Developmental Cell, Vol. 9, 605–615, November, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.devcel.2005.09.017

A Role for SIR-2.1 Regulation of ER Stress Response Genes in Determining *C. elegans* Life Span

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Summary

C. elegans **SIR-2.1, a member of the Sir-2 family of NAD$^+$-dependent protein deacetylases, has been shown to regulate nematode aging via the insulin/IGF pathway transcription factor *daf-16*. Treatment of *C. elegans* with the small molecule resveratrol, however, extends life span in a manner fully dependent upon *sir-2.1*, but independent of *daf-16*. Microarray analysis of worms treated with resveratrol demonstrates the transcriptional induction of a family of genes encoding prion-like glutamine/asparagine-rich proteins involved in endoplasmic reticulum (ER) stress response to unfolded proteins. RNA interference of *abu-11*, a member of this ER stress gene family, abolishes resveratrol-mediated life span extension, and overexpression of *abu-11* extends the life span of transgenic animals. Furthermore, SIR-2.1 normally represses transcription of *abu-11* and other ER stress gene family members, indicating that resveratrol extends life span by inhibiting *sir-2.1*-mediated repression of ER stress genes. Our findings demonstrate that *abu-11* and other members of its ER stress gene family are positive determinants of *C. elegans* life span.**

Introduction

Numerous genes have been identified that regulate the life span of model organisms (Braeckman et al., 2001; Guarente and Kenyon, 2000; Hekimi and Guarente, 2003; Kenyon, 2005), including genes that encode Sir2 proteins (Blander and Guarente, 2004), components of the insulin/IGF signaling pathway (Friedman and Johnson, 1988; Kenyon et al., 1993; Kimura et al., 1997), and heat shock proteins (Nollen and Morimoto, 2002; Wu, 1995). Certain heat shock proteins and the heat shock transcription factor have been shown to be longevity determinants in *D. melanogaster* (Morrow et al., 2004; Tatar et al., 1997) and *C. elegans* (Hsu et al., 2003; Lithgow et al., 1995; Morley and Morimoto, 2004), perhaps by counteracting the presence of misfolded proteins in the cytoplasm and nucleus arising from oxidative or other types of protein damage.

SIR2 and its orthologs represent a conserved group of genes encoding NAD$^+$-dependent protein deacetylases (Imai et al., 2000; Landry et al., 2000; North and Verdin, 2004) that are responsive to metabolic changes in the cellular environment, including nutrient/energy availability and cellular stress (Lin and Guarente, 2003). Increased dosage of Sir2 homologs from *S. cerevisiae* (Kaeberlein et al., 1999), *D. melanogaster* (Rogina and Helfand, 2004), and *C. elegans* (Tissenbaum and Guarente, 2001) extend life span, suggesting that Sir2 genes are conserved regulators of the aging process. In mammals, there are seven Sir2 orthologs SIRT1–7 (Frye, 2000). SIRT1 has been implicated in diverse metabolic functions, including adipogenesis (Picard et al., 2004), myogenesis (Fulco et al., 2003), and apoptosis (Brunet et al., 2004; Cohen et al., 2004; Luo et al., 2001; Motta et al., 2004; Vaziri et al., 2001).

In *C. elegans*, life span extension conferred by increased dosage of *sir-2.1* requires the forkhead transcription factor *daf-16*, a member of the *C. elegans* insulin/IGF signaling pathway (Tissenbaum and Guarente, 2001). Signaling through this pathway begins at the insulin receptor DAF-2 and continues through a series of kinases that ultimately phosphorylate DAF-16, resulting in its cytoplasmic retention (Kenyon, 2005). Downregulation of this pathway, by mutations in *daf-2* for example, results in nuclear localization of DAF-16 and the induction of genes that confer longevity (reviewed in Braeckman et al., 2001).

Small molecules that alter SIR2 enzymatic activity have been identified (reviewed in Porcu and Chiarugi, 2005). Nicotinamide (Bitterman et al., 2002), a product of the Sir2 deacetylase reaction, and NADH (Lin et al., 2004; Schmidt et al., 2004) are potent inhibitors of SIR2 deacetylase activity in vivo. Resveratrol (3, 4′, 5-trihydroxystilbene), a plant-derived polyphenolic compound, is among a number of small molecules that were initially shown to activate the catalytic activity of mammalian ortholog SIRT1 and yeast Sir2p (Howitz et al., 2003). Resveratrol has been observed to have therapeutic benefits, including cancer prevention (Aggarwal et al., 2004), cardio protection (Hung et al., 2000; Sato et al., 2002), and antifungal effects (reviewed in Pervaiz, 2003). Recent studies suggest that the activation of Sir2 by resveratrol may result from the fluorescently tagged substrate used in the in vitro assay for Sir2 deacteylase activity (Borra et al., 2005; Kaeberlein et al., 2005). Still, there is significant in vivo evidence to suggest that resveratrol extends life span in a Sir2-dependent manner (Howitz et al., 2003; Wood et al., 2004).

