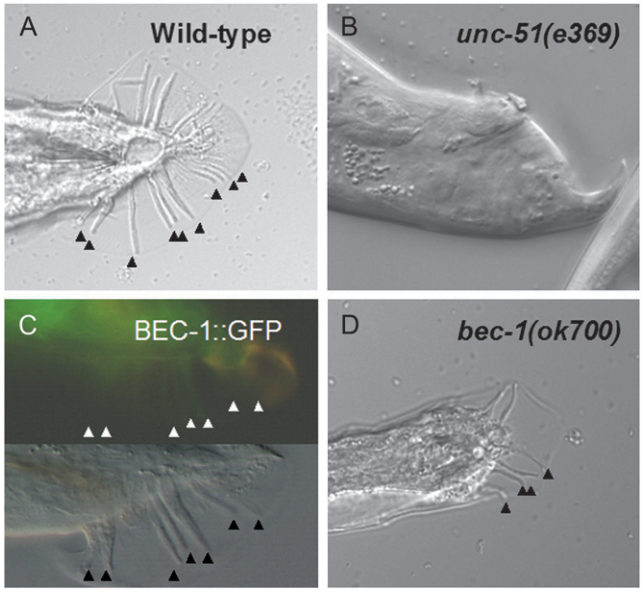

Figure 4.—UNC-51 and BEC-1 are required for male tail development. (A) Tail structure of wild-type adult male. (B) Tail phenotype of an *unc-51(e369)* male. In this animal the rays are completely missing. (C) p*bec-1*::BEC-1::GFP is expressed in wild-type male. Corresponding fluorescence (top) and Nomarski (bottom) images. (D) Male tail structure in a *bec-1 (ok691); Ex*[p*bec-1*::BEC-1::GFP] adult. Arrowheads indicate the rays on one side. *bec-1* mutants with affected tail always lost GFP expression in the tail.

shown to influence cell size in mice; in Atg5-deficient animals cell size is not reduced in response to food withdrawal (Hosokawa *et al.* 2006). Furthermore, autophagy genes are also implicated in TOR (target of rapamycin) kinase-mediated cell growth control in Drosophila (Scott *et al.* 2004). Together, these data point to autophagy, an evolutionarily conserved cellular degradative pathway, as a possible mechanism to take part in size regulation of cells and organs in divergent animal phyla (Figure 6). This is in good accordance with the pivotal role of autophagy in regulated turnover of subcellular constituents (cytoplasmic macromolecules and organelles) (Klionsky and Emr 2001).

Further studies are needed to determine whether other *C. elegans* autophagy genes are also involved in cell growth control. However, at present only a very limited number of autophagy genes are available as mutant alleles and, what we did not show here, their RNAi-mediated silencing is often ineffective or leads to weak reactions (see also Kovács *et al.* 2004).

The characterization of the autophagic process itself in *C. elegans* is still in a very preliminary stage (Meléndez *et al.* 2003; Kovács *et al.* 2004). Feeding defective mutant worms also have a shorter body length and are proposed to have increased autophagy (Morck and Pilon 2006). This suggests that, similar to its possible dual role in neuronal cell survival and loss (Takács-Vellai *et al.* 2006), both deregulation and hyperactivation of autophagy genes cause reduction in cell size. Thus, fine tuning of autophagy gene activity is critical for maintaining normal cell size. The regulation of cell growth and proliferation are tightly integrated. Our study suggests that identifying autophagy genes as key modulators of cell size will be essential for understanding how uncontrolled cell growth leads to cancer.

