

The TOR pathway interacts with the insulin signaling pathway to regulate *C. elegans* larval development, metabolism and life span

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Summary

The highly conserved target-of-rapamycin (TOR) protein kinases control cell growth in response to nutrients and growth factors. In mammals, TOR has been shown to interact with raptor to relay nutrient signals to downstream translation machinery. We report that in *C. elegans*, mutations in the genes encoding CeTOR and raptor result in dauer-like larval arrest, implying that CeTOR regulates dauer diapause. The *daf-15* (raptor) and *let-363* (CeTOR) mutants shift metabolism to accumulate fat, and raptor mutations extend adult life span. *daf-15*

transcription is regulated by DAF-16, a FOXO transcription factor that is in turn regulated by *daf-2* insulin/IGF signaling. This is a new mechanism that regulates the TOR pathway. Thus, DAF-2 insulin/IGF signaling and nutrient signaling converge on DAF-15 (raptor) to regulate *C. elegans* larval development, metabolism and life span.

Key words: TOR, Raptor, *daf-15*, Dauer formation, Aging, Insulin

Introduction

In multicellular organisms, the control of growth depends on the integration of genetic and environmental cues (Conlon and Raff, 1999). Cell growth in response to nutrients is controlled by the highly conserved TOR (target of rapamycin) protein kinases (Schmelzle and Hall, 2000). TOR belongs to a family of phosphatidylinositol kinase-related kinases. It is specifically inhibited by the macrolide rapamycin, which forms a complex with the prolyl isomerase FKBP12 to interact with TOR (Heitman et al., 1991; Stan et al., 1994; Chen et al., 1995; Choi et al., 1996). TOR kinase regulates various cellular processes, including initiation of mRNA translation, ribosome synthesis, expression of metabolism-related genes and autophagy (Schmelzle and Hall, 2000). The mechanism by which nutrients regulate the TOR signaling pathway remains poorly understood.

Recently, TOR was reported to interact with raptor (regulatory associated protein of mTOR) to transduce nutrient signals to the downstream translation machinery in mammals (Kim et al., 2002; Hara et al., 2002). Raptor associates in a near stoichiometric ratio with mTOR to form a complex that functions as the nutrient sensor (Kim et al., 2002). It was proposed that raptor acts as a scaffold to bridge TOR with its putative phosphorylation targets (Abraham, 2002; Kim et al., 2002; Hara et al., 2002).

Insulin/IGF signaling is also essential for growth and body size (Oldham and Hafen, 2003). Both TOR and insulin/IGF signaling regulate a common set of effectors involved in control of cell growth, including the translation initiation factor 4E-binding protein (4EBP1) and the S6 ribosomal protein kinase (Schmelzle and Hall, 2000). TOR is a potential downstream component of the insulin/IGF signaling (Oldham

and Hafen, 2003), but it is not clear how these two signaling pathways interact.

In *C. elegans*, insulin/IGF signaling regulates larval development and adult life span (Kenyon et al., 1993; Kimura et al., 1997). The first-stage (L1) larva responds to overcrowding and limited food by arresting development as a long-lived dauer larva, an alternative to the growing third-stage, L3 (Riddle and Albert, 1997). Pre-dauer L2 larvae accumulate fat in preparation for a prolonged period of non-feeding. Dauer larvae have a constricted pharynx, shrunken intestinal lumen, and a specialized cuticle. These traits are reversed when dauer larvae resume development to the adult in response to food.

daf-15 mutants are Daf-c (dauer-formation constitutive). At the second molt, they arrest development non-conditionally as dauer-like L3 larvae, but feeding is not completely suppressed. Electron microscopic observation of *daf-15(m81)* showed that some tissues assume dauer morphology and others do not (Albert and Riddle, 1988). Head shape, cuticle and intestinal ultrastructure are non-dauer, whereas sensory structure and excretory gland morphology are intermediate between that of dauer and nondauer stages. *daf-15* larvae are neither able to complete dauer morphogenesis nor develop to the adult.

We report that *daf-15* encodes the *C. elegans* ortholog of raptor, and that a mutation in *let-363*, the gene encoding CeTOR (Long et al., 2002), also results in dauer-like larval arrest. Hence, raptor and TOR are required for dauer morphogenesis and for maturation to the adult. *daf-15* and *let-363* mutants shift metabolism to accumulate fat, as do pre-dauer larvae. Life spans of *daf-15* heterozygous adults are significantly extended. We also show that *daf-15* transcription is regulated by *daf-2* insulin/IGF signaling. Thus, DAF-15 is a

point of integration of insulin/IGF signaling and nutrient signaling pathways to regulate *C. elegans* larval development, metabolism and longevity.

Materials and methods

Strains

Strains were maintained at 20°C as described by Brenner (Brenner, 1974). Genotypes of animals used were: wild-type N2 Bristol, *unc-24(e138) +/+ daf-15(m81)*, *unc-24(e138)/+*, + *dpy-20(e1282)/daf-15(m81) +*, *dpy-20(e1282)/+*, *unc-24(e138) daf-15(m634)/nT1*, *unc-24(e138)/nT1*, *let-363(h111) +/+ dpy-5(e61)*, *unc-63(e384) +/+ let-363(h111)*; + *dpy-20(e1282)/daf-15(m81) +*, *unc-63(e384)/+*; + *dpy-20(e1282)/daf-15(m81) +*, *daf-2(e1370)*, *daf-2(e1370)*; + *dpy-20(e1282)/daf-15(m81) +*, *daf-2(e1370)*; *dpy-20(e1282)/+*, *daf-16(mgDf47)*, *daf-16(mgDf47)*; + *dpy-20(e1282)/daf-15(m81) +* and *daf-16(mgDf47)*; *dpy-20(e1282)/+*.

Life span

For heterozygous strains, wild-type L4 larvae were picked from populations grown at 20°C and transferred to 25°C for survival tests. For some controls (*unc-63/+*; + *dpy-20/daf-15 +*, *daf-2*; *dpy-20/+* and *daf-16*; *dpy-20/+*), the genotype of single animals grown at 25°C was determined by progeny testing. Animals with the correct genotype were pooled. The date when the animals were shifted to 25°C was designated as day 1. SPSS Windows Version 11.5 was used for data analysis.