In this study, we examine the mechanism of life span extension conferred by resveratrol in *C. elegans*. We find that life span extension by this compound is dependent upon *sir-2.1*, yet is independent of *daf-16*, identifying a longevity pathway that is mediated by *sir-2.1* but is independent of the insulin/IGF pathway. Genome-wide microarrays demonstrate that resveratrol upregulates a family of stress response genes encoding prion-like glutamine/asparagine-rich proteins that are thought to aid protein folding in the endoplasmic reticulum (ER) (Urano et al., 2002). At least one of these ER stress genes, *abu-11,* is necessary and sufficient for the longevity conferred by resveratrol. Expression of *abu-*

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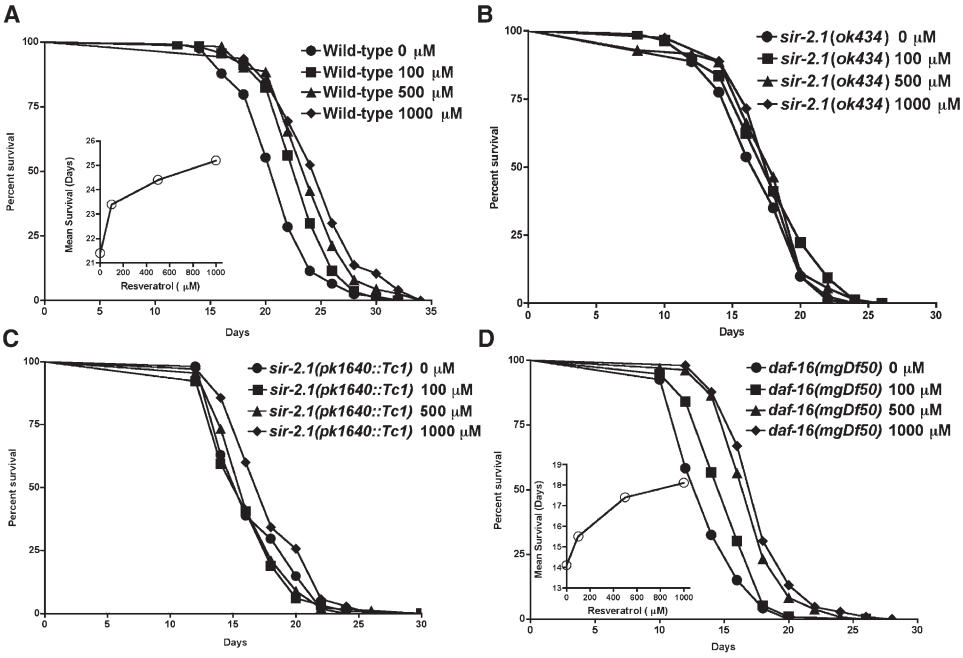

Figure 1. Resveratrol Extends *C. elegans* Life Span by a *sir-2.1*-Dependent and *daf-16*-Independent Mechanism

(A) Resveratrol extends wild-type life span in a dosage-dependent manner. 0 μM control (m = 21.4, M = 32, n = 123); 100 μM (m = 23.4, M = 32, n = 114, p < 0.0001, 9.3%); 500 μM (m = 24.4, M = 34, n = 113, p < 0.0001, 14.0%); 1000 μM (m = 25.2, M = 34, n = 124, p < 0.0001, 17.8%).

(B) Resveratrol does not extend life span of the LG231 *sir-2.1*(*ok434*) deletion mutant. 0 μM (m = 17.3, M = 24, n = 80); 100 μM (m = 18.1, M = 26, n = 85, p = 0.0500 [ns], 4.6%); 500 μM (m = 17.9, M = 26, n = 71, p = 0.0857 [ns], 3.5%); 1000 μM (m = 18.2, M = 24, n = 81, p = 0.1695 [ns], 5.2%).

(C) Resveratrol does not extend life span of the NL4251 *sir-2.1*::*pk1640* transposon insertion mutant. 0 μM (m = 16.8, M = 26, n = 55); 100 μM (m = 16.5, M = 30, n = 116, p = 0.3208 [ns], –1.8%); 500 μM (m = 16.9, M = 30, n = 90, p = 0.8441 [ns], 0.6%); 1000 μM (m = 18.2, M = 30, n = 81, p = 0.0711 [ns], 8.3%).

