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RLE-1, an E3 Ubiquitin Ligase, Regulates *C. elegans* Aging by Catalyzing DAF-16 Polyubiquitination

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SUMMARY

The forkhead transcription factor, DAF-16, a downstream target of the insulin/IGF-I signaling pathway in *C. elegans*, is indispensable both for lifespan regulation and stress resistance. The molecular mechanisms involved in regulating DAF-16 transcriptional activation remain undefined. Here, we have identified an E3 ubiquitin ligase, RLE-1 (regulation of longevity by E3), which regulates aging in *C. elegans*. Disruption of RLE-1 expression in *C. elegans* increases lifespan; this extension of lifespan is due to elevated DAF-16 protein but not to changes of *daf-16* mRNA levels. We have also found that RLE-1 catalyzes DAF-16 ubiquitination, leading to degradation by the proteasome. Elimination of RLE-1 expression in *C. elegans* causes increased transcriptional activation and sustained nuclear localization of DAF-16. Overexpression of DAF-16 in *rle-1* mutants increases worm lifespan, while disruption of DAF-16 expression in *rle-1* mutants reverses their longevity. Thus, RLE-1 is an E3 ubiquitin ligase of DAF-16 that regulates *C. elegans* aging.

INTRODUCTION

The insulin/IGF-1 (insulin-like growth factor-1) pathway, which includes the DAF-2 transmembrane receptor, a series of intracellular kinases, and the DAF-16 forkhead-family transcription factor, is involved in aging in *C. elegans* (reviewed by Gami and Wolkow, 2006). Animals with weak *daf-2* mutations age more slowly than wild-type animals and live much longer (Riddle, 1977; Kimura et al., 1997; Tissenbaum and Ruvkun, 1998). Furthermore, mutation of the downstream *age-1* gene, which encodes a protein similar to the mammalian p110 catalytic subunit of PI3K, leads to a 65% increase in mean lifespan (Friedman and Johnson, 1988; Paradis et al., 1999). These effects have been shown to depend on the activity of DAF-16, which has similarity to a family of mammalian forkhead transcription factors (Lin et al., 1997). All known *daf-2* mutant phenotypes are completely dependent upon DAF-16 (Kenyon et al., 1993; Gottlieb and Ruvkun, 1994; Larsen et al., 1995; Dorman et al., 1995; Tissenbaum and Ruvkun, 1998; Lee et al., 2001). A null mutation in *daf-16* suppresses the phenotypes of *daf-2* and *age-1*, indicating that the insulin/IGF-I receptor-like signaling pathway regulates aging by modulating gene expression (Hsu et al., 2003; Lee et al., 2003).

The function of DAF-16, a transcriptional activator, is regulated by the insulin/IGF pathway. Activation of AKT by insulin/IGF results in the phosphorylation of DAF-16, and this phosphorylation event likely inhibits the nuclear translocation and transcriptional activity of DAF-16 in a DAF-2-dependent manner (Henderson and Johnson, 2001; Lee et al., 2001, 2003; Lin et al., 2001; Kops et al., 2002; Hsu et al., 2003). Motta et al. reported that the mammalian NAD-dependent deacetylase, SIRT1, deacetylates and represses the activity of the forkhead transcription factor Foxo3a, the mammalian homologue of DAF-16; this result suggests a similar mechanism may exist in *C. elegans* (Motta et al., 2004). Moreover, the c-Jun N-terminal kinase (JNK) is a positive regulator of DAF-16, both for lifespan regulation and stress resistance (Oh et al., 2005). Thus, multiple protein kinases of the DAF-2 signaling pathway converge to negatively regulate DAF-16 activity by changing its localization within the cell. However, the exact mechanisms involved in regulating the cytoplasmic-nuclear shuttling of DAF-16 are relatively unknown.

Protein ubiquitination has emerged as one of the key posttranslational regulatory mechanisms affecting many aspects of biological function, including cell-cycle control, immune response, apoptosis, and neuronal development (Nakata et al., 2005; Zhong et al., 2005). Ubiquitin is a highly conserved 76 amino acid polypeptide that can be covalently conjugated to the lysine sites of a target protein. Ubiquitin conjugation to a substrate requires a cascade of at least three different enzymatic reactions. First, ubiquitin is activated by E1, the ubiquitin-activating enzyme. Next, ubiquitin is transiently transferred to E2, the ubiquitin-conjugating enzyme. Finally, an E3 ubiquitin ligase transfers the activated ubiquitin molecule from the

E2 enzyme to a lysine residue on the substrate (reviewed by Hershko and Ciechanover, 1998). Ubiquitin conjugation generally mediates protein degradation via the 26S proteasome (Pickart, 2001). Recently, the systemic analysis by RNA interference of genes involved in longevity in *C. elegans* revealed that many genes in the ubiquitin pathway are involved in the regulation of lifespan (Hamilton et al., 2005).

Here, we have identified an E3 ubiquitin ligase, RLE-1, that functions as an E3 ubiquitin ligase for DAF-16. Overexpression of RLE-1 promotes DAF-16 ubiquitination. Disruption of the *rle-1* gene causes increased longevity in *C. elegans*, with elevated DAF-16 expression and increased DAF-16 transcriptional activity. Overexpression of DAF-16 in *rle-1* mutants further increases *C. elegans* lifespan, while *daf-16;rle-1* double mutants show a reversal of this increased longevity. Our findings indicate that RLE-1 regulates *C. elegans* lifespan by controlling the transcriptional activation of DAF-16 via the ubiquitin pathway.

RESULTS

Disruption of RLE-1 Expression Increases the Lifespan of *C. elegans*

Our laboratory is currently interested in studying an E3 ubiquitin ligase, Roquin, which has been demonstrated to play important roles in autoimmunity in the mouse (Vinuesa et al., 2005). To investigate the functional roles of Roquin family proteins, we obtained a genetically modified *C. elegans* allele, cxTi510, which was generated by the Segalat laboratory (University of Lyon, France) by using the gene trap approach with the Tc5 vector (http://www.wormbase.org/db/gene/variation?name=cxTi510;class=Variation). We back crossed the *rle-1* (*cxTi510*) animals six times with wild-type (N2) animals to eliminate any additional copies of Tc5, confirmed the genotype of the *rle-1* mutants by PCR, and named this strain KB6. The exact position of the insertion of the Tc5 vector was determined (Figure S1A, see the Supplemental Data available with this article online), with DNA sequencing revealing the Tc5 transposon is inserted into the fourth exon of the *rle-1* gene (Figure S1B). PCR using primers corresponding to genomic DNA from each side of the Tc5 insertion failed to detect the bands in the *rle-1* mutant that are present in wildtype DNA (Figure S1A). This insertion apparently disrupts the expression of the C-terminal proline-rich region of the RLE-1 protein. We further confirmed the lack of expression of RLE-1 in *rle-1* mutants by western blotting with an anti-RLE-1 antibody that we generated (Figures S2A and S2B). A 130 kDa band, which corresponds to the predicted molecular weight of the RLE-1 protein, was detected in wild-type but not in *rle-1* animals (Figure S2C). These data indicate that the KB6 strain is a homozygous *rle-1* mutant (*rle-1*$^{-/-}$).

Next, we analyzed the lifespan of the *rle-1* mutant. Lifespans were determined at 25°C, with the day of hatching (first larval stage, L1) used as the first time point. Under the same conditions, the *rle-1* mutants live much longer than wild-type (N2) animals. The average lifespan of *rle-1* worms is 23.9 ± 1.2 days, while N2s have an average lifespan of 19.1 ± 1.1 days ($p < 0.001$) (Figure 1A and Table 1). When lifespan was analyzed beginning from the L4 larval stage, *rle-1* animals still live significantly longer than N2 animals ($p < 0.01$) (Figure S3). These results indicate that loss of RLE-1 increases *C. elegans* lifespan.

We also determined that *rle-1* mutants have fewer progeny over a longer period of time when compared to N2s. As shown in Figure 1B, there is a 2 day delay in producing embryos for the *rle-1* worms. In addition, the total numbers of embryos produced by the *rle-1* mutants are reduced about 40%–50% when compared to those produced by wild-type animals. Disruption of RLE-1 expression also resulted in partial (about 20%–30%) embryonic lethality (Figure 1C). Moreover, the *rle-1* mutants grow more slowly and even as adults are thinner and slightly shorter than N2s (Figure 1D). These phenotypes are similar to those observed in *daf-16::gfp* transgenic animals (Henderson and Johnson, 2001), in which DAF-16 is overexpressed, suggesting that RLE-1 could regulate this signaling pathway.