Sudan black staining

Heterozygous mutant strains were grown at 20°C and dauer-like segregants (*daf-15* and *let-363* homozygotes or *daf-15*; *daf-d* double mutants) were hand-picked 1 day after dauer-like arrest. *daf-2(e1370)* dauer larvae grown at 25°C were harvested one day after dauer arrest. Control animals [N2 and *daf-16(mgDf47)*] were grown at 20°C on ample food. Staining was performed as described by Kimura et al. (Kimura et al., 1997).

daf-15 cloning

SNP mapping (Wicks et al., 2001) was used to localize *daf-15*. Cosmid rescue was done as described previously (Jia et al., 2002). To test three candidate genes carried by cosmid C10C5 with dsRNA treatment, part of each gene was amplified by PCR using the following primers (underlined): C10C5.1, 5' CGT GCG TCG TCA AAT TGC TG 3', 5' CAC TCC ATG TCT CAG TGG TG 3'; C10C5.2, 5' GGA TTC GGT GGA CTC GGT CA 3', 5' GTT GGC AAG GAT TCT CAC TCT 3'; C10C5.6, 5' TGA CTT CGA ACA TGT GCT GAC 3', 5' TCT CGC AGT ATC ATC GAC CAT 3'. The PCR fragments were cloned into the pGEMT vector (Promega), and the Riboprobe Combination System-SP6/T7 (Promega) was used to transcribe RNA in vitro according to the manufacturer's protocol. Double-stranded RNA was synthesized and injected as described by Fire et al. (Fire et al., 1998).

To confirm the exon-intron junctions predicted by genefinder, 5' and 3' regions of the *daf-15* cDNA were isolated by RT-PCR of total RNA from mixed-stage N2 animals. The cDNA fragments were cloned into pGEM-T (Promega) and sequenced. To identify lesions in *m81* and *m634*, DNA from homozygous mutant animals [segregants from *unc-24(e138) daf-15(m81)/nT1* or *unc-24(e138) daf-15(m634)/nT1*, respectively] was amplified using gene-specific primers. Resulting PCR fragments were cloned into pGEM-T and sequenced.

daf-15::gfp (green fluorescent protein)

Gene-specific primers were used to amplify 1.8 kb of *daf-15* promoter plus DNA from the ATG start codon to 67 bp inside exon 3 (5' CCC AAG CTT GGA ATT TCC AAA ACG GTC GAG 3' and 5' ACG

CGT CGA CCT ACT TCC TGC GAT ATC TTC GAC 3'). The PCR fragment was digested and cloned into the *HindIII/SalI* sites of *gfp* vector pPD95.75. The *daf-15::gfp* plasmid DNA (100 ng/μl) and the pRF4 *rol-6(su1006)* marker plasmid (100 ng/μl) were microinjected into the ovaries of N2 adults to generate transgenic lines. Images are from *mEx157 [rol-6(su1006) daf-15p::Exon1-3::gfp]*. Use of GFP as a reporter was described by Chalfie et al. (Chalfie et al., 1994).

daf-16 RNAi treatment was performed by feeding according to Kamath et al. (Kamath et al., 2000) to examine whether the expression of *daf-15::gfp* is regulated by DAF-16. L4 transgenic *daf-2* animals carrying *daf-15::gfp* and *rol-6* were grown on food carrying vector only, or *daf-16* RNAi food, for 24 hours at 20°C. The adult animals were transferred to fresh plates and shifted to 25°C. GFP expression was examined in L2-stage transgenic progeny.

To construct the *daf-16* RNAi plasmid, 1.1 kb of the *daf-16*-coding region was amplified from *C. elegans* genomic DNA using primers 5'-CGG GAT CCG TCA CAA TCT GTC TC-3' and 5'-CCC AAG CTT GAA GTT AGT GCT TGG C-3'. The PCR product was cloned into the L4440 vector between the *BamHI* and *HindIII* sites, then transformed into HT115. This RNAi construct effectively suppressed the Daf-c phenotype of *daf-2(e1370)* mutants.

Semi-quantitative RT-PCR

daf-2(e1370) and *daf-16(mgDf47)*; *daf-2(e1370)* mutant animals were prepared as described by Lee et al. (Lee et al., 2003). Total RNA was extracted with an acid guanidinium thiocyanate-phenol-chloroform mixture (Chomczynski and Sacchi, 1987). mRNA was purified using a PolyAtract mRNA Isolation System (Promega). All RT-PCR was performed with SuperScript One-Step RT-PCR for Long Template (Invitrogen). The primers used for *daf-15* and *rpl-21* are: *daf-15*, 5' TGA CTT CGA ACA TGT GCT GAC 3' and 5' TCT CGC AGT ATC ATC GAC CAT 3'; *rpl-21*, 5' ATG ACT AAC TCC AAG GGT C 3' and 5' TCA CGC AAC AAT CTC GAA AC 3'.

Electrophoretic mobility shift assays (EMSA)

To perform EMSA, we first amplified a *daf-16* cDNA using gene specific primers (5' CGG GAT CCA TGA ACG ACT CAA TAG ACG AC 3', and 5' CCC AAG CTT CAA ATC AAA ATG AAT ATG CTG C 3') from total RNA of mixed-stage N2. The PCR fragment was digested and cloned into the *BamHI/HindIII* sites of protein expression vector pET-28a (Novagen) with a His tag at the C terminus. The construct was confirmed by DNA sequencing.

His-tagged DAF-16 was expressed in *E. coli* strain BL21 (DE3) after induction by 0.25 mM isopropyl-thio-β-D-galactopyranoside at 20°C for 2 hours. The fusion protein was purified using Ni-NTA agarose (Qiagen) according to the manufacturer's protocol. The DNA-binding abilities of purified His-tagged DAF-16 were tested by EMSA. The sequence of the *daf-15* probe (with the IRS in bold) was 5'- TTT TGC ACG AAA **TAT TTT TTC** TTA AAC TCG -3', and the sequence of the mutated probe (with base-pair changes underlined) was 5'- TTT TGC ACG AAA **GAG GGT TTC** TTA AAC TCG -3'. Oligonucleotides for the sense and antisense strand of each probe were annealed, and the double-stranded probes were end-labeled using T4 polynucleotide kinase (Fermentas) following the manufacturer's protocol. 10 nM ³²P-labeled probe, 200 ng His-tagged DAF-16 protein and 10 ng salmon sperm DNA (as a non-specific competitor) were used for each reaction. For competition experiments, a fivefold or a tenfold excess of either wild-type or mutant probe was added to each reaction. The DNA and protein were mixed and incubated at room temperature for 15 minutes, and the products separated on 5% PAGE gels. Gels were dried prior to autoradiography.