(D) Resveratrol extends life span of the GR1307 *daf-16*(*mgDf50*) mutant. 0 μM (m = 14.1, M = 20, n = 120); 100 μM (m = 15.5, M = 26, n = 113, p < 0.0001, 9.9%); 500 μM (m = 17.4, M = 26, n = 133, p < 0.0001, 23.4%); 1000 μM (m = 18.1, M = 28, n = 106, p < 0.0001, 28.4%).

C. elegans life span assays were performed on NGM + DMSO + FUdR plates containing resveratrol at the specified concentrations. Mean day of life span (m), maximum day of life span (M), number of animals (n), log-rank observed significance levels (p), and percentage difference in mean relative to no resveratrol control are reported for each assay. All life spans were repeated and showed similar effects. Ns, not significant.

11, as well as several other members of this gene family, is repressed by *sir-2.1*. This implies that resveratrol inhibits SIR-2.1 action on these genes, challenging earlier reports that resveratrol is an activator of Sir2 catalytic activity. Our findings identify these ER stress proteins as possible determinants of organismal life span, and they show that their activation is the likely mechanism by which resveratrol extends life span in *C. elegans*.

Results

Life Span Extension in *C. elegans* by Resveratrol Requires *sir-2.1*, but Not *daf-16*

Resveratrol extended *C. elegans* life span in a dosage-dependent manner, increasing both the mean and maximum life span of wild-type worms (Figure 1A). A nearly 18% increase in mean life span was obtained with 1 mM resveratrol. We next determined whether *sir-2.1* was required for life span extension by resveratrol, as it is in yeast and *Drosophila* (Wood et al., 2004). Two independent *sir-2.1* mutant strains were assayed for life span in the presence of varying concentrations of resveratrol. While wild-type worms demonstrated a significant increase in life span in the presence of 1 mM resveratrol (Figure 1A), neither *sir-2.1* mutant strain showed a significant change in life span at any of the concentrations of resveratrol tested (Figures 1B and 1C), demonstrating that the effect of resveratrol on worm life span is completely dependent on *sir-2.1*.

The life span extension by a *sir-2.1* transgene is fully dependent upon the insulin/IGF pathway and its forkhead transcription factor, *daf-16* (Tissenbaum and Guarente, 2001). Given that life span extension by resveratrol is dependent upon *sir-2.1*, we postulated that resveratrol worked by phenocopying the mechanism of life span extension in *sir-2.1* transgenic animals. We tested this idea by determining whether life span extension by resveratrol was dependent upon *daf-16*. Surprisingly, when *daf-16* mutant animals were assayed for life span on media containing resveratrol, a robust increase in life span was noted in a dosage-dependent manner (Figure 1D). At the highest concentration, *daf-16* worms lived an average of 28.4% longer than non-treated worms. These results demonstrate that the mechanism of life span extension by resveratrol differs from that of *sir-2.1* overexpression, thus identifying a separate pathway of life span regulation, which is dependent upon *sir-2.1* but independent of *daf-16*.

In order to determine whether resveratrol-induced life

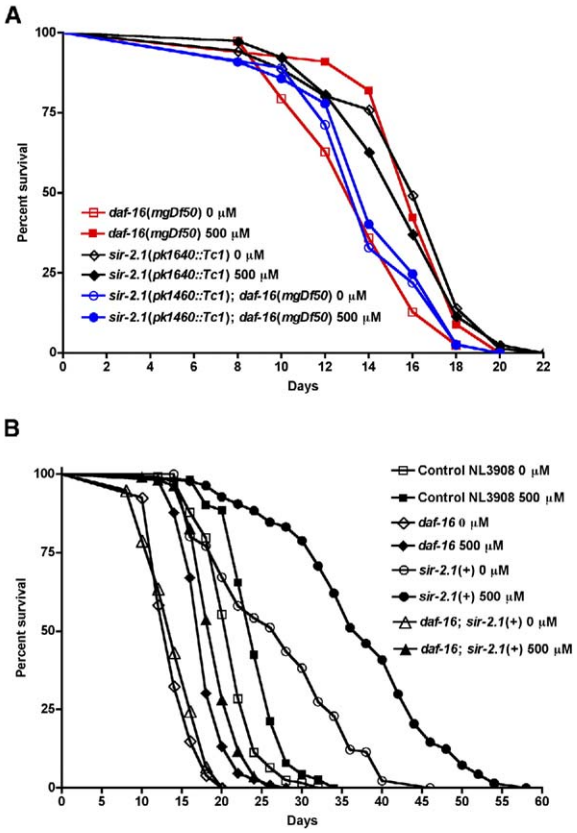

Figure 2. Resveratrol Treatment Extends *C. elegans* Life Span by a Different Mechanism from that of *sir-2.1* Overexpression

Open symbols are control animals; closed symbols are worms treated with 500 μM resveratrol. Reported p values are relative to the 0 μM control for each strain.