RLE-1 Is Involved in Stress Resistance and Dauer Formation in *C. elegans*

Previous studies have demonstrated a correlation between increased longevity and stress resistance (Kenyon et al., 1993; Tissenbaum and Ruvkun, 1998; Murakami and Johnson, 2001; Lithgow and Walker, 2002). Because a loss of RLE-1 expression increases the lifespan of *C. elegans*, we hypothesized that RLE-1 might be involved in stress resistance. We therefore conducted physiological tests to compare the responses of the *rle-1* mutants to those of wild-type animals when challenged with heat shock, ultraviolet (UV) damage, and pathogens. The *rle-1* mutation results in significantly increased resistance both to heat shock (Figure S4A) and to UV damage (Figure S4B). RLE-1 is the homolog of the mammalian Roquin, which is involved in regulating the immune response and in autoimmunity (Vinuesa et al., 2005). It is therefore possible that RLE-1 is involved in the innate immune response of *C. elegans*. To support this hypothesis, we found *rle-1* mutants survive significantly longer when exposed to the pathogenic bacterium *P. aeruginosa* (Figure S4C). In addition, disruption of RLE-1 expression results in partial dauer formation when these animals are grown at 25°C (Figure S4D). Thus, the physiological evidence supports an involvement of RLE-1 in multiple responses to stress in *C. elegans*. Mutation of DAF-2, the sole insulin/IGF-1 receptor, negatively regulates DAF-16 transcriptional activation and causes longevity and stress resistance (Riddle 1977; Kenyon et al., 1993; Murakami and Johnson, 2001). Collectively, our results suggest that RLE-1 may be involved in crosstalk with the insulin/IGF pathway in regulating lifespan and stress resistance in *C. elegans*.

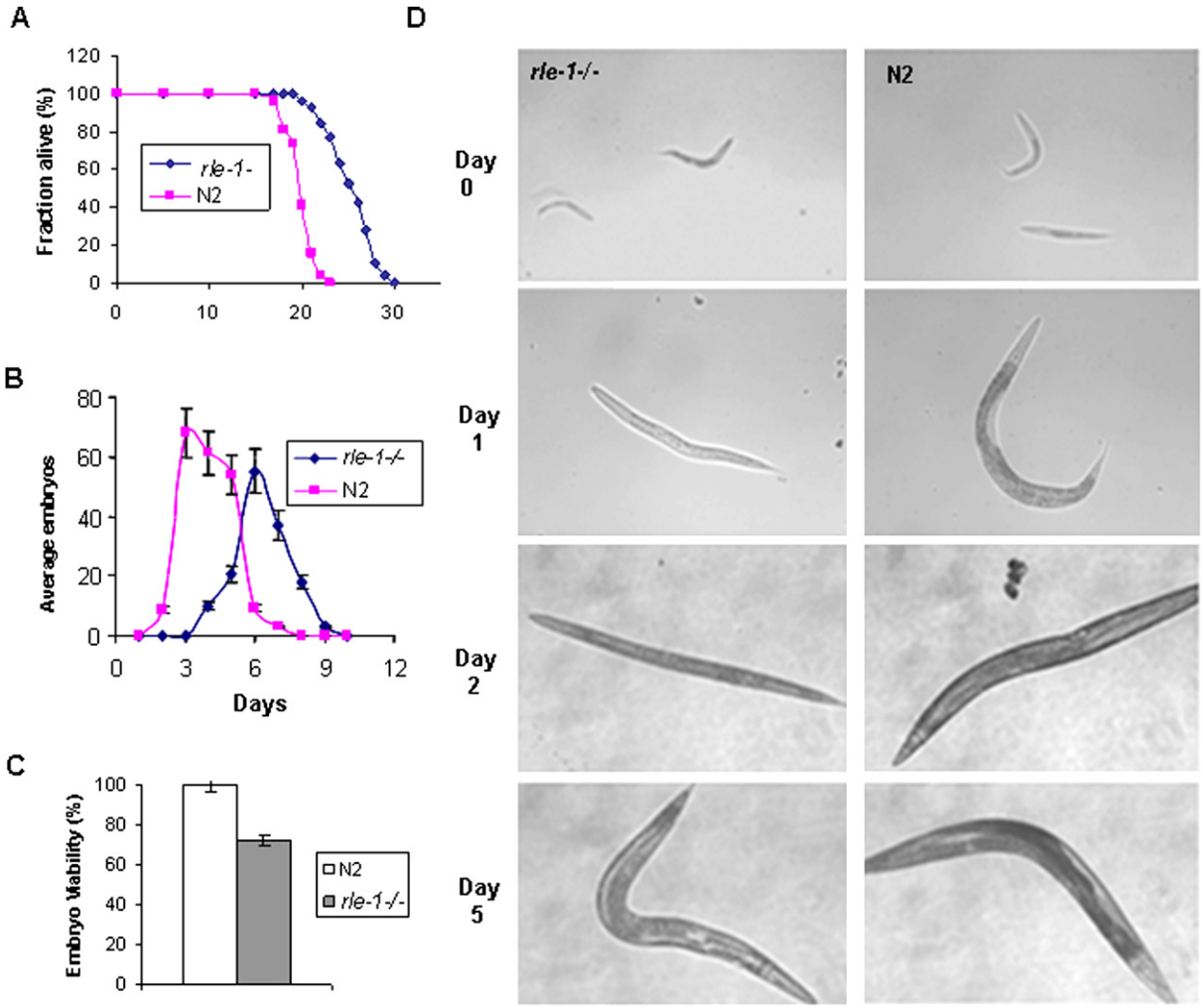

Figure 1. Analysis of the Phenotype of *rle-1* Mutants

(A) Survival curves of *rle-1*$^{-/-}$ and N2 animals. Forty N2 and *rle-1*$^{-/-}$ animals were collected as L1 larvae. These animals were transferred to agar media containing 20 μg/ml of FUDR when they reached the L4 larval stage. Animals were kept at 25°C, and then viability was assessed every 2 days for the first 10 days and then daily thereafter.

(B) Fertility of *C. elegans*. Each of 10 L4 larvae were collected and transferred onto a new plate. Animals were kept at 20°C. The production of embryos was counted daily. The error bars represent variation of the fertility of 20 different animals.

(C) Embryonic lethality study. Gravid N2 or *rle-1*$^{-/-}$ animals were transferred onto new plates to allow them to lay eggs for 4 hr. Unhatched embryos and L1 larvae were counted 24 hours later, with the number of viable larvae divided by that of unhatched eggs to determine the percentage of viability. The error bars represent variation of three independent experiments (mean ± SEM).

(D) Delayed development of *rle-1* mutants. Both *rle-1*$^{-/-}$ and N2 animals were collected at the L1 stage and cultured at 20°C. Every 24 hr, several representative animals were selected, fixed onto a slide, and photographed. Images representative of the majority of animals were selected.

DAF-16 Protein but Not Its mRNA Levels Are Increased in *rle-1* Mutants

The forkhead family transcription factor DAF-16 plays important roles in regulating lifespan in *C. elegans*. Overexpression of a fusion protein of DAF-16 tagged with GFP increases the lifespan of transgenic animals (Henderson and Johnson, 2001). We thus asked whether *rle-1* worms have elevated DAF-16 expression. As shown in Figure 2A, more DAF-16 was detected in the *rle-1* mutant (lanes 2 and 4) than in N2 wild-type lysates (lanes 1 and 3). As a loading control, the protein levels of Actin were comparable in these lysates. Quantification of the densities of DAF-16 bands from three independent experiments indicated that the protein level of DAF-16 in *rle-1* mutants was significantly higher than that in N2 animals (Figure 2B), while the mRNA levels of *daf-16* were comparable between *rle-1* mutants and wild-type animals, as determined both by RT-PCR (Figure 2C) and real-time quantification PCR (Figure 2D). These results indicate that a mutation in the gene that encodes RLE-1 causes increased protein but not increased mRNA for DAF-16, suggesting that RLE-1 is involved in the longevity of *C. elegans*

Table 1. The Lifespan of Animals

Strains	Genotype	Number of Worms Tested	Mean Lifespan ± SEM (days)	p Value against N2	p Value against Specified Group
N2	wild-type	183	19.1 ± 1.1		
KB6	*rle-1*$^{-/-}$	168	23.9 ± 1.2	<0.001	0.16[a]
TJ356	*daf-16::gfp*	116	23.1 ± 0.8	<0.005	0.02[b]
KB7	*rle-1*$^{-/-}$;*daf-16::gfp*	137	25.4 ± 1.6	<0.001	
N2	wild-type	183	19.1 ± 1.1		
KB6	*rle-1*$^{-/-}$	168	23.9 ± 1.2	<0.001	<0.001[c]
CF1038	*daf-16*$^{-/-}$	107	14.3 ± 0.5	<0.001	0.08[d]
KB8	*rle-1-;daf-16*$^{-/-}$	125	15.7 ± 0.9	<0.001	
N2	wild-type	78	20.5 ± 1.2		
KB6	*rle-1* mutant	91	25.1 ± 1.4	<0.001	
CF1308	*daf-16*$^{-/-}$	87	13.5 ± 0.6	<0.001	
CF1308-HZ*	daf-16$^{+/-}$	58	19.1 ± 0.8	0.13	
KB9	*rle-1*$^{-/-}$;*daf-16*$^{+/-}$	63	22. 7 ± 0.9	<0.01	<0.005[e]

p values represent the probability that the estimated survival function of the experimental group of animals is equal to that of the control group. p values are determined by using the logrank (Mantel-Cox) statistics. p values less than 0.05 are considered statistically significant, demonstrating that the two survival functions are different. *CF1308-HZ, CF1308-heterozygous.
[a] TJ356.
[b] KB7.
[c] CF1038.
[d] KB9.
[e] CF1038-HZ.

through regulation of DAF-16 expression at the posttranscriptional level.