Mutagenesis of IRS elements in the promoter and first intron of *daf-15*

Mutations were introduced with the QuickChange site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions. Primers (5'- GAC TCG AAT AAA TAA AGA GGG TTT

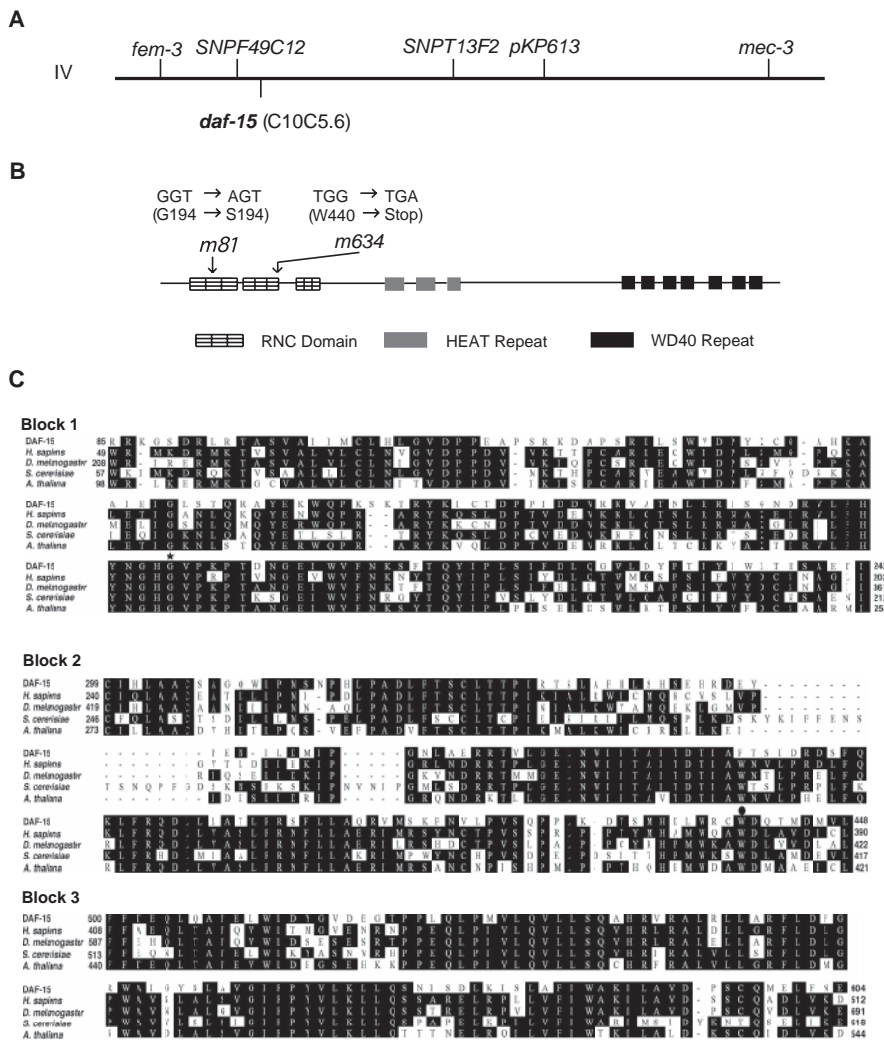


Fig. 1. *daf-15* encodes the *C. elegans* raptor. (A) Physical map of the *daf-15* region of chromosome IV (corresponding 0.6 map units). (B) A schematic structure of DAF-15 raptor. The predicted RNC domain, HEAT (Huntingtin, elongation factor 3, the regulatory A subunit of PP2A and Tor1p) repeats and WD40 repeats are indicated. Cosmid C10C5 partially rescues a *daf-15* mutant, but lacks the last three exons of *daf-15* encoding the last four WD40 repeats. Sequence alterations in *m81* and *m634* are shown. (C) Alignment of the three blocks of the DAF-15 RNC domain with its orthologs. Identical amino acids are in black boxes. The star in block 1 indicates the G194S change in *m81*. W440 (black dot) is changed to a TGA stop in *m634*. The numbers beside the sequences indicate the position of the beginning and ending amino acids in the corresponding protein. The GenBank Accession Number of the *daf-15* cDNA sequence is AY396716.

TAA ATT AAG ATA TTC G -3' and 5'- GGA ATA TCT TAA TTT AAA ACC CTC TTT ATT TAT TCG AGT C -3') were used to mutate the IRS that is 237 bp upstream of the ATG. Primers (5'- GCA ATT TTG CAC GAA AGA GGG TTT CTT AAA CTC GGT TTC C -3' and 5'- GGA AAC CGA GTT TAA GAA ACC CTC TTT CGT GCA AAA TTG C -3') were used to mutate the IRS in the first intron at +127. The mutated nucleotides are underlined in the primer sequences. The mutations were confirmed by sequencing.

Results

***daf-15* encodes the *C. elegans* ortholog of raptor**

Genetic mapping had shown that *daf-15* is between *fem-3* and *mec-3* on chromosome IV (I. M. Caldicott, PhD thesis, University of Missouri, 1995). We refined its position to a 477 kb region between single nucleotide polymorphism (SNP) markers *SNPF49C12* and *SNPT13F2* (Fig. 1A). Genomic cosmid clones corresponding to this region were injected individually into the germline of *unc-24(e138) daf-15(m81)/nT1* to test for complementation of *daf-15*. Cosmid C10C5 was found to partially rescue the *daf-15* Daf-c phenotype, as judged by the segregation of homozygous *Unc-24* progeny that bypassed larval arrest and matured to sterile adults.

We used RNA-mediated interference (RNAi) to test loss-of-function phenotypes for genes carried on cosmid C10C5. The RNAi was administered to wild-type N2 young adults by injection of in vitro synthesized double-stranded RNA (Fire et al., 1998). Inactivation of C10C5.6 induced the *daf-15* Daf-c phenotype in 100% of the progeny observed, indicating that C10C5.6 is *daf-15*. Cosmid C10C5 lacks the last three out of 20 *daf-15* exons, explaining the partial failure of this cosmid to rescue the mutant phenotype.

daf-15 encodes the *C. elegans* ortholog of raptor and exhibits sequence conservation with protein family members from mammals, *Drosophila*, *Arabidopsis* and yeast (Kim et al., 2002; Hara et al., 2002; Wedaman et al., 2003). The gene spans ~8.3 kb from the predicted ATG initiator site to the TAA terminator. All predicted intron-exon junctions were confirmed by RT-PCR. The 5.4 kb cDNA (GenBank Accession Number AY396716) encodes a predicted 205 kDa protein.

The N-terminal RNC (raptor N-terminal conserved) domain consists of three highly conserved blocks (Fig. 1B,C). All of the raptor family members, including DAF-15, share 34-52% amino acid identity (67-79% similarity) in this domain (Kim et al., 2002) (Fig. 1C). An ethylmethane sulfonate-induced GC-to-AT mutation in exon 5 was identified in *daf-15(m81)*. It changes an invariant amino acid (G194S) in the first block of the RNC domain (Fig. 1B,C). G194 is the second glycine in a sequence of ten amino acids (LFHYNGHGV) that is invariant in all raptor family members (Fig. 1C), indicating that this domain is functionally important. It might be a key region in mediating raptor's interaction with TOR or other proteins. The *daf-15(m634)* allele carries a nonsense mutation in exon 8 that changes TGG (W440) to a TGA stop, resulting in a truncated protein retaining only the first two blocks of the RNC domain (Fig. 1B,C). In addition to the RNC domain, all raptor orthologs have three HEAT repeats followed by seven WD40

repeats in the C-terminal third of the protein (Fig. 1B). Both HEAT and WD40 domains mediate protein-protein interactions, implying that raptor could be a scaffold for the TOR kinase to interact with regulatory proteins (Kim et al., 2002; Hara et al., 2002; Wedaman et al., 2003).