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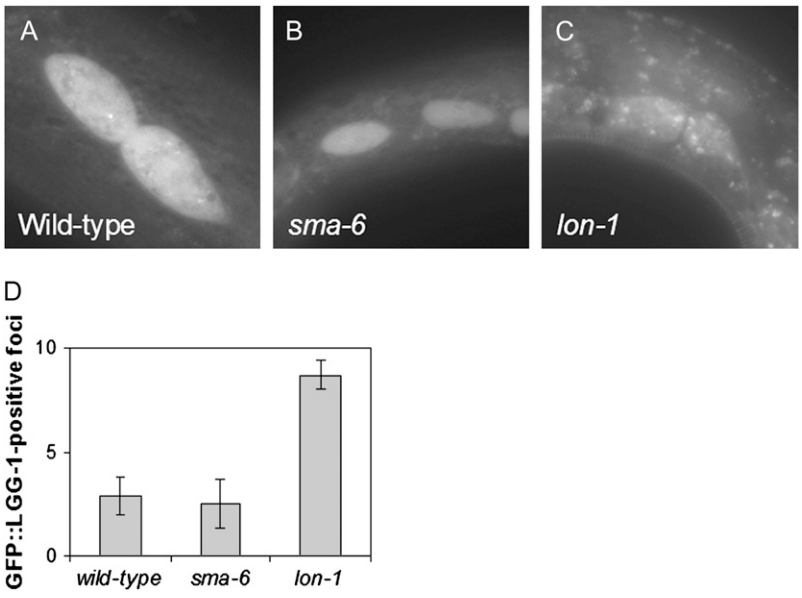

Figure 5.—Intracellular accumulation of LGG-1 in hypodermal seam cells is affected by TGF-β signaling. (A) Expression of GFP::LGG-1 in the seam cells of wild-type animal. GFP foci are supposed to label autophagosomal structures. (B) Expression of the GFP::LGG-1 reporter in the seam cells of *sma-6(e1482)* mutant L3 larva. (C) GFP::LGG-1 accumulates in punctate areas in the seam cells of *lon-1(e185)* mutant L3 larva. (D) Quantification of GFP::LGG-1-positive foci in individual seam cells of wild-type, *sma-6(e1482)*, and *lon-1(e185)* mutant animals. The number of punctate area per seam cell is shown.

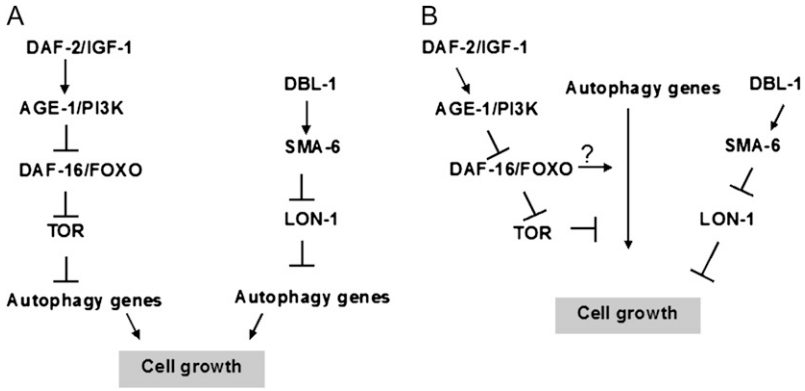

FIGURE 6.—Two alternative models for how autophagy affects cell growth in *C. elegans*. (A) If autophagy genes mediate the effect of both insulin/IGF-1 and TGF-β signaling to control cell growth, *i.e.*, insulin/IGF-1 and TGF-β signaling converge on autophagy to regulate cell size, *unc-51* and *bec-1* should function downstream of these two hormonal systems. (B) Because mutations in *bec-1* and *unc-51* do not completely suppress the Lon phenotype of *daf-2* and certain TGF-β mutant strains, it is also possible that autophagy genes act in parallel to insulin/IGF-1 and TGF-β signaling in cell growth control. The question marks indicate whether the effect of the insulin/IGF-1 signaling pathway on body size is solely the result of changes in autophagy. Arrows indicate positive regulatory interactions, bars represent inhibitions. DAF-2, IGF-1 receptor; AGE-1, phosphatidylinositol-3-OH kinase; DAF-16, FOXO forkhead transcription factor; SMA-6, type I TGF-β receptor; DBL-1, TGF-β ligand for SMA-6; LON-1, cytoplasmic transducer of TGF-β signaling.

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