RLE-1 Interacts with DAF-16 In Vitro and in *C. elegans*

To determine the physiological function of RLE-1, we cloned RLE-1 from *C. elegans* by RT-PCR. The full-length *rle-1* cDNA encodes a 1,014 amino acid protein (Figure S5A). The RLE-1 protein contains an N-terminal RING finger, indicative of an E3 ubiquitin ligase. RLE-1 also has an ROQ domain, which has high homology with the mammalian Roquin (Figure S5). The functions of the ROQ domain are not known; however, it is predicted to mediate protein-protein interactions. The C terminus of

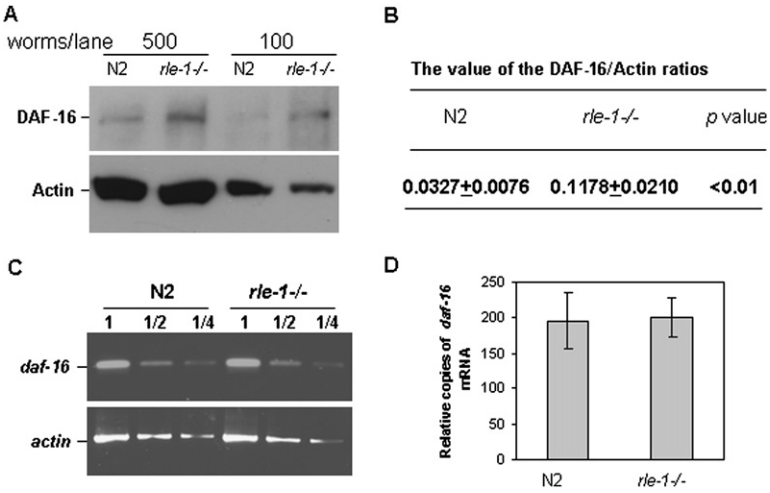

Figure 2. Expression of DAF-16 in *C. elegans*

(A) Increased DAF-16 protein expression. Adult animals were collected, and lysates from N2s and *rle-1*$^{-/-}$ animals were prepared. Protein from 500 or 100 animals in each lane was analyzed by SDS-PAGE. The expression of DAF-16 was detected by western blotting (top panel). The same membrane was reprobed with anti-Actin antibody (bottom panel).

(B) The ratio of DAF-16 to Actin. The densities of western blotting bands of DAF-16 and Actin from three independent experiments were measured by PhosphoImage software, and ratios were calculated. Data were analyzed using the Student's T test.

(C) *daf-16* mRNA levels in *C. elegans*. Total RNA was isolated from N2 and *rle-1*$^{-/-}$ animals. mRNA was reverse transcribed into cDNA. The level of *daf-16* cDNA was analyzed by PCR (top panel). The mRNA level of *actin* was analyzed as a control (bottom panel). Primers used here are shown in Table S1.

(D) Analysis of *daf-16* mRNA by real-time PCR. The cDNAs from (C) were used for real-time PCR analysis. Error bars represent three independent experiments (mean ± SEM).

RLE-1 contains a long proline-rich region, a motif which has also been demonstrated to mediate protein-protein interactions. Moreover, RLE-1 contains two coiled-coil domains, motifs which often function as protein-protein interaction domains (Newman and Keating, 2003).

To investigate the physiological roles of RLE-1 in regulating DAF-16 expression, we examined whether RLE-1 interacts with DAF-16. DAF-16 and Flag-tagged RLE-1 expression plasmids were cotransfected into HEK293 cells. RLE-1 was detected by coimmunoprecipitation and western blotting when it was coexpressed with DAF-16 but not in the control lane of RLE-1 alone, suggesting that DAF-16 can interact with RLE-1 (Figure 3A). More interestingly, RLE-1 was detected in the anti-DAF-16 immunoprecipitate when lysates of wild-type worms but not of *rle-1* mutants were used (Figure 3B). These results indicate that RLE-1 interacts with DAF-16 in vivo. To support this conclusion, we further demonstrated by confocal microscopy that RLE-1 colocalizes with DAF-16 in *C. elegans* (Figure S6A) as well as in HEK293 cells (Figure S6B).

Since the *rle-1* mutation likely results in a deletion of the C-terminal 491 amino acids of RLE-1, we hypothesized that the C-terminal region of RLE-1 is important for its interaction with DAF-16. Analysis of the cDNA sequence of *rle-1* revealed a convenient restriction enzyme site, *Kpn I*, by which a truncated RLE-1 was generated that removed the C-terminal 473 amino acids (RLE-1/ΔC). RLE-1/ΔC is similar in length (586 aa) to the size of the truncated RLE-1 protein that could be produced in the *rle-1* mutants (558 aa). As shown in Figure 3C, deletion of the C terminus of RLE-1 completely abolished its interaction with DAF-16, indicating the RLE-1 C terminus is required for the interaction of RLE-1 with DAF-16.

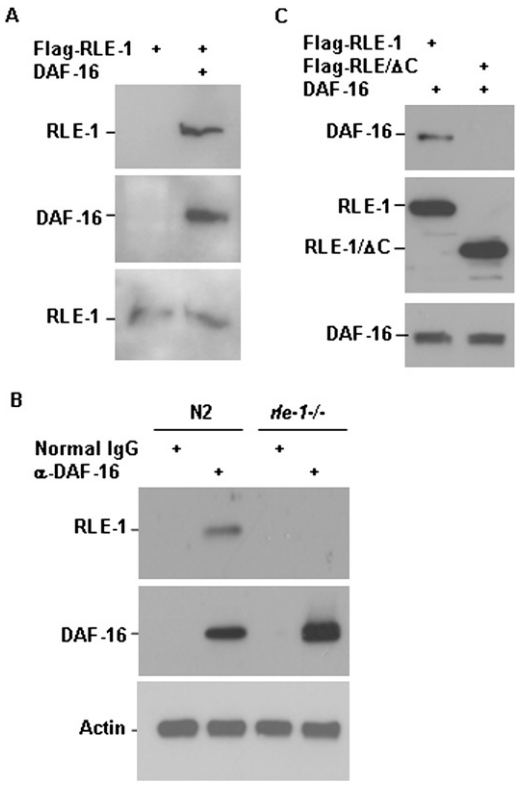

Figure 3. RLE-1 Interacts with DAF-16

(A) RLE-1 interacts with DAF-16 in vitro. Flag-tagged RLE-1 expression plasmids were cotransfected without (lane 1) or with DAF-16 (lane 2). DAF-16 protein was immunoprecipitated with an anti-DAF-16 antibody. The interaction of RLE-1 was detected by western blotting with anti-Flag (top panel). The same membrane was reprobed with anti-DAF-16 (middle panel). RLE-1 expression in the whole cell lysates was detected by anti-Flag (bottom panel).

(B) RLE-1 interacts with DAF-16 in animals. Whole-cell lysates were prepared from N2 or *rle-1*$^{-/-}$ animals. DAF-16 protein was immunoprecipitated by anti-DAF-16 antibody. The interaction of RLE-1 in these immunoprecipitates was detected by western blotting with an anti-RLE-1 antibody. Normal goat IgG was used as a negative control for immunoprecipitations (top panel). The same membrane was reprobed with anti-DAF-16 antibody (middle panel). The protein levels of Actin in whole-cell lysates were used as loading controls (bottom panel).

(C) The C terminus of RLE-1 is required for its interaction with DAF-16. DAF-16 was cotransfected with RLE-1 or RLE-1/ΔC. The interaction of DAF-16 with RLE-1 or its deletion mutant was analyzed by coimmunoprecipitation and western blotting as in (A).