LET-363 and DAF-15 comprise the TOR/raptor complex in *C. elegans*

Having implicated loss of raptor function in dauer-like larval arrest, we next examined TOR, which is encoded by *let-363*. The *let-363* mutants were reported to arrest development at the L3 stage, exhibiting a phenotype thought to result from global inhibition of mRNA translation (Long et al., 2002). As the *let-363* mutation was originally isolated on a chromosome marked with *dpy-5(e61)*, we separated it from the closely linked *dpy* marker in order to observe the mutant morphology in a wild-type genetic background. The *let-363* larvae are similar to *daf-15* (dauer-like both in morphology and movement), consistent with a similarity in gene function. Blast searches of the *C. elegans* genome revealed only one raptor (Kim et al., 2002; Hara et al., 2002) and one TOR (Long et al., 2002), as previously reported. We propose that LET-363 and DAF-15 comprise the TOR/raptor complex in *C. elegans*.

let-363 is expressed in most, if not all, cells from the early embryo to adulthood (Long et al., 2002). We constructed a *daf-15::gfp* reporter that fuses the *gfp* sequence in frame with the first 71 amino acids of DAF-15. This reporter was expressed in many cells and tissues, including the nervous system, the intestine, gonadal distal tip cells, the excretory cell, hypodermal cells, and pharyngeal and body wall muscles (Fig. 2). The expression was observed in all stages, including starvation-induced dauer larvae. An overall expression pattern similar to that of *let-363* is consistent with the idea that DAF-15 and LET-363 interact.

CeTOR/DAF-15 raptor signaling regulates metabolism and life span

Fat accumulation is one characteristic of *C. elegans* dauer larvae (Kimura et al., 1997). We examined fat accumulation in *let-363(h111)* and *daf-15(m81)* homozygous mutants using Sudan black staining. Wild-type L3 animals accumulated relatively little fat (Fig. 3A), whereas both *let-363* and *daf-15* accumulated numerous large fat droplets, similar to *daf-2(e1370)* dauer larvae (Fig. 3B-D).

Mutants with reduced insulin/IGF signaling have increased adult longevity (Kenyon, 1997; Tatar et al., 2001; Holzenberger et al., 2003). In *C. elegans*, these include mutants in the *daf-2* insulin receptor (Kimura et al., 1997) and *age-1* PI3 kinase (Morris et al., 1996). As neither *daf-15* nor *let-363* homozygotes reach maturity, we tested the life spans of heterozygous adults. We hypothesized that the loss of one dose of *let-363* or *daf-15* might decrease the activity of this pathway enough to affect life span, as is the case with the mouse IGF receptor (Holzenberger et al., 2003). We tested life spans at 25°C, a temperature at which many *daf-2* longevity mutants show the greatest life span extension (Larsen et al., 1995; Gems et al., 1998).

A heterozygous *daf-15* mutation extends both mean and maximum adult life span. The mean life span of *daf-15(m81)* +/+ *dpy-20(e1282)* adults was increased by 30% compared with *dpy-20*+ controls, and maximum life span increased by

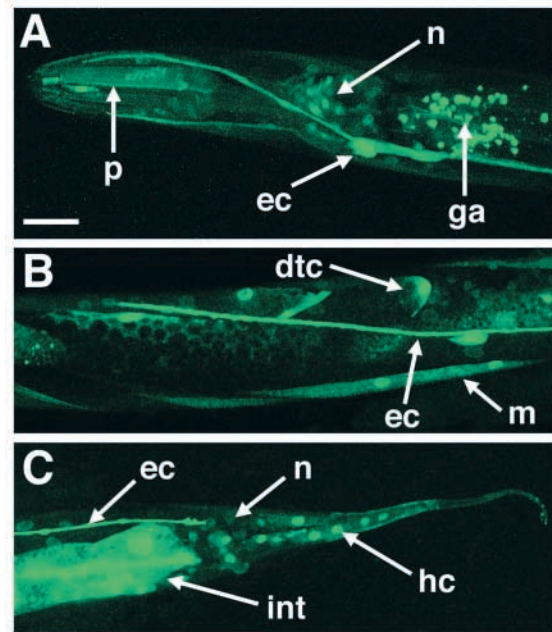


Fig. 2. *daf-15p::gfp* expression in N2 L4 larvae. Confocal images of selected planes at the same magnification show (A) head, (B) mid-body and (C) tail regions. n, nervous system; p, pharynx; ec, excretory cell; ga, gut autofluorescence; dtc, distal tip cell; m, body wall muscle; int, intestine; hc, hypodermal cell. Scale bar: 20 μ m.

4 \pm 0 days (mean \pm s.d.), or 19% (Table 1; Fig. 4A). When *dpy-20* was replaced with another balancing marker, *unc-24(e138)*, mean life span was extended 13% by *daf-15(m81)* (Table 1; Fig. 4B). Mean life span of *unc-24(e138) daf-15(m634)/nT1* was 33% longer than that of the control strain *unc-24(e138)/nT1* (Table 1; Fig. 4C).

We did not observe obvious life span extension in *let-363(h111)* heterozygous mutants balanced by *dpy-5(e61)*, the duplication *hDp2* or the translocation *szT1* (data not shown). Thus, we examined whether *let-363* could enhance the life span of *daf-15* in double heterozygotes. In only one of three independent trials, the mean life span of + *unc-63(e384)/let-363(h111)* +; + *dpy-20(e1282)/daf-15(m81)* + ($n=62$) was increased by 20% ($P<0.05$, log-rank test) relative to *unc-63(e384)/+*; + *dpy-20(e1282)/daf-15(m81)* + controls ($n=55$), but the maximum life span was not extended. Possibly, LET-363 activity was not reduced sufficiently in heterozygotes for a reproducible effect on life span. Nevertheless, the effect of *daf-15/+* on life span shows that CeTOR/DAF-15 raptor signaling modulates adult life span in addition to regulating larval development and metabolism. Recently, inactivation of *let-363* in the adult by RNAi treatment was reported to extend longevity (Vellai et al., 2003).