(A) Loss of *sir-2.1* abolishes resveratrol-induced life span extension of the short-lived *daf-16* mutant. GR1307 *daf-16*(*mgDf50*): 0 μM (m = 13.8, M = 20, n = 78); 500 μM (m = 16.5, M =20, n = 78, p < 0.0001, 19.6%). NL4251 *sir-2.1*(*pk1460::Tc1*): 0 μM (m = 16.1, M = 22, n = 71); 500 μM (m = 15.7, M = 22, n = 78, p = 0.2543 [ns], –0.2%). LG267 *daf-16*(*mgDf50*); *sir-2.1*(*pk1460::Tc1*): 0 μM (m = 14.4, M = 20, n = 73); 500 μM (m = 14.4, M = 20, n = 77, p = 0.5460 [ns], 0.0%). Similar results were obtained with LG265 *sir-2.1*(*ok434*); *daf-16*(*mgDf50*) mutants and their respective controls (data not shown).

(B) Resveratrol extends life span of the *sir-2.1* transgenic worms even in the absence of *daf-16.* GR1307 *daf-16*(*mgDf50*): 0 μM (m = 14.1, M = 20, n = 120); 500 μM (m = 18.1, M = 28, n = 106, p < 0.0001, 28.4%). NL3908 *pkIs1641*(*unc-119*[+]): 0 μM (m = 21.4, M = 32, n = 123); 500 μM (m = 24.4, M = 34, n = 113, p < 0.0001, 14.0%). NL3909 *pkIs1642*(*unc-119*[+] *sir-2.1*[+]): 0 μM (m = 27.0, M = 46, n = 131); 500 μM (m = 37.5, M = 58, n = 137, p < 0.0001, 38.9%). LG248 *daf-16*(*mgDf50*); *pkIs1642*(*unc-119*[+] *sir-2.1*[+]): 0 μM (m = 14.2, M = 20, n = 118); 500 μM (m = 19.5, M = 26, n = 110, p < 0.0001, 37.3%). Life span assays in (B) were performed once.

span extension in *daf-16* worms is dependent on *sir-2.1*, as it is in wild-type worms, we tested whether resveratrol could extend the life span of *sir-2.1*; *daf-16* double mutants. As shown in Figure 2A, resveratrol significantly extended the life span of *daf-16* mutant animals (19.6%) relative to untreated animals, yet it did not extend either the *sir-2.1* single mutant or the *sir-2.1*; *daf-16* double mutant. This result demonstrates that the life span extension by resveratrol in both wild-type and *daf-16* backgrounds is mediated via *sir-2.1*, and this finding strengthens the surmise that *sir-2.1* can participate in a pathway of life span regulation independent of *daf-16*.

We also examined the effect of resveratrol on *sir-2.1* transgenic strains. As shown in Figure 2B, *sir-2.1* transgenic animals carrying an integrated low-copy array have a robustly extended life span. Moreover, resveratrol treatment of these already long-lived animals further increases the mean life span by 39%, extending the maximum age to nearly 2 months. Life span extension was also seen in resveratrol-treated *daf-16* and wild-type animals, as expected. In the absence of resveratrol, loss of *daf-16* abolished the life span extension of low-copy *sir-2.1* overexpression, reducing the life span to the level of the *daf-16* mutant (Figure 2B). In the presence of resveratrol, however, the *daf-16* mutant carrying the *sir-2.1* transgene lived an average of 37% longer. The life span effect of resveratrol is additive with that due to overexpression of *sir-2.1*, consistent with the idea that resveratrol and overexpression of *sir-2.1* extend life span by distinct pathways.

Genome-Wide Transcriptional Profiling of Resveratrol-Treated *C. elegans*

To identify genetic components that may contribute to resveratrol-mediated life span extension, we carried out genome-wide microarray analysis, comparing the transcriptional profiles of worms grown in the presence and absence of resveratrol. Since resveratrol treatment resulted in life span extension in both wild-type and *daf-16* mutant worms, microarray analysis was performed with both strains independently. Microarray data were analyzed for genes that were regulated by resveratrol ($p < 0.01$) in either wild-type or *daf-16* strains (Table S1; see the Supplemental Data available with this article online). The most highly induced coordinately regulated gene, *cyp-13A6*, encoding a cytochrome P450 protein, was upregulated 23-fold in wild-type and 8-fold in *daf-16* mutant worms. Cytochrome P450 proteins have been shown to be involved in the metabolism of resveratrol (Piver et al., 2004). Furthermore, resveratrol has been shown to be an enzymatic inhibitor of some cytochrome P450 proteins (Piver et al., 2001), and it affects the expression of P450 proteins in cancer cells (Liu et al., 2004).