RLE-1 Is an E3 Ubiquitin Ligase for DAF-16

Since RLE-1 contains a RING finger, which has been demonstrated as an E3 ubiquitin ligase domain (Zheng et al., 2000), we proposed that RLE-1 regulates DAF-16 expression via its E3 activity. To test this hypothesis, an in vivo ubiquitination assay was performed (Gao et al., 2006). When DAF-16 was coexpressed with HA-Ub and RLE-1, higher molecular weight ladders were detected in the anti-DAF-16 immunoprecipitates, which indicates that DAF-16 is modified by ubiquitin (Figure 4A, lane 2). Treatment of these transfected HEK293 cells with the proteasome inhibitor, MG132 (Saito et al., 1992), significantly increased the ubiquitination of DAF-16 (Figure 4A, lane 3). These results suggest that RLE-1 is an E3 ubiquitin ligase of DAF-16 and that the proteasome pathway is involved in DAF-16 degradation. A direct interaction is required for RLE-1-mediated DAF-16 ubiquitination because overexpression of RLE-1/ΔC failed to induce DAF-16 ubiquitination (Figure 4B). In addition, a functional RING finger, which binds to ubiquitin-carrying E2s (Zheng et al., 2000), is also required for RLE-1-mediated ubiquitin conjugation of DAF-16. Mutation of the active cysteine site in the RING finger, RLE-1/C34A, essentially abolished its E3 ubiquitin ligase activity for DAF-16 ubiquitination (Figure 4C) without affecting the interaction of RLE-1 with DAF-16 (Figure 4D). These results collectively demonstrate that RLE-1 is an E3 ubiquitin ligase for the *C. elegans* DAF-16.

RLE-1 Induces DAF-16 Degradation and Suppresses the Transcriptional Activity of DAF-16

The fact that the protein but not the mRNA level of *daf-16* is increased in *rle-1* mutants suggests that RLE-1 is involved in the protein degradation of DAF-16. To test this hypothesis, we found that coexpression of RLE-1 and

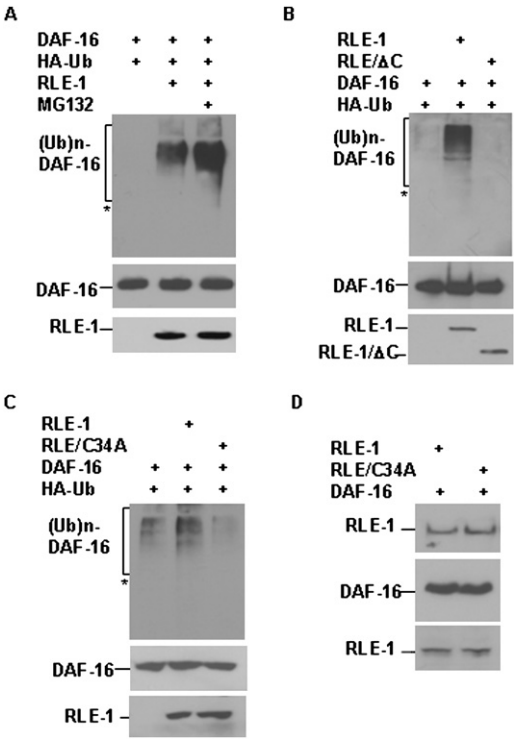

Figure 4. RLE-1 Is an E3 Ligase for DAF-16 Ubiquitination

(A) RLE-1 induces DAF-16 ubiquitination. DAF-16 and HA-Ub expression plasmids were cotransfected with or without RLE-1 into 293T cells. Cells were treated with or without MG132 for 1 hr before harvesting. DAF-16 protein was immunoprecipitated with anti-DAF-16, and ubiquitin conjugation to DAF-16 was detected with anti-HA (top panel). The same membrane was reprobed with anti-DAF-16 (middle panel). The expression of RLE-1 in whole cell lysates was determined by western blotting with anti-Flag antibody (bottom panel).

(B) Effects of the C terminus deletion of RLE-1 on ubiquitination of DAF-16. DAF-16 and HA-Ub were cotransfected with RLE-1 or RLE-1/ΔC. DAF-16 was immunoprecipitated with anti-DAF-16, and ubiquitinated DAF-16 was detected by anti-HA (top panel). The same membrane was reprobed with anti-DAF-16 antibody (middle panel). The expression of RLE-1 and RLE-1/ΔC in the whole-cell lysates was analyzed by western blotting with anti-Flag antibody (bottom panel).

(C) The effect of a RING finger mutation on DAF-16 ubiquitination. DAF-16, HA-Ub, and RLE-1 or RLE-1/C34A were transfected into HEK293 cells. Ubiquitin conjugation of DAF-16 was analyzed as described in (A).

(D) Interaction of DAF-16 with the RING finger mutant of RLE-1. HEK293 cells were transfected with DAF-16 and RLE-1 or RLE-1/C34A. The interaction of DAF-16 with RLE-1 or RLE-1/C34A was analyzed as described in Figure 3A. Asterisk indicates the size of unubiquitinated forms of DAF-16.

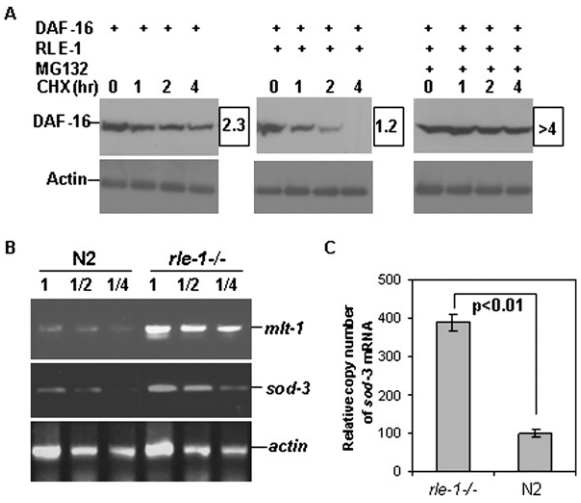

Figure 5. Effect of RLE-1 on the Protein Stability and Transcriptional Activation of DAF-16

(A) RLE-1-mediated protein degradation of DAF-16. DAF-16 was transfected with or without the RLE-1 expression plasmid. Forty-eight hours after transfection, cells were treated with or without cycloheximide (CHX, 50 μg/ml) for different amounts of time as indicated. Some DAF-16 and RLE-1 cotransfected cells were further treated with 50 μM MG132. The protein stability of DAF-16 was analyzed by western blotting. The densities of each band were measured by PhosphoImage software, and the half-life of DAF-16 was calculated as indicated. The same membrane was reprobed with an Actin antibody as a control (bottom panel).

(B) Disruption of RLE-1 expression increases DAF-16 transcriptional activation. mRNA was isolated from N2 or *rle-1*$^{-/-}$ animals. The levels of *mlt-1* and *sod-3* mRNA were examined by RT-PCR with the *mlt-1* and *sod-3* primers as shown in Table S1. The mRNA level of *actin* was used a control.

(C) Increased *sod-3* transcription in *rle-1*$^{-/-}$ animals. The cDNA in (B) was used for real-time PCR analysis. Error bars represent the results of three independent experiments.

DAF-16 in cultured cells dramatically accelerated the degradation of DAF-16 by shortening its half-life to about 1.2 hours, down from 2.3 hours for DAF-16 alone (Figure 5A). Overexpression of RLE-1 even reduces the levels of DAF-16 protein when less DAF-16 plasmid is cotransfected (Figure S7). These results indicate that RLE-1 induces DAF-16 degradation. Ubiquitination can mediate protein degradation via either the proteasome or the lysosome pathway, or both (reviewed by Hershko and Ciechanover, 1998). Our findings that RLE-1-mediated ubiquitination of DAF-16 is increased in the presence of the proteasomal inhibitor, MG132 (Figure 4A), suggest that ubiquitination may induce DAF-16 degradation via the proteasome pathway. The protein stability of DAF-16 was thus analyzed in the presence of MG132. Treatment of cells with MG132 prevented RLE-1-mediated degradation of DAF-16 (Figure 5A, right panel). These results suggest that RLE-1 induces DAF-16 degradation via the proteasome pathway.