Genetic interaction between DAF-2/insulin and CeTOR signaling pathways

Insulin signaling can modulate the TOR pathway in mammals (Schmelzle and Hall, 2000), and in *C. elegans* the DAF-2 insulin/IGF pathway functions to inhibit its major target, DAF-16, a FOXO family transcription factor (Lin et al., 1997; Ogg et al., 1997). We examined the effect of the *daf-16(mgDf47)* null mutation (Ogg et al., 1997) on the *daf-15* Daf-c phenotype,



Fig. 3. *daf-15* and *let-363* metabolic defect. Fat accumulation was assayed by Sudan black staining in L3 larvae grown at 20°C; *daf-2* dauer larvae were formed constitutively at 25°C. (A) Wild-type N2, (B) *daf-2(e1370)*, (C) *daf-15(m81)*, (D) *let-363(h111)*, (E) *daf-16(mgDf47)*, (F) *daf-16(mgDf47); daf-15(m81)*. All mutants except for the *daf-16* single mutant accumulate abnormally high levels of fat relative to wild type. The entire experiment was performed twice, with at least 30 animals observed each time. Fat accumulation in *daf-15(m81)* and *let-363(h111)* was also confirmed by Nile red staining (data not shown).

and found *daf-15* to be epistatic. That is, the *daf-16; daf-15* double mutants exhibit the *daf-15* dauer-like phenotype, confirming previous work using the *daf-16(m26)* allele (Albert and Riddle, 1988). *daf-16* also failed to suppress *daf-15* fat

accumulation (Fig. 3). Compared with the single *daf-16(mgDf47)* mutant (which is similar to wild-type L3 larvae) *daf-16(mgDf47); daf-15(m81)* double mutants accumulate fat (Fig. 3E,F). Taken together with data for *let-363*, the *let-363/daf-15* pathway could be downstream of, and regulated by, the *daf-2* pathway. Alternatively, it may be a new pathway that functions in parallel with *daf-2* signaling to regulate *C. elegans* larval development and metabolism.

We also examined the genetic interactions between *daf-15* and *daf-2* with respect to life span. The mean life span of *daf-2(e1370); + dpy-20/daf-15(m81) +* was increased by 12% relative to the *daf-2(e1370); dpy-20(e1282)/+* controls ($P < 0.01$, log-rank test), and the maximum life span was increased by 12 ± 5 days (mean \pm s.d.) (Table 1; Fig. 4D). These results suggest that *daf-15* may act in parallel to *daf-2*, but the interpretation must be guarded, because we used *daf-15/+* strains to test the genetic interactions. Reduction, but not elimination, of *daf-15* function leads to increased life span.

daf-16 activity, which is required for *daf-2* life span extension (Kenyon, 1997), is also required for increased *daf-15/+* longevity. As *daf-16* life span is shorter than that of N2 (Larsen et al., 1995; Gems et al., 1998), we were not surprised that the mean life span of *daf-16(mgDf47); dpy-20/+* decreased by 13% relative to *dpy-20/+* (Table 1). The mean life span of *daf-16(mgDf47); + dpy-20/daf-15(m81) +* was 39% shorter than that of *+ dpy-20/daf-15(m81) +*, and was similar to that of *daf-16(mgDf47); dpy-20/+* (Table 1; Fig. 4E). Hence, loss of *daf-16* function suppressed the effect of *daf-15* on longevity, but did not suppress the *daf-15* Daf-c or fat deposition phenotypes. The latter results indicate that *daf-15* acts either downstream or in parallel with *daf-16*, raising questions about the mechanism by which *daf-16* suppresses *daf-15/+* longevity.

DAF-16 negatively regulates *daf-15* transcription

Loss of DAF-16 transcription factor activity may suppress *daf-15/+* mutant life span by enhancing transcription of the wild-type allele. We performed semi-quantitative RT-PCR to

Table 1. Heterozygous *daf-15* adult life span and effects of other mutations

| Strain | Mean \pm s.e.m.* (entire population) | Mean \pm s.e.m.* (last quartile) | Maximum life span [†] | n [‡] | P [§] |
|--|---|---------------------------------------|-----------------------------------|----------------|---------------------|
| Heterozygous <i>daf-15</i> | | | | | |
| <i>dpy-20/+</i> | 12.6 \pm 0.3 | 16 \pm 0.2 | 21, 21 | 67, 106 | |
| <i>+ dpy-20/daf-15(m81) +</i> | 16.4 \pm 0.3 | 18 \pm 0.4 | 25, 25 | 88, 80 | <0.0001 |
| <i>unc-24/+</i> | 15.1 \pm 0.4 | 18 \pm 0.2 | 19, 23 | 21, 84 | |
| <i>unc-24 +/+ daf-15(m81)</i> | 17.1 \pm 0.3 | 19 \pm 0.4 | 23, 24 | 70, 84 | <0.0001 |
| <i>unc-24/nT1</i> | 11.2 \pm 0.3 | 15 \pm 0.3 | 25, 26 | 95, 144 | |
| <i>unc-24; daf-15(m634)/nT1</i> | 14.9 \pm 0.2 | 17 \pm 0.2 | 27, 24 | 94, 162 | <0.0001 |
| Interaction with <i>daf-15(m81)/+</i> | | | | | |
| <i>unc-63/+; + dpy-20/daf-15 +</i> | 11.3 \pm 0.5 | 15 \pm 0.6 | 20, 21 | 55, 44 | |
| <i>+ unc-63/let-363 +; + dpy-20/daf-15 +</i> | 11.4 \pm 0.3 | 15 \pm 0.4 | 23, 21 | 62, 71 | 0.6349 [¶] |
| <i>daf-2; dpy-20/+</i> | 27.9 \pm 1.1 | 36 \pm 1.2 | 48, 44 | 65, 32 | |
| <i>daf-2; + dpy-20/daf-15 +</i> | 31.4 \pm 0.9 | 39 \pm 1.1 | 64, 52 | 63, 97 | 0.0063 |
| <i>daf-16; dpy-20/+</i> | 10.9 \pm 0.2 | 13 \pm 0.2 | 18, 16 | 95, 75 | |
| <i>daf-16; + dpy-20/daf-15 +</i> | 10.0 \pm 0.2 | 12 \pm 0.4 | 18, 17 | 82, 79 | 0.0776 |

*s.e.m., standard error of mean. Two independent trials were pooled.

[†]Maximum life span for each trial.

[‡]Population size for each trial.

[§]P values (log-rank test) for mean life span of the entire population compared to corresponding control.

[¶]Increased life span was observed in a third trial (see text).