Strikingly, a novel family of genes comprising *pqn* (prion-like Q/N proteins) and *abu* (activated in blocked unfolded protein response, see below) genes was also induced by treatment with resveratrol (Table 1). PQN and ABU proteins are members of a prion-like glutamine/asparagine-rich protein family. Many of the *abu*/*pqn* genes listed in Table 1 are also coregulated in other experimental and developmental conditions, as evidenced by the fact that they are clustered together in the relatively small and distinct mountain 29 of the *C. elegans* topographical expression map (Kim et al., 2001).

The *abu* class of genes, which are a subset of the *pqn* gene family, have been implicated in a pathway of protein folding in the ER that functions in parallel with the canonical unfolded protein response (UPR) pathway (see Discussion). Eleven *abu* genes are induced in response to ER stress caused by the protein glycosylation inhibitor tunicamycin in worms in which the UPR

Table 1. *C. elegans pqn*-Related Genes Regulated by Resveratrol Treatment and SIR-2.1 Overexpression

ORF Name	Gene Name	Induction in Wild-Type by Resveratrol	Induction in *daf-16* by Resveratrol	Repression in SIR-2.1 Overexpressor	Protein Description				Mountain
					DUF 1096 (+/–)	DUF 139 (#)	Signal Peptide	Transmembrane Domain	
Y105C5A.5	*pqn-78*	1.97 ± 0.77	2.66 ± 0.27	–1.27 ± 0.47	+	10	+	+	16
Y105C5A.3	*pqn-76*	2.05 ± 0.79	2.61 ± 0.32	–2.01 ± 0.50	+	10	+	+	29
F19G12.7	*pqn-30/ abu-2*	1.79 ± 0.62	2.44 ± 0.38	–1.29 ± 0.39	+	19	+	+	29
Y73F8A.9	*pqn-91*	1.86 ± 0.81	2.42 ± 0.38	–1.41 ± 0.27	+	14	+	+	29
Y105C5A.4	*pqn-77/ abu-5*	1.98 ± 0.79	2.42 ± 0.29	–0.90 ± 0.26	+	10	+	+	29
F10F2.9	*pqn-29*	ns	2.41 ± 0.18	–0.41 ± 0.12	–	—	+	–	NA
F31A3.1	*abu-3*	1.94 ± 0.59	2.35 ± 0.39	–1.02 ± 0.39	–	9	+	–	9
M02G9.2	M02G9.2	ns	2.22 ± 0.37	ns	+	8	+	+	NA
F07H5.8	F07H5.8	ns	2.15 ± 0.43	–1.85 ± 0.34	–	22	–	–	29
ZK1067.7	*pqn-95*	ns	1.97 ± 0.11	ns	–	8	+	–	14
W02A2.3	*pqn-74*	ns	1.82 ± 0.29	–1.47 ± 0.22	–	—	+	–	NA
F35D11.2	*pqn-35*	ns	1.82 ± 0.15	–0.68 ± 0.13	–	—	–	–	26
T01D1.6	*pqn-61/ abu-11*	1.60 ± 0.66	1.78 ± 0.30	ns	+	10	+	–	29
T22B2.6	T22B2.6	ns	1.66 ± 0.10	ns	–	1	+	–	29
C03A7.4	*pqn-5*	2.05 ± 0.57	ns	–0.84 ± 0.28	+	9	+	–	29
R09B5.5	*pqn-54*	2.07 ± 0.54	1.54 ± 0.40	–1.02 ± 0.27	+	9	+	–	29
C03A7.7	*pqn-6/ abu-6*	2.23 ± 0.40	ns	–1.28 ± 0.37	+	9	+	–	29
C03A7.8	*pqn-7/ abu-7*	2.14 ± 0.50	ns	–1.12 ± 0.26	+	9	+	–	29
C03A7.14	*pqn-4/ abu-8*	2.14 ± 0.47	ns	–1.12 ± 0.37	+	9	+	–	29

Shown are the mean ± SEM of base$_2$ log-fold changes in wild-type (n = 4, $p < 0.05$) and *daf-16* (n = 4, $p < 0.001$) strains after treatment with resveratrol and in the SIR-2.1 low-copy overexpression strain NL3909 (n = 4, $p < 0.014$) relative to control NL3908 for *pqn* (prion-like glutamine [Q]/asparagines [N]), *abu* (activated in blocked unfolded protein response), and other *pqn*/*abu*-related genes (containing domain of unknown function [DUF] 1096 and/or DUF139 domains). *abu* genes are categorized as ER stress response genes based on experimental evidence (Urano et al., 2002). *pqn* genes encoding prion-like Q/N-rich domain-bearing proteins are predicted based on the DIANA prediction algorithm (Michelitsch and Weissman, 2000). NH_2-terminal Q-rich domains are annotated as DUF1096 (Interpro accession IPR009475), and C-X3-C-X3-C cysteine-rich repeats are annotated as DUF139 (Interpro accession IPR003341) in WormBase. The presence of a signal peptide sequence was determined by using the SignalP 3.0 program (www.cbs.dtu.dk/services/SignalP [Bendtsen et al., 2004]). The presence of transmembrane domains was determined with the PHD program (www.predictprotein.org [Rost, 1996]). The location of genes within the *C. elegans* global expression mountain system (Kim et al., 2001) is also annotated when available. ns, not significant; NA, not available.