We next examined how RLE-1-mediated ubiquitination regulates the transcriptional activation of DAF-16. To determine the physiological functions of RLE-1-mediated DAF-16 ubiquitination in *C. elegans*, we compared the transcriptional activity of DAF-16 between wild-type worms and *rle-1* mutants. Several target genes of DAF-16 have been identified in *C. elegans*, including *sod-3* and *mlt-1* (Essers et al., 2005). These target genes play important roles in regulating the lifespan of *C. elegans*. Under normal conditions with plenty of food, both *sod-3* and *mlt-1* are expressed at very low levels in N2s, as

demonstrated by the weak bands corresponding to *sod-3* and *mlt-1* detected after 40 cycles of PCR amplification. In contrast, the mRNA levels of both *sod-3* and *mlt-1* were dramatically increased in *rle-1* mutants (Figure 5B). As controls, the mRNA levels of both *actin* and *daf-16* were comparable between N2 worms and *rle-1* mutants (Figure 5B, bottom panel, Figure 2C, and Figure S2D). Results from the real-time PCR experiments indicated that *sod-3* transcript levels are at least four-fold higher in *rle-1* mutants than in wild-type (Figure 5C). These results indicate that in *C. elegans* RLE-1 functions as a negative regulator of DAF-16 transcriptional activation.

DAF-16 Exhibits Sustained Nuclear Localization in *rle-1* Mutants after Heat Stress

In the AKT pathway, activation by growth factors induces DAF-16 phosphorylation and inhibits DAF-16 nuclear translocation, while stress or withdrawal of growth factors triggers the dephosphorylation and relocalization of DAF-16 to the nucleus (Lin et al., 1997). Some evidence has shown that nuclear DAF-16 is exported back to the cytoplasm when the stress is released. The mechanisms of the sequestration of DAF-16 away from the nucleus are still not known. To investigate whether RLE-1-mediated ubiquitination is involved in this process, we analyzed changes over time in the subcellular localization of DAF-16 in *rle-1* mutants. A transgenic *C. elegans* strain that expresses a fusion protein of DAF-16 with GFP (*daf-16::gfp* transgenic animals) under the control of the native *daf-16* promoter has been previously used as a tool for investigation of the tissue distribution and subcellular localization of DAF-16 (Henderson and Johnson, 2001). We thus bred the *rle-1* mutant with *daf-16::gfp* transgenic animals, resulting in an *rle-1*$^{-/-}$*;daf-16::gfp* strain. Under normal conditions when food is plentiful, both the tissue distribution and subcellular localization of DAF-16 were similar for the *rle-1;daf-16::gfp* and the *daf-16::gfp* animals, although the *rle-1*$^{-/-}$*;daf-16::gfp* animals generally show brighter green fluorescence than *daf-16::gfp* alone when observed by fluorescence microscopy (Figure S8A, top panel), indicating the protein level of the DAF-16::GFP fusion protein is higher when RLE-1 expression is disrupted. These in vivo results further support our findings that RLE-1-mediated ubiquitination induces DAF-16 degradation.

We then subjected animals to the stress of heat and found the DAF-16::GFP fluorescence became concentrated as bright dots in both strains after treatment of these animals at 35°C for 30 min (Figure S8A). These green dots colocalized well with DNA, indicating DAF-16 had translocated into the nuclei after stress (Figure S8C). We then examined the relocalization of DAF-16 from the nucleus back to the cytoplasm when these worms were returned to 20°C. Within 20 min, most of the concentrated DAF-16 fluorescence had disappeared, concomitant with a rapid increase of diffuse fluorescence in the *daf-16::gfp* animals (Figure S8). A complete disappearance of the concentrated dots of DAF-16 fluorescence in the *daf-16::gfp* transgenic animals took about 30–40 min; this disappearance was delayed to about 70–80 min in the *rle-1* mutants (Figure S8B). These results indicate that RLE-1 may be involved in regulating the cytoplasmic sequestration of DAF-16.

RLE-1 Regulates the Longevity of *C. elegans* in a DAF-16-Dependent Manner

To analyze how the regulatory functions of RLE-1-mediated DAF-16 ubiquitination affect *C. elegans* longevity, we first asked whether overexpression of DAF-16 in *rle-1* mutants further increases the lifespan of *C. elegans*. We thus compared the lifespan of *daf-16::gfp* transgenic animals with *rle-1;daf-16::gfp* animals with the *rle-1* mutants and N2 animals as controls. Both *rle-1* mutant and *daf-16::gfp* animals have a similar lifespan when cultured at 25°C, which is about 20% greater than that of N2 animals. When RLE-1 expression was disrupted in *daf-16::gfp* animals by crossing *rle-1* mutants into the *daf-16::gfp* background, the lifespan of *daf-16::gfp* increased an additional 10% ($p = 0.02$) (Figure 6A and Table 1). These results indicate that disruption of RLE-1 adds to the overexpression of DAF-16 to further extends *C. elegans* lifespan.

To more thoroughly investigate whether RLE-1 and DAF-16 regulate *C. elegans* lifespan in the same pathway, we generated RLE-1 and DAF-16 double mutants by breeding the *rle-1*$^{-/-}$ *(KB6)* strain with *daf-16*$^{-/-}$ (*CF1038*) animals (Lin et al., 2001). Disruption of DAF-16 expression in *rle-1* mutants reduced their longevity, indicating that RLE-1 strictly depends on DAF-16 to regulate *C. elegans* lifespan (Figure 6B and Table 1). Given the fact that both DAF-16 protein expression and its transcriptional activation are increased in *rle-1*$^{-/-}$ animals, we collectively conclude that the longevity of *rle-1* mutants is caused by the gain of DAF-16 function. If this is true, reduction of DAF-16 expression by removing one copy of *daf-16* gene should suppress the *rle-1* mutant phenotypes. To support this hypothesis, we have found that removing one copy of the *daf-16* gene, in *rle-1*$^{-/-}$*;daf-16*$^{+/-}$ animals, partially suppressed the *rle-1*$^{-/-}$ mutant lifespan extension. However, when compared to N2 (or *daf-16*$^{+/-}$) animals, *rle-1*$^{-/-}$*;daf-16*$^{+/-}$ worms still live slightly longer (Figure 6C and Table 1), suggesting that other protein(s) involved in *C. elegans* aging may also be regulated by RLE-1. Taken together, our results indicate that RLE-1 regulates *C. elegans* aging in a DAF-16-dependent manner.

DISCUSSION

The complex signaling networks mediated by insulin/IGF-I play important roles in aging from *C. elegans* to humans (reviewed by Hekimi and Guarente, 2003). Our studies add a new layer of complexity to the signal transduction pathways in aging regulation by revealing crosstalk between insulin/IGF signaling and the ubiquitin pathway. In this study, we have identified an E3 ubiquitin ligase, RLE-1, involved in regulating the longevity of *C. elegans* and have demonstrated that RLE-1 is an E3 ubiquitin ligase for the forkhead transcription factor, DAF-16,

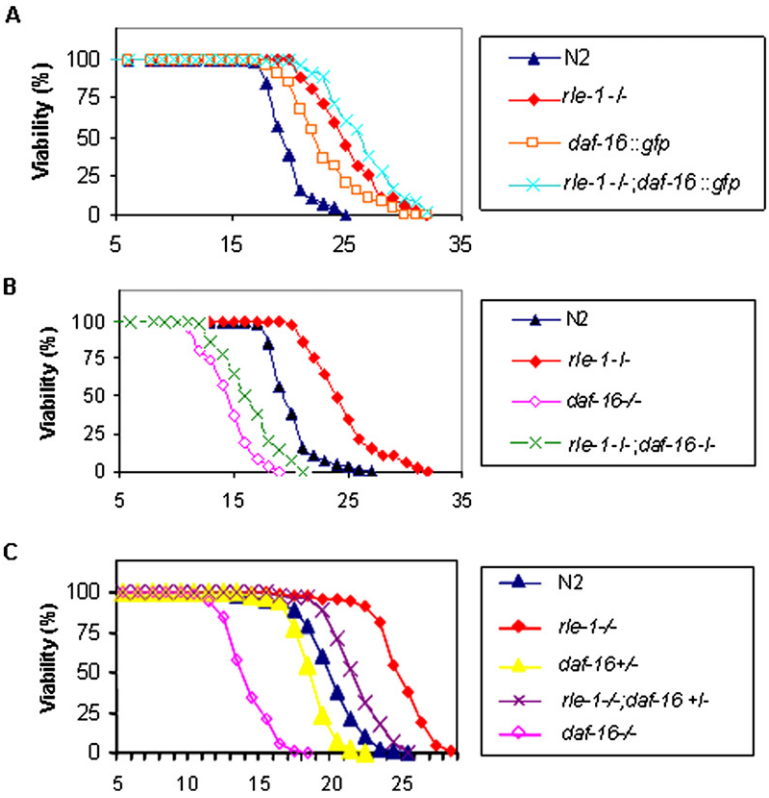

Figure 6. Regulation of the Lifespan by RLE-1 Depends on DAF-16

The curves shown represent the sum of all animals examined in two independent experiments. Lifespans were determined at 25°C. (A) The *daf-16::gfp* transgene on the *rle-1* mutant background further increased the lifespan of *C. elegans* animals. *daf-16::gfp* transgenic animals were bred with *rle-1* mutant animals (*rle-1;daf-16::gfp*); their lifespan was compared with N2, *rle-1*$^{-/-}$, and *daf-16::gfp* animals. (B) Disruption of DAF-16 expression in *rle-1* mutants reverses the longevity of *C. elegans*. *rle-1*$^{-/-}$ worms were bred with *daf-16* mutants; their lifespan was analyzed and compared with the lifespan of N2, *rle-1*, and *daf-16* mutants. (C) The lifespan of *rle-1* mutants with reduced DAF-16 expression. *rle-1*$^{-/-}$ males were selected and bred with *rle-1*$^{-/-}$;*daf-16*$^{-/-}$ animals to produce *rle-1*$^{-/-}$;*daf-16*$^{+/-}$ animals. Their lifespan was analyzed and compared with *daf-16*$^{+/-}$, *daf-16*$^{-/-}$, and *rle*$^{-/-}$ animals.

a critical component of the insulin/IGF signaling pathway (Lin et al., 1997; Ogg et al., 1997).