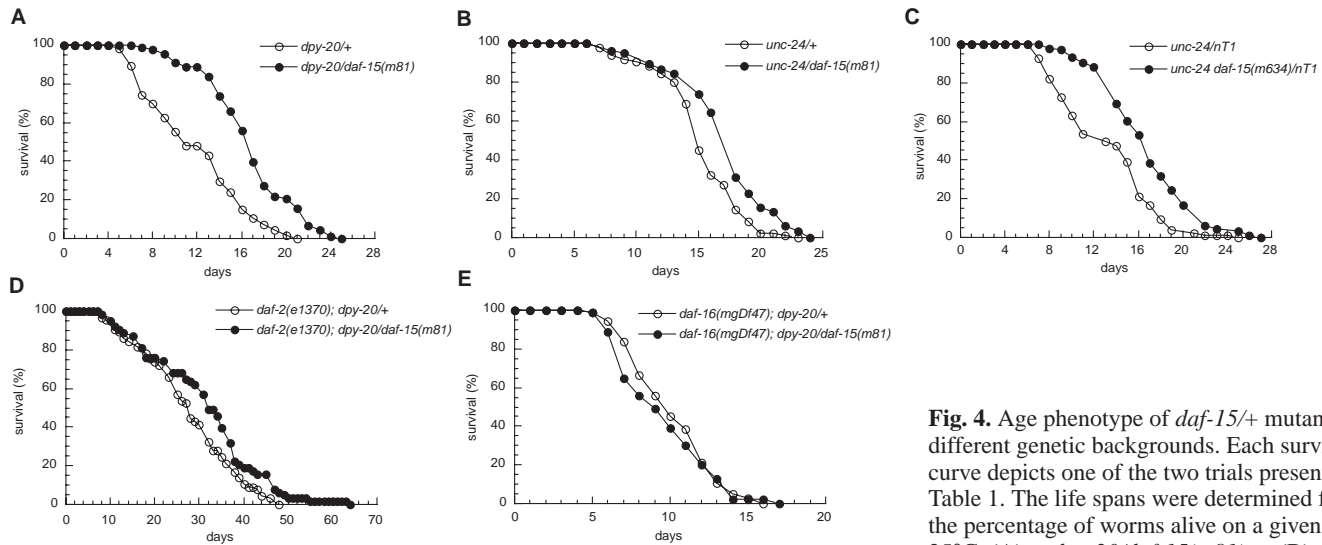


Fig. 4. Age phenotype of *daf-15/+* mutants in different genetic backgrounds. Each survival curve depicts one of the two trials presented in Table 1. The life spans were determined from the percentage of worms alive on a given day at 25°C. (A) + *dpy-20/daf-15(m81)* +, (B) *unc-24* + *daf-15(m81)* +, (C) *unc-24 daf-15(m634)/nT1*, (D) *daf-2*; + *dpy-20/daf-15(m81)* + and (E) *daf-16*; + *dpy-20/daf-15(m81)* +. The *daf-15/+* strains show extended longevity, but the *m81/+* longevity phenotype is suppressed by *daf-16*.

+ *daf-15(m81)*, (C) *unc-24 daf-15(m634)/nT1*, (D) *daf-2*; + *dpy-20/daf-15(m81)* + and (E) *daf-16*; + *dpy-20/daf-15(m81)* +. The *daf-15/+* strains show extended longevity, but the *m81/+* longevity phenotype is suppressed by *daf-16*.

compare levels of *daf-15* mRNA in *daf-2(e1370)* (in which DAF-16 is activated) and *daf-16(mgDf47); daf-2(e1370)*, in which there is no DAF-16 activity. The *daf-15* mRNA level was reduced in *daf-2* (Fig. 5A, lane 1) compared with *daf-16; daf-2* (Fig. 5A, lane 2), indicating that DAF-16 negatively regulates the expression of *daf-15*.

We examined 1.8 kb of sequence 5' of the *daf-15* ATG translation initiation site, as well as the first intron for possible DAF-16 binding sites, TTGTTTAC (Lee et al., 2003; Murphy et al., 2003), and for insulin response sequences (IRS) (O'Brien et al., 2001). No DAF-16-binding site was found, but there are two

PEPCK-like IRS (TRTTTKK) in the 5' region at -1281 and -237, and one in the first intron at +127. Thus, if DAF-16 directly regulates transcription of *daf-15*, it may be via IRS sites.

DAF-16 can bind specifically to the *daf-15* IRS in vitro. We performed electrophoretic mobility shift assays (EMSA) as shown in Fig. 5B. His-tagged DAF-16 protein expressed in bacteria binds directly to the *daf-15* IRS (lane 2), but not to the mutant probe with four consensus T residues changed to G (lane 5). The binding was effectively self-competed with fivefold and tenfold excesses of unlabeled *daf-15* IRS probes (lanes 3 and 4). As expected, the formation of protein-DNA complex was not competed by the corresponding concentrations of unlabeled mutant probes (lanes 6 and 7).

To determine whether DAF-16 binds IRS elements in vivo, we mutated the two IRS elements most proximal to the ATG start codon in the *daf-15p::gfp* reporter construct. One is 237 bp upstream of the ATG, and the other is in the first intron at +127. The wild-type and the mutant *gfp* reporter constructs were introduced into *daf-2(e1370)* mutants at a concentration of 1 ng/μl. The GFP expression was observed only in the excretory cell in some transgenic animals. We reduced *daf-16* activity with RNAi (Kamath et al., 2000) to compare the expression pattern of *daf-15p::gfp* (wild-type and mutant IRS) in L2 larvae (Table 2). We compared *daf-2(e1370)* grown on control vector food versus *daf-2(e1370)* grown on *daf-16* RNAi food. The RNAi treatment was judged to be effective because it suppressed the *Daf-c* phenotype.

Treatment with *daf-16* RNAi enhanced wild-type *gfp* reporter expression. In one transgenic line (line 2), GFP signals were observed in excretory cells of 68% of the animals ($n=50$)

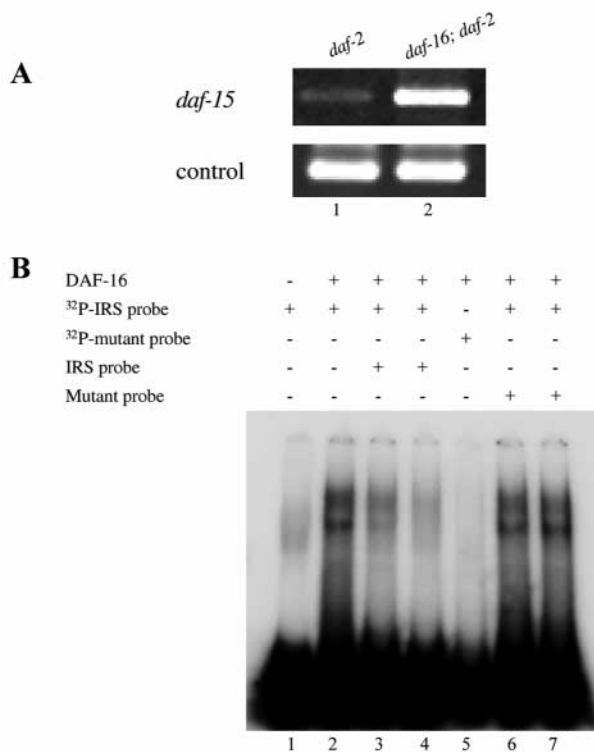


Fig. 5. DAF-16 negatively regulates the expression of *daf-15*. (A) Semi-quantitative RT-PCR shows that expression of *daf-15* is negatively regulated by DAF-16. The control *rpl-21* gene (encoding the large ribosomal subunit L21 protein) was equally represented in both RNA preparations. The experiment was performed three times using three independent RNA preparations. (B) EMSA indicates DAF-16 binds specifically to the *daf-15* IRS in vitro (see text).