pathway is compromised (Urano et al., 2002). Sequences of some of the PQN and ABU proteins demonstrating their similarity are shown in Figure S1 (see the Supplemental Data available with this article online). ABU proteins contain amino-terminal ER signal sequences necessary for translocation across the ER membrane (Table 1). ABU1–9 contain putative transmembrane domains and short cytoplasmic tails, while ABU-10 and ABU-11 appear to be luminal. Three other genes found to be induced by resveratrol, M02G9.2, F07H5.8, and T22B2.6, are not classified as *pqn* or *abu* genes, but they are included in Table 1 because of similarities of their domain architecture to *pqn* genes. F07H5.8 and T22B2.6 are also coregulated with *pqn* genes in mountain 29.

As a validation of the microarray results, RT-PCR analysis was performed on total RNA isolated from wild-type and *daf-16* worms grown in the presence or absence of resveratrol. *cyp-13A6* transcript is upregulated by resveratrol, as demonstrated by the fact that its PCR product was generated at higher dilutions of cDNA from resveratrol-treated worms compared to untreated in both wild-type (Figure 3A, lane 7 versus lane 1) and *daf-16* samples (Figure 3B, lane 6 versus lane 1). *act-1* (actin) served as a control for equivalency of input cDNA in these PCR reactions. The RT-PCR assay also confirmed an increase in *abu-11* RNA in the resveratrol-treated worms (Figure 3A, lane 7 and Figure 3B, lane 6) compared to untreated worms (Figure 3A, lane 2 and Figure 3B, lane 1), but the effect was not as strong as for *cyp-13A6*, consistent with the array data.

To test more definitively whether resveratrol induced *abu-11* RNA, Northern analysis was performed on total RNA isolated from two independent sets of samples of wild-type (Figure 3C) and *daf-16* (Figure 3D) worms grown in the presence or absence of resveratrol. In these blots probed with *abu-11*-specific probes, transcripts were 3- to 4-fold higher in total RNA isolated from resveratrol-treated wild-type (Figure 3C) and *daf-16* (Figure 3D) worms relative to control, untreated samples. Total RNA samples for Northern analysis were verified for equal loading by hybridization to *act-1* (actin) probe (Figure 3C) and visualization of 28s and 18s rRNA by ethidium bromide staining (Figure 3D).

Life Span Extension by Resveratrol Treatment Is Dependent upon the ER Stress Gene *abu-11*

We hypothesized that the *pqn*/*abu* family of genes induced by resveratrol and by ER stress could be mediators of life span extension by resveratrol. Seven ER stress genes, *pqn-76*, *pqn-78*, *abu-2*, *pqn-91*, *abu-5*, *abu-11*, and *pqn-54*, upregulated in both wild-type and