RLE-1 Is an E3 Ubiquitin Ligase of DAF-16

Disruption of RLE-1 expression results in longevity and delays in development in *C. elegans*. In *C. elegans*, the transcription factor DAF-16 promotes longevity in response to reduced insulin/IGF-1 signaling or to germline ablation (Lin et al., 1997). Overexpression of DAF-16 in *C. elegans daf-16::gfp* transgenic animals also delays development and results in longevity and in partial embryonic lethality (Henderson and Johnson, 2001; Murakami and Johnson, 2001). These are almost identical phenotypes to the phenotypes of the *rle-1(KB6)* strain. The fact that DAF-16 protein expression is elevated in *rle-1* mutants further strengthens our hypothesis that RLE-1 may regulate *C. elegans* aging by controlling the protein levels of DAF-16.

We then investigated the mechanism by which RLE-1 regulates DAF-16 expression. We reasoned that since RLE-1 is an E3 ubiquitin ligase, and that the *daf-16* mRNA levels are comparable between N2 and *rle-1* mutants, RLE-1 might promote DAF-16 degradation. Indeed, expression of RLE-1 accelerated DAF-16 degradation via the proteasome pathway as the proteasome-specific inhibitor, MG132, blocked DAF-16 degradation. RLE-1 mediates DAF-16 degradation via the ubiquitin pathway because RLE-1 directly interacts with and induces ubiquitin conjugation of DAF-16. RLE-1 belongs to the family of RING finger-containing E3 ubiquitin ligases. The RING-finger was defined as E3 ubiquitin ligase, with the RING proteins recruiting the activated E2s (Zheng et al., 2000). The active site cysteine within the RING finger receives ubiquitin from the corresponding E2 and transfers it to the protein substrate. Mutation of the conserved cysteine in the RING finger of RLE-1 essentially abolishes its DAF-16 ubiquitination activity.

E3 ubiquitin ligases recruit substrates through their protein-protein interaction domains or motifs or via adaptor proteins (Hershko and Ciechanover, 1998). The C terminus of RLE-1, which is highly proline rich, is responsible for recruiting DAF-16 for ubiquitination as deletion of this C terminus abolished its interaction with DAF-16. RLE-1 also contains a ROQ domain and two coiled-coiled domains, the precise roles of which remain to be defined by further structure-function studies. In addition to these recognizable domains, other regions in this very large protein may serve additional unknown functions.

Regulation of DAF-16 Transcription Activation by RLE-1

To our knowledge, RLE-1 is the first E3 ubiquitin ligase found to regulate the DAF-16 transcription factor and to result in longevity. Reduction-of-function mutations affecting the insulin/IGF-1-like receptor DAF-2, or components of a downstream PI3-kinase/PDK/AKT pathway, double the animal's lifespan (Larsen et al., 1995; Morris et al., 1996; Lin et al., 1997; Kimura et al., 1997; Paradis and Ruvkun, 1998). This lifespan extension requires DAF-16. Our findings of elevated DAF-16 protein

expression in *rle-1*$^{-/-}$ animals indicate that RLE-1 regulates the aging process by controlling the protein levels of DAF-16. As a critical transcription factor involved in development, aging, dauer formation, and response to stress, under normal conditions DAF-16 is synthesized but remains in the cytoplasm and thus is inactive as a transcription factor (Lin et al., 1997). When worms are stressed, as with high temperatures, DAF-16 translocates into the nucleus to induce the transcription of stress-response genes. However, continuous accumulation of DAF-16 in *C. elegans* delays development, reduces the number of embryos produced, and results in embryonic lethality (Henderson and Johnson, 2001). Our findings that RLE-1 colocalizes with DAF-16 in the cytoplasm and induces DAF-16 degradation provide the molecular mechanism for the control by RLE-1 of DAF-16 protein levels. A similar mechanism has been observed in the regulation of the mammalian NRF2 protein, a key transcription factor that responds to oxidative stress (Cullinan et al., 2004; Kobayashi et al., 2004; Zhang et al., 2004). The protein levels of NRF2 are regulated by a Kelch-BTB protein, Keap1, at the posttranscriptional level via the ubiquitin pathway. Oxidative stress releases NRF2 from Keap1 and allows its nuclear translocation, which then results in the transcription of stress-response genes (Cullinan et al., 2004; Zhang et al., 2004).

In wild-type *C. elegans*, the AKT-1 and AKT-2 proteins phosphorylate DAF-16, inhibiting its nuclear localization (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001). In DAF-2 pathway mutants, DAF-16 accumulates in the nuclei of many cell types, where it leads to changes in the expression of a wide variety of metabolic, stress response, antimicrobial, and novel genes, thereby extending lifespan (Lee et al., 2001; McElwee et al., 2004; Murphy et al., 2003). Under normal conditions, disruption of RLE-1 expression increases the protein levels of DAF-16 in the cytoplasm without resulting in its nuclear translocation. However, after stress sustained nuclear localization of DAF-16 occurs in *rle-1* mutants. One possible explanation is that it takes longer for DAF-16 to export back to the cytoplasm in *rle-1* animals because the protein levels of DAF-16 in *rle-1* mutants are higher. We still cannot exclude the possibility that RLE-1-mediated DAF-16 ubiquitination functions as an exportation signal for DAF-16 or induces nuclear DAF-16 degradation. Our laboratory is currently investigating the roles of RLE-1 in regulating DAF-16 nuclear localization.

RLE-1-Mediated Ubiquitination in Aging

Some evidence has emerged implicating protein ubiquitination with aging. A systematic RNAi screen for longevity genes identified many genes that are involved in protein ubiquitination and degradation in *C. elegans* (Hamilton et al., 2005). Skp2, an oncogenic subunit of the Skp1/Cul1/F-box protein ubiquitin complex, interacts with, ubiquitinates, and promotes the degradation of FOXO1, a forkhead transcription factor involved both in longevity and in tumor suppression (Huang et al., 2005). To our knowledge, RLE-1 is the first E3 ubiquitin ligase isolated to date that is directly involved in aging in *C. elegans*. Disruption of DAF-16 expression reverses the longevity of *C. elegans* in *rle-1*$^{-/-}$ animals, demonstrating that RLE-1 regulates the lifespan of *C. elegans* in a DAF-16-dependent manner. However, we also observed that *daf-16;rle-1* double mutants live slightly longer than *daf-16* mutants. Therefore, RLE-1 may also regulate other proteins, possibly by ubiquitination, to further impact the aging of *C. elegans*.

Protein ubiquitination is a process that is regulated by upstream signal molecules (Hershko and Ciechanover, 1998; Fang and Liu, 2001). RLE-1-mediated DAF-16 ubiquitination is thus possibly regulated by insulin/IGF-1 signaling in *C. elegans*. In an attempt to investigate whether RLE-1 acts in the canonical DAF-2 to DAF-16 pathway, we have tried to generate *daf-2;rle-1* double mutants. However, all of the thirty confirmed *daf-2;rle-1* animals were sterile (data not shown), indicating that addition of the *rle-1* mutant to the *daf-2* mutant background further impaired the reproductive capacity of *C. elegans*. Our laboratory is currently investigating how RLE-1-induced DAF-16 ubiquitination crosstalks with insulin/IGF-1 signaling. Preliminary studies have suggested that RLE-1 interacts with DAF-16 in a phosphorylation (possibly by AKT)-dependent manner (B.G. and D.F., unpublished data). Given that AKT-mediated phosphorylation of DAF-16 prevents DAF-16 from entering the nucleus (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001), and the fact that RLE-1 colocalizes with DAF-16 in the cytoplasm in *C. elegans*, these new findings suggest that the RLE-1-mediated DAF-16 ubiquitination is regulated by the DAF-2/DAF-16 signaling pathway.