Table 2. DAF-16 regulates *daf-15* expression in vivo

| Strain | Excretory Cells | | | Muscles | Intestine | Neurons | Hypo [§] | DTC [¶] | n ^{**} |
|--------------------------------|-------------------|---------------------|---------------------|---------|-----------|---------|-------------------|------------------|-----------------|
| | Weak [*] | Medium [†] | Strong [‡] | | | | | | |
| Wild-type GFP reporter | | | | | | | | | |
| Transgenic line 1 | | | | | | | | | |
| <i>daf-2</i> | 8 | 5 | 0 | 2 | 0 | 0 | 0 | 0 | 50 |
| <i>daf-2 (daf-16 RNAi)</i> | 19 | 6 | 1 | 3 | 6 | 1 | 0 | 0 | 50 |
| Transgenic line 2 | | | | | | | | | |
| <i>daf-2</i> | 8 | 4 | 1 | 2 | 2 | 0 | 0 | 0 | 50 |
| <i>daf-2 (daf-16 RNAi)</i> | 17 | 15 | 2 | 7 | 8 | 4 | 2 | 1 | 50 |
| IRS mutant GFP reporter | | | | | | | | | |
| Transgenic line 3 | | | | | | | | | |
| <i>daf-2</i> | 2 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 50 |
| <i>daf-2 (daf-16 RNAi)</i> | 6 | 3 | 3 | 3 | 4 | 3 | 3 | 0 | 50 |
| Transgenic line 4 | | | | | | | | | |
| <i>daf-2</i> | 3 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 50 |
| <i>daf-2 (daf-16 RNAi)</i> | 12 | 6 | 1 | 1 | 1 | 0 | 0 | 0 | 50 |

*A weak signal in the excretory cell body.

†A weak signal in both excretory cell body and canals or a clear signal in the excretory cell body.

‡A strong signal in both excretory cell body and canals.

§Hypodermis cells.

¶Distal tip cells.

**Total number of animals scored.

(Table 2). By contrast, only 26% of animals without RNAi showed GFP expression in excretory cells (Table 2). Furthermore, 44% of RNAi treated animals showed GFP expression in additional tissues such as intestine, muscles, neurons and DTCs (Table 2), whereas only 8% of control animals showed GFP expression in intestine and muscles. This suggests that DAF-16 inhibits *daf-15* transcription in vivo, which is consistent with the semi-quantitative RT-PCR results. However, the expression of the mutant *gfp* reporter, in which two IRS elements were mutated, was still regulated by DAF-16 activity. In one transgenic line (line 4), the GFP expression in excretory cells was observed in 8% of control animals, but the percentage was increased to 38% in *daf-16* RNAi treated animals (Table 2). The one unmutated IRS element at -1281 upstream of the ATG may function sufficiently to mediate this regulation. Alternatively, DAF-16 may regulate the expression of *daf-15* indirectly. Nevertheless, the results show that TOR and DAF-2 signaling pathways interact.

Taken together, our data indicate that DAF-16 negatively regulates *daf-15* transcription. Hence, the *daf-16* mutation may suppress *daf-15/+* life span extension by derepressing transcription of the wild-type gene. In homozygous mutants, however, *daf-16* cannot suppress the Daf-c and metabolic defect of *daf-15* because there is no wild-type DAF-15 produced. The homozygotes form dauer-like larvae and accumulate fat independently of DAF-16.

Discussion

A conserved TOR signaling pathway in *C. elegans*

TOR kinase is a structurally conserved, central controller of cell growth. As in yeast, *C. elegans* TOR is an upstream regulator of overall mRNA translation (Long et al., 2002). CeTOR mutations result in larval arrest. In this study, we identified another conserved component of TOR signaling, DAF-15 (raptor), mutations in which result in dauer-like larval

arrest. To examine the *let-363* phenotype, we removed the closely linked *dpy* mutation from the *let-363* chromosome, and showed that it had a Daf-c and fat accumulation phenotype very similar to *daf-15*. Expression of these two genes is highly overlapping, consistent with the idea that LET-363 and DAF-15 interact to form the TOR/raptor complex in *C. elegans*.

In *Drosophila*, *Tor* is also required for normal growth. *Tor* homozygotes arrested development as larvae (Zhang et al., 2000; Oldham et al., 2000). Loss of *Tor* resulted in lipid vesicle aggregation in the larval fat body (Zhang et al., 2000), a fat accumulation phenotype similar to that observed in *let-363* and *daf-15* mutants. Thus, the function of TOR signaling is conserved between *C. elegans* and *Drosophila*.

We have shown that raptor modulates *C. elegans* adult life span. Similar results have been reported recently (Vellai et al., 2003) using the *let-363(h111) dpy-5(e61)* mutant, but these results are difficult to interpret because the mutant larvae do not mature to the adult. Hence, the relationship of their survival to adult aging is not clear. Nevertheless, Vellai et al. (Vellai et al., 2003) did report that the *let-363* arrested larvae accumulate fat, which is consistent with our results. Extension of adult life span in *daf-15/+* heterozygotes clearly established a role for TOR/raptor signaling in the normal modulation of longevity. Reduction of TOR signaling extends longevity, but loss of function is lethal. Study of other dauer-like mutants may reveal additional components of the TOR/raptor pathway, and should contribute to the understanding of how nutrient availability influences life span in other organisms.

Insulin/IGF and nutrient signaling converge at DAF-15 (raptor)

Insulin/IGF signaling controls cellular and organismal growth. The TOR pathway also regulates a variety of processes contributing to cell growth. Disruption of either pathway in *C. elegans* results in larval arrest. Previous work has shown that in the *daf-2(e1370); daf-15(m81)* double mutant, the *daf-15*

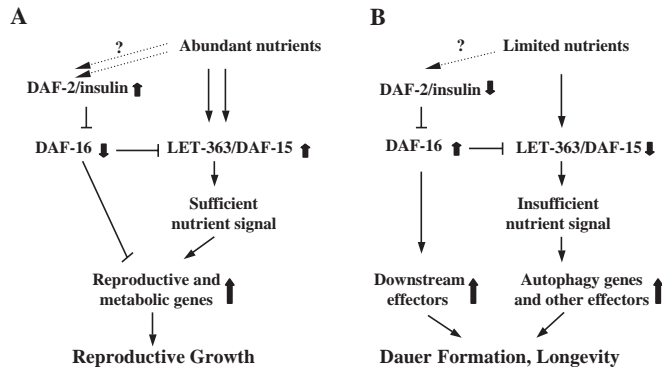


Fig. 6. A model for regulation of *C. elegans* larval development, metabolism and longevity. Proposed wild-type functions are shown, with arrows indicating stimulation of activities and T-bars indicating inhibition, but the steps do not indicate direct protein interactions. Bold arrows represent changes in wild-type activities. Both *daf-15* and *daf-2* pathways are essential for larval development. Null mutations result in larval lethality. (A) When food is abundant, LET-363/DAF-15 transduces a sufficient nutrient signal to permit growth to the reproductive adult. This signal is also required for DAF-2/insulin signaling to stimulate growth. Disruption of either pathway will cause larval arrest at the second molt. Food availability could also regulate the DAF-2/insulin pathway. (B) When nutrients are limited, the LET-363/DAF-15 signal is insufficient to prevent larval arrest. With concomitant down regulation of the DAF-2 pathway, animals will enter the dauer stage. When DAF-16 activity is high, TOR activity is low, and vice versa. However, in a *daf-15* or *let-363* mutant TOR activity is low even when insulin/IGF signaling is high, resulting in activation of some target functions, such as autophagy, but the reduced activity of DAF-16 fails to activate other functions needed to complete dauer morphogenesis. *daf-15* is epistatic to *daf-2* because some targets of TOR that are essential for dauer morphogenesis fail to be activated in the absence of DAF-15 function. Essentially, knockout of LET-363/DAF-15 activity results in dauer-like arrest regardless of DAF-2 signaling because LET-363/DAF-15 is required for both dauer and non-dauer development. TOR regulates autophagy, which is required for dauer morphogenesis and for the increased longevity of *daf-2* adults, but TOR activity is also required for maturation to the adult. DAF-16 and LET-363/DAF-15 have other downstream targets not shown here.