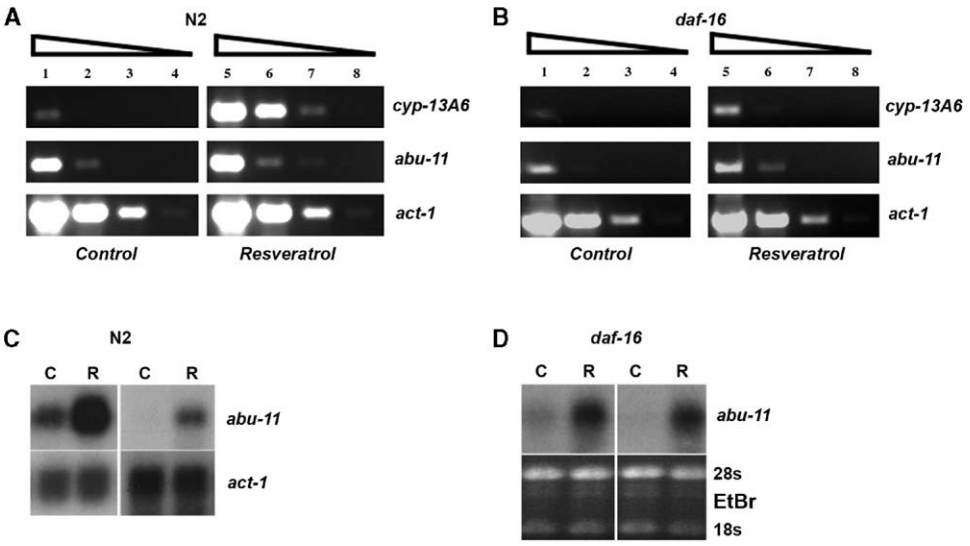

Figure 3. Resveratrol Treatment Induces *abu-11* Transcription

(A–D) Synchronized wild-type and GR1307 *daf-16*(*mgDf50*) L1-stage worms were grown in the absence (C) or presence (R) of 1000 μM resveratrol to young adults. Purified total RNA was isolated and used for (A and B) RT-PCR or (C and D) Northern analysis to detect *cyp-13A6*, *abu-11*, and *act-1* (actin) transcripts. RT-PCR products were run on agarose gels stained with ethidium bromide. PCR products in (A) and (B), lanes 1–4 and 5–8, are generated from stepwise 10-fold serially diluted cDNA by using gene-specific primers. Northern gels were prepared with 10 μg total RNA from two independent worm preparations, transferred to membranes, and blotted with gene-specific radioactive probes for *abu-11* and *act-1*. Ethidium bromide staining of agarose gel shows 28s and 18s rRNA samples as loading controls in (D).

daf-16 strains (Table 1), were chosen for functional analysis. Bacterial feeding strains that induce gene-specific RNAi were utilized to determine if any of the selected ER stress genes, when downregulated, could abolish resveratrol-mediated life span extension in the *daf-16* strain. Seven sets of RNAi life span assays were performed, including one set utilizing the control bacterial strain MV175, which contained vector alone.

Resveratrol treatment of *daf-16* worms on control RNAi bacteria caused a robust 23% extension in mean life span (Figure 4, all panels). Although RNAi of *pqn-76/78* (Figure 4A), *pqn-91* (Figure 4B), and *pqn-54* (Figure 4C) shortened life span in the absence of resveratrol, they still allowed for significant ($p < 0.0001$) life span extension by resveratrol. RNAi of *abu-11*, however, completely abolished resveratrol-mediated life span extension in both wild-type (Figure S2A) and *daf-16* (Figure 4D; Figure S2B) worms, while it shortened the life span in the absence of the compound. RNAi of *abu-2* (Figure 4E) and *abu-5* (Figure 4F) showed no effect on the life span of untreated worms, but *abu-5* RNAi partially blunted the extension by resveratrol. These findings show that *abu-11*, and to a lesser extent *abu-5*, are necessary for resveratrol to extend life span.

C. elegans sir-2.1 Regulates Expression of ER Stress Genes

Since resveratrol has been reported to be a Sir2 activator, we hypothesized that SIR-2.1 might be a positive transcriptional regulator of ER stress genes and is activated by resveratrol. By such a model, we expected to observe reduced transcription of these genes in *sir-2.1* mutants. Total RNA isolated from wild-type and *sir-2.1* mutant worms was analyzed by Northern analysis probing for *abu-11* transcript. Surprisingly, in *sir-2.1*(*ok434*) mutants, *abu-11* transcription was 15- to 20-fold higher relative to wild-type worms (Figure 5A), indicating that SIR-2.1 is a repressor of this gene. A similar increase was evident in the *pk1460::Tc1* insertion mutant of *sir-2.1* (data not shown). Furthermore, the elevated level of *abu-11* transcript in the *sir-2.1* mutant background remained unchanged with resveratrol treatment (Figure 5A). Northern probes were also designed to examine the transcript levels of other ER stress-related genes induced by resveratrol in the microarray. Similar to induction of *abu-11*, transcripts for *abu-7/8* and *pqn-5/6* were highly induced in *sir-2.1* mutant worms (Figure 5B). Transcripts for *pqn-76/77/78/79* (Figure 5C) and *abu-2* (Figure 5D) were also found to be elevated (ca. 2-fold) in the *sir-2.1* mutant background.

Although these data suggested that SIR-2.1 is a repressor of the *pqn* genes, it seemed possible that the mutant showed induction because of some secondary effect, for example, elevated endogenous stress in the ER. To test in a different way whether SIR-2.1 was a repressor of the *pqn* genes, we assessed whether overexpression would reduce transcript levels below those in a wild-type control. Microarray analysis of gene expression in the *sir-2.1* low-copy transgenic strain NL3909 relative to its control NL3908 (Table S1) indeed confirmed that the majority of *pqn/abu* genes induced by resveratrol are repressed below control levels by overexpression of SIR-2.1 (Table 1).