It is important to point out that the *rle-1*$^{-/-}$ (*KB6*) strain has been generated by a gene transposon approach, such that insertion of the Tc5 vector may only interrupt the expression of the C terminus of RLE-1. A truncated form of an N-terminal RLE-1 may still be present in these *rle-1* mutants since RT-PCR can still detect the 5′-end of *rle-1* mRNA (Figure S2D). Western blotting did not detect RLE-1 protein in the *rle-1* mutants; however, the polyclonal anti-RLE-1 antibody only recognizes the C terminus of RLE-1 since these antibodies were produced by immunizing mice with a GST-fusion protein of the RLE-1 C terminus; therefore, a truncated N-terminal RLE-1 protein could be produced by the *rle-1(KB6)* strain. Therefore, a *C. elegans* mutant strain in which the full-length RLE-1 expression is disrupted may need to be generated for further investigation of RLE-1 functions.

RLE-1 appears to be an E3 ubiquitin ligase of DAF-16 that specifically regulates the longevity of *C. elegans*. In the future, it will be imperative to understand how insulin/IGF-1 signaling regulates RLE-1-mediated DAF-16 ubiquitination, both under normal conditions and when *C. elegans* confronts various stressful conditions. Since the insulin/IGF-1 pathway is well conserved from *C. elegans* to humans, knowledge obtained from these studies will shed light on the molecular mechanisms of insulin/IGF-1 signaling in the aging of *C. elegans* and perhaps also in humans.

EXPERIMENTAL PROCEDURES

C. elegans Strains, Plasmids, and Antibodies

All strains of *C. elegans* used in this study are listed in Table S1. *rle-1* and *daf-16* double heterozygous *C. elegans* (F1) were obtained by breeding *rle-1*$^{-/-}$ with *daf-16*$^{-/-}$. The F2 offspring of these double heterozygous animals were genotyped to find *rle-1*$^{-/-}$;*daf-16*$^{-/-}$ animals. *rle-1*$^{-/-}$;*daf-16*$^{+/-}$ animals were produced by breeding *rle-1*$^{-/-}$;*daf-16*$^{+/+}$ males with *rle-1*$^{-/-}$;*daf-16*$^{-/-}$ hermaphrodites (6:1 ratio) of L4 larvae to produce *rle-1*$^{-/-}$;*daf-16*$^{+/-}$ (F1). F1 animals were used for the analysis of lifespan. Since it is possible that *rle-1*$^{-/-}$;*daf-16*$^{-/-}$ hermaphrodites produce offspring without mating, some F1 animals may have an *rle-1*$^{-/-}$;*daf-16*$^{-/-}$ genotype. Therefore, their genotypes were confirmed by PCR during lifespan analysis.

rle-1 cDNA was amplified by RT-PCR with mRNA from N2 animals with the primers shown in Table S1, and RLE-1 expression plasmids were generated by insertion of the full-length cDNA into the pCMV-Flag vector (Sigma, Atlanta, GA). An RLE-1/C34A point mutation was generated by a PCR-based gene mutation approach, and the N-terminal truncated mutant was generated by *Kpn1* and *Xba1* digestion. The DAF-16 expression plasmid (1006c10) was a gift from the Japanese National Institute of Genetics (http://www.nig.ac.jp/). Anti-HA, anti-DAF-16, and anti-Actin antibodies are from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Flag monoclonal antibody is from Sigma (Atlanta, GA). The anti-RLE-1 polyclonal antibody was produced in our laboratory by immunization of mice with a GST-RLE-1 fusion protein.

Lifespan Analysis and Stress Assays

Lifespan was determined at 25°C in the presence of 5-fluorodeoxyuridine (FUDR) to inhibit progeny development. The animals were cultured on standard agar plates at 10 animals per plate. Day of hatching (L1) was used as the first time point. Animals were considered dead when they ceased moving and did not respond to prodding. Survival curves, mean lifespan, and p values were determined nonparametrically; log-rank tests were used to assess the similarity between these two groups.

Stress Assays

Sensitivity to thermal stress was determined as described (Lithgow and Walker, 2002). Briefly, L1 larvae were cultured on nematode growth medium (NGM) plates for 4 days to allow them to reach adulthood. Then, 50 adult animals were incubated at 37°C on fresh NGM plates. Animals were checked every hour for viability. Similarly, 50 adult worms were placed on bacteria-free plates and exposed to UV light (40 J/m^2) as described (Murakami and Johnson, 2001). The animals were then transferred to NGM plates and examined for their survival during subsequent culture at 20°C. For the killing assay, approximately 25 L4-stage wild-type N2 or *rle-1* mutants were placed on each of three agar plates with *P. aeruginosa* PA14 lawns under standard slow-killing assay conditions as described (Tan et al., 1999). The fraction of live animals was determined at each time point; animals were considered dead when they did not respond to gentle touch.

Dauer Formation Assay

Synchronous adults were left to lay eggs at 20°C for three hours. These plates were transferred to 25°C for 3 days and then the number of adults and dauer larvae were counted. Dauer larvae were additionally scored by their resistance to 1% SDS exposure for 30 min.

RT-PCR and Quantitative Real-Time PCR Analysis

Total RNA was isolated from *rle-1* mutants or N2 animals and then subjected to reverse transcription with oligo-dT primer and the AMV Reverse Transcriptase (NEB, Boston, MA). For real-time PCR, the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) was used. Reactions were loaded on 96-well thin wall plates and sealed with optical quality sealing tape. Each reaction was run on an iCycler iQ Multi-Color Real Time PCR detection system (Bio-Rad, Hercules, CA) under the following conditions: 94°C for 5 min, followed by 94°C for 30 s, 55°C for 30 s, 72°C for 30 s for 40 cycles, and then 72°C for 3 min. Samples were run in triplicate, and relative copy numbers were determined. All primers used in this study are listed in Table S2.

Coimmunoprecipitation and Western Blotting

Transient transfections were performed by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions in 6 cm^2 dishes with 1–3 μg of total DNA used per transfection. Transfected cells were settled to pellet and resuspended in 1× Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 5 mM NaPiP, 2 mM Na_3VO_4, and 1× protease inhibitor cocktail). Cells were lyzed for 10 min at 4°C, and insoluble materials were removed by centrifugation at 15,000 × g (4°C, 10 min). For immunoprecipitation, lysates (~1 × 10^7 cells) were mixed with antibodies (1 μg) for 2 hr, followed by the addition of 30 μl of protein G-Sepharose beads (Amersham, Piscataway, NJ) for an additional 2 hr at 4°C. Immunoprecipitates were washed four times with 1× Nonidet P-40 lysis buffer and boiled in 20 μl of 2× Laemmli's buffer. Samples were subjected to 8% or 10% SDS-polyacrylamide gel electrophoresis analysis and electrotransferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were probed with the indicated primary antibodies (usually at 1 μg/ml), followed by horseradish peroxidase-conjugated secondary antibodies. Membranes were then washed and visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ). When necessary, membranes were stripped by incubation in stripping buffer (62.5 mM Tris-HCl [pH 5.7], 100 mM 2-mercaptoethanol, and 2% SDS) for 1 hr at 70°C with constant agitation, washed, and then reprobed with additional antibodies as indicated.

Immunostaining and Confocal Microscopy

Protocols for immunochemistry were as described (Kuznicki et al., 2000). Animals were splayed with a 25 gauge needle, put onto slides, and then fixed for 10–15 min in methanol at −20°C followed by 10–15 min in acetone at −20°C. Primary antibodies were diluted 1:200 in PBS with 2% BSA, added to the fixed animals, and incubated for 2 hr. Secondary antibodies conjugated to Alexa Fluor 594 (Molecular Probes, Eugene, OR) were diluted 1:3000. Confocal images were collected by using an Olympus IX70 microscope coupled to a BioRad 2000 confocal system at the UMC Cytology Core Facility. Other fluorescent images were gathered on a Zeiss Auxioplan microscope with a SPOT CCD camera.

Supplemental Data

Supplemental Data include eight figures and two tables and are available at http://www.developmentalcell.com/cgi/content/full/12/2/235/DC1/.

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REFERENCES

Cullinan, S.B., Gordan, J.D., Jin, J., Harper, J.W., and Diehl, J.A. (2004). The Keap1-BTB protein is an adaptor that bridges Nrf2 to

a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. Mol. Cell. Biol. *24*, 8477–8486.

Dorman, J.B., Albinder, B., Shroyer, T., and Kenyon, C. (1995). The age-1 and daf-2 genes function in a common pathway to control the lifespan of *Caenorhabditis elegans*. Genetics *141*, 1399–1406.