mutation was epistatic to *daf-2* (Albert and Riddle, 1988). Here, we show that *daf-15* is also epistatic to *daf-16* for both Daf-c and metabolic phenotypes. Therefore, the TOR pathway is required for insulin/IGF signaling to control *C. elegans* development and metabolism. These results are consistent with those in *Drosophila* that show a TOR mutation to be epistatic to PTEN (Zhang et al., 2000; Oldham et al., 2000), which acts downstream of the insulin receptor.

Our study reveals a possible mechanism by which insulin signaling regulates the TOR pathway. DAF-16, the major target of insulin signaling, can negatively regulate *daf-15* transcription, either directly by binding PEPCK-like IRS in the regulatory region of *daf-15* or indirectly through other unknown transcription factors. Mammalian TOR controls the translation machinery via activation of the p70^{S6K} protein kinase and via inhibition of the translation inhibitor 4E-BP (Schmelzle and Hall, 2000). However, this mechanism apparently is not used in *C. elegans*, because Cep70 RNAi did not phenocopy loss of CeTOR function and search of the *C.*

elegans genome failed to detect a 4E-BP ortholog (Long et al., 2002). Recently, FOXO, the *Drosophila* DAF-16 ortholog, was reported to positively regulate the transcription of 4E-BP (Puig et al., 2003). Repression of *daf-15* transcription in *C. elegans* by DAF-16 may result in inhibition of translation, as does regulation of 4E-BP transcription in *Drosophila*.

TOR is a nutrient checkpoint in yeast, *Drosophila* and mammalian cells. In yeast, rapamycin treatment mimics the effects of starvation. In *Drosophila*, amino acid deprivation phenocopies the *Tor* mutant phenotype. CeTOR-deficient *C. elegans* larvae also share some features with starved L3 larvae (Long et al., 2002). We propose that LET-363 and DAF-15 couple the DAF-2 signal and nutrient availability to regulate *C. elegans* development and longevity. When nutrients are abundant, LET-363/DAF-15 relays a nutrient sufficiency signal to downstream effectors and *daf-2* signaling enhances transcription of *daf-15*, which further stimulates TOR signaling to respond to the elevated nutrients (Fig. 6A). However, when nutrients are limited, reduction of TOR activity and DAF-16-mediated repression of DAF-15 transcription activate genes for dauer arrest (Fig. 6B). It is notable that the TOR pathway is required both for growth and dauer formation. *let-363* and *daf-15* developmental arrest is accompanied by fat accumulation and a dauer-like morphology, but neither mutant is able to complete dauer morphogenesis.

Autophagy may be a major target for TOR/raptor signaling

As inhibition of TOR activity induces autophagy in yeast and mammalian cells (Schmelzle and Hall, 2000), autophagy may be a major target for TOR to regulate *C. elegans* life span as well as dauer formation (Fig. 6B). Recently, autophagy has been shown to be required for dauer morphogenesis (Melendez et al., 2003). This suggests that autophagy may be induced in *daf-15* and *let-363*, while insulin/IGF signaling in these mutants may prevent activation of other essential dauer functions. RNAi directed against autophagy genes renders constitutively formed *daf-2* dauer larvae incapable of completing morphogenesis (Melendez et al., 2003), mimicking the phenotype of a *daf-2; daf-15* double mutant (Albert and Riddle, 1988). Autophagy has been proposed to delay aging of post-mitotic cells by removal of damaged mitochondria (Brunk and Terman, 2002). Indeed, *bec-1*, the *C. elegans* ortholog of the yeast and mammalian autophagy gene (*APG6/VSP30/beclin1*), is essential for life span extension in *daf-2* mutants (Melendez et al., 2003).

Caloric restriction extends life span in *C. elegans* (Klass, 1977; Lakowski and Hekimi, 1998) and in a wide spectrum of other organisms. As the TOR pathway primarily responds to nutrient availability, caloric restriction may extend life span by decreasing TOR activity. Interestingly, it has been reported that caloric restriction and reduced insulin signaling may exhibit their aging effects at least partly by their common stimulatory action on autophagy (Bergamini et al., 2003). If insulin/IGF and nutrient signaling converge at raptor, TOR signaling could be a central pathway mediating caloric restriction.

Raptor is a potential therapeutic target for PTEN-deficient tumors

Given that the TOR and insulin pathways are highly conserved

among divergent species, crosstalk between these pathways via a DAF-16 ortholog may exist in humans. In *C. elegans*, DAF-16 functions downstream of the AGE-1 phosphoinositide 3-kinase (PI3K), which mediates *daf-2* signaling (Morris et al., 1996). The DAF-18 PTEN phosphatase (Ogg and Ruvkun, 1998), a tumor suppressor in humans, indirectly activates DAF-16 by dephosphorylating PIP3. As DAF-16 inhibits raptor transcription, a PTEN null mutation would be expected to increase TOR activity and promote cell growth, potentially contributing to the growth of PTEN-deficient tumors in humans. Thus, TOR inhibitors such as rapamycin could inhibit growth of these tumors. Indeed, PTEN-null cells are sensitive to inhibition of TOR by a rapamycin derivative (CCI-779) (Neshat et al., 2001; Podsypanina et al., 2001), which is currently in phase II clinical trials as an anticancer agent (Huang and Houghton, 2003). Therefore, further work in *C. elegans* may help elucidate the molecular mechanism of the anticancer effect of agents like CCI-779. DAF-16 repression of DAF-15 transcription also suggests that agonists of FOXO transcription factors in humans are potential anticancer agents. A conditional *daf-15* mutant could be used to screen for such candidate agents predicted to enhance the *Daf-c* phenotype of the mutant.

Conclusion

We found that *daf-15* encodes the *C. elegans* raptor, and we established roles for TOR/raptor in controlling *C. elegans* development, metabolism and life span. Additionally, we identified a mechanism by which insulin/IGF signaling regulates the TOR pathway via DAF-16. The role of the TOR pathway in aging may contribute to the understanding of how insulin/IGF signaling and nutrient availability influence life span.

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