Essers, M.A., de Vries-Smits, L.M., Barker, N., Polderman, P.E., Burgering, B.M., and Korswagen, H.C. (2005). Functional interaction between beta-catenin and FOXO in oxidative stress signaling. Science *308*, 1181–1184.

Fang, D., and Liu, Y.C. (2001). Proteolysis-independent regulation of PI3K by Cbl-b-mediated ubiquitination in T cells. Nat. Immunol. *2*, 870–875.

Friedman, D.B., and Johnson, T.E. (1988). A mutation in the age-1 gene in *Caenorhabditis elegans* lengthens life and reduces hermaphrodite fertility. Genetics *118*, 75–86.

Gami, M.S., and Wolkow, C.A. (2006). Studies of *Caenorhabditis elegans* DAF-2/insulin signaling reveal targets for pharmacological manipulation of lifespan. Aging Cell *5*, 31–37.

Gao, B., Lee, S.M., and Fang, D. (2006). The tyrosine kinase c-Abl protects c-Jun from ubiquitination-mediated degradation in T cells. J. Biol. Chem. *281*, 29711–29718.

Gottlieb, S., and Ruvkun, G. (1994). daf-2, daf-16 and daf-23: genetically interacting genes controlling Dauer formation in *Caenorhabditis elegans*. Genetics *137*, 107–120.

Hamilton, B., Dong, Y., Shindo, M., Liu, W., Odell, I., Ruvkun, G., and Lee, S.S. (2005). A systematic RNAi screen for longevity genes in *C. elegans*. Genes Dev. *19*, 1544–1555.

Hekimi, S., and Guarente, L. (2003). Genetics and the specificity of the aging process. Science *299*, 1351–1354.

Henderson, S.T., and Johnson, T.E. (2001). daf-16 integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans*. Curr. Biol. *11*, 1975–1980.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. *67*, 425–479.

Hsu, A.L., Murphy, C.T., and Kenyon, C. (2003). Regulation of aging and age-related disease by DAF-16 and heat-shock factor. Science *300*, 1142–1145.

Huang, H., Regan, K.M., Wang, F., Wang, D., Smith, D.I., van Deursen, J.M., and Tindall, D.J. (2005). Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. Proc. Natl. Acad. Sci. USA *102*, 1649–1654.

Kenyon, C., Chang, J., Gensch, E., Rudner, A., and Tabtiang, R. (1993). A *C. elegans* mutant that lives twice as long as wild type. Nature *366*, 461–464.

Kimura, K.D., Tissenbaum, H.A., Liu, Y., and Ruvkun, G. (1997). daf-2, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. Science *277*, 942–946.

Kobayashi, A., Kang, M.I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., Igarashi, K., and Yamamoto, M. (2004). Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol. Cell. Biol. *24*, 7130–7139.

Kops, G.J., Dansen, T.B., Polderman, P.E., Saarloos, I., Wirtz, K.W., Coffer, P.J., Huang, T.T., Bos, J.L., Medema, R.H., and Burgering, B.M. (2002). Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature *419*, 316–321.

Kuznicki, K.A., Smith, P.A., Leung-Chiu, W.M., Estevez, A.O., Scott, H.C., and Bennett, K.L. (2000). Combinatorial RNA interference indicates GLH-4 can compensate for GLH-1; these two P granule components are critical for fertility in *C. elegans*. Development *127*, 2907–2916.

Larsen, P.L., Albert, P.S., and Riddle, D.L. (1995). Genes that regulate both development and longevity in *Caenorhabditis elegans*. Genetics *139*, 1567–1583.

Lee, R.Y., Hench, J., and Ruvkun, G. (2001). Regulation of *C. elegans* DAF-16 and its human ortholog FKHRL1 by the daf-2 insulin-like signaling pathway. Curr. Biol. *11*, 1950–1957.

Lee, S.S., Kennedy, S., Tolonen, A.C., and Ruvkun, G. (2003). DAF-16 target genes that control *C. elegans* life-span and metabolism. Science *300*, 644–647.

Lin, K., Dorman, J.B., Rodan, A., and Kenyon, C. (1997). daf-16: An HNF-3/forkhead family member that can function to double the lifespan of *Caenorhabditis elegans*. Science *278*, 1319–1322.

Lin, K., Hsin, H., Libina, N., and Kenyon, C. (2001). Regulation of the *Caenorhabditis elegans* longevity protein DAF-16 by insulin/IGF-1 and germline signaling. Nat. Genet. *28*, 139–145.

Lithgow, G.J., and Walker, G.A. (2002). Stress resistance as a determinate of *C. elegans* lifespan. Mech. Ageing Dev. *123*, 765–771.

McElwee, J.J., Schuster, E., Blanc, E., Thomas, J.H., and Gems, D. (2004). Shared transcriptional signature in *Caenorhabditis elegans* Dauer larvae and long-lived daf-2 mutants implicates detoxification system in longevity assurance. J. Biol. Chem. *279*, 44533–44543.

Morris, J.Z., Tissenbaum, H.A., and Ruvkun, G. (1996). A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in *Caenorhabditis elegans*. Nature *382*, 536–539.

Motta, M.C., Divecha, N., Lemieux, M., Kamel, C., Chen, D., Gu, W., Bultsma, Y., McBurney, M., and Guarente, L. (2004). Mammalian SIRT1 represses forkhead transcription factors. Cell *116*, 551–563.

Murakami, S., and Johnson, T.E. (2001). The OLD-1 positive regulator of longevity and stress resistance is under DAF-16 regulation in *Caenorhabditis elegans*. Curr. Biol. *11*, 1517–1523.

Murphy, C.T., McCarroll, S.A., Bargmann, C.I., Fraser, A., Kamath, R.S., Ahringer, J., Li, H., and Kenyon, C. (2003). Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. Nature *424*, 277–283.

Nakata, K., Abrams, B., Grill, B., Goncharov, A., Huang, X., Chisholm, A.D., and Jin, Y. (2005). Regulation of a DLK-1 and p38 MAP kinase pathway by the ubiquitin ligase RPM-1 is required for presynaptic development. Cell *120*, 407–420.

Newman, J.R., and Keating, A.E. (2003). Comprehensive identification of human bZIP interactions with coiled-coil arrays. Science *300*, 2097–2101.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G.I., Lee, L., Tissenbaum, H.A., and Ruvkun, G. (1997). The Forkhead transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. Nature *389*, 994–999.

Oh, S.W., Mukhopadhyay, A., Svrzikapa, N., Jiang, F., Davis, R.J., and Tissenbaum, H.A. (2005). JNK regulates lifespan in *Caenorhabditis elegans* by modulating nuclear translocation of forkhead transcription factor/DAF-16. Proc. Natl. Acad. Sci. USA *102*, 4494–4499.

Paradis, S., and Ruvkun, G. (1998). *Caenorhabditis elegans* Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. Genes Dev. *12*, 2488–2498.

Paradis, S., Ailion, M., Toker, A., Thomas, J.H., and Ruvkun, G. (1999). A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in *Caenorhabditis elegans*. Genes Dev. *13*, 1438–1452.

Pickart, C.M. (2001). Mechanisms underlying ubiquitination. Annu. Rev. Biochem. *70*, 503–533.

Riddle, D.L. (1977). A genetic pathway for dauer larva formation in *C. elegans*. Stadler Genet. Symp. *9*, 101–120.

Saito, Y., Tsubuki, S., Ito, H., Ohmi-Imajo, S., and Kawashima, S. (1992). Possible involvement of clathrin in neuritogenesis induced by a protease inhibitor (benzyloxycarbonyl-Leu-Leu-Leu-aldehyde) in PC12 cells. J. Biochem. (Tokyo) *112*, 448–455.

Tan, M.W., Mahajan-Miklos, S., and Ausubel, F.M. (1999). Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model

mammalian bacterial pathogenesis. Proc. Natl. Acad. Sci. USA *96*, 715–720.

Tissenbaum, H.A., and Ruvkun, G. (1998). An insulin-like signaling pathway affects both longevity and reproduction in *Caenorhabditis elegans*. Genetics *148*, 703–717.

Vinuesa, C.G., Cook, M.C., Angelucci, C., Athanasopoulos, V., Rui, L., Hill, K.M., Yu, D., Domaschenz, H., Whittle, B., Lambe, T., et al. (2005). A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. Nature *435*, 452–458.

Zhang, D.D., Lo, S.C., Cross, J.V., Templeton, D.J., and Hannink, M. (2004). Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. Mol. Cell. Biol. *24*, 10941–10953.

Zheng, N., Wang, P., Jeffrey, P.D., and Pavletich, N.P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. Cell *102*, 533–539.

Zhong, Q., Gao, W., Du, F., and Wang, X. (2005). Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. Cell *121*, 1085–1095.