C. elegans abu-11 Modulates Life Span

If resveratrol extends life span by upregulating ER stress proteins, then increased expression of one or more of these proteins by transgenes per se may trigger a longer life span. To test this idea, we created transgenic animals containing multicopy *abu-11* ex-

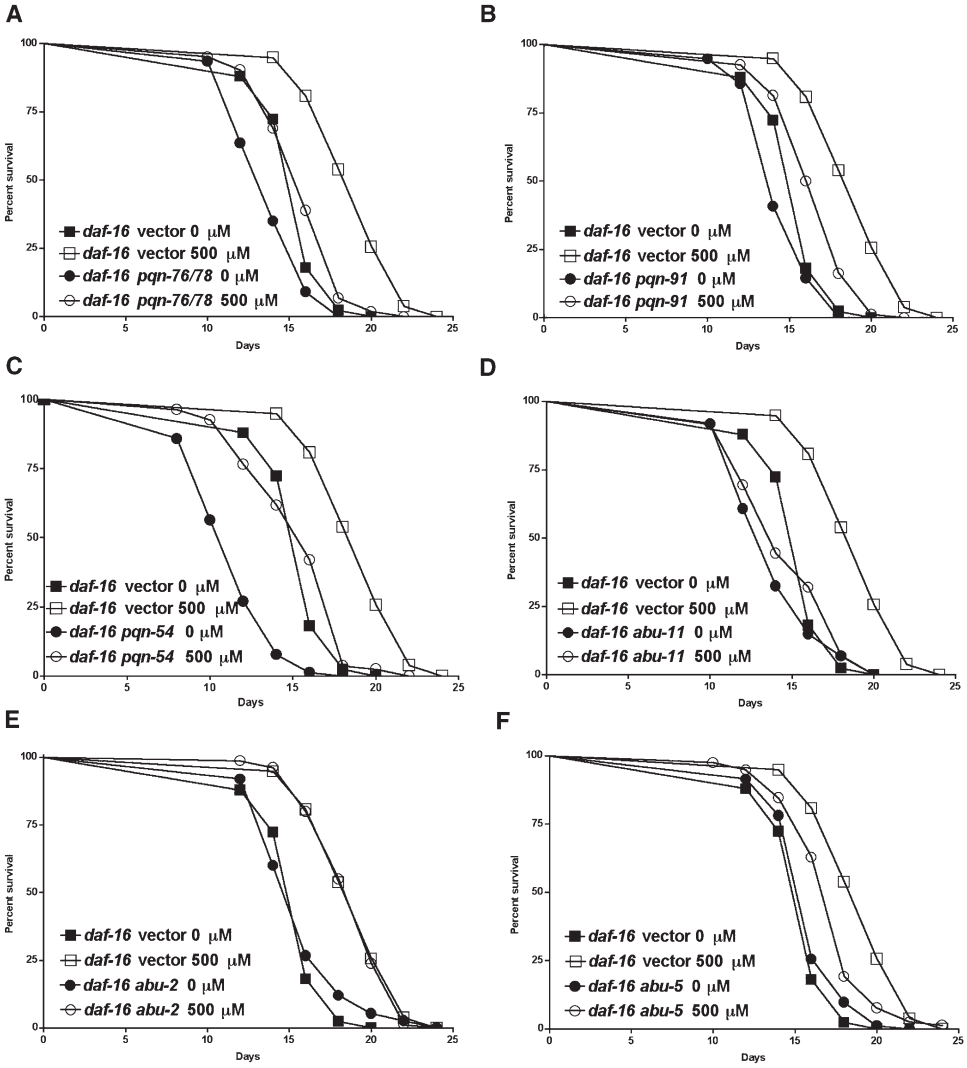

Figure 4. Life Span of *daf-16* Worms Treated with Resveratrol and Subjected to RNAi of Selected Prion-like Q/N-Rich Genes

(A–F) Survival curves of GR1307 *daf-16*(*mgDf50*) worms fed *E. coli* containing either vector L4440 or gene-specific dsRNA trigger-producing vectors. Life spans were performed on NGM + Ap + Tc media with (open symbol) and without (closed symbol) 500 M resveratrol. p values and percentage difference in life span are relative to untreated worms for each gene tested. (A–F) vector: 0 μM (m = 15.6, M = 20, n = 83); 500 μM (m = 19.2, M = 24, n = 78, p < 0.0001, 23.1%). (A) *pqn-76/78* RNAi: 0 μM (m = 14.0, M = 18, n = 77); 500 μM (m = 16.0, M = 22, n = 103, p < 0.0001, 14.3%). (B) *pqn-91* RNAi: 0 μM (m = 14.7, M = 18, n = 76); 500 μM (m = 16.8, M = 22, n = 80, p < 0.0001, 14.3%). (C) *pqn-54* RNAi: 0 μM (m = 11.6, M = 18, n = 78); 500 μM (m = 15.5, M = 22, n = 81, p < 0.0001, 33.6%). (D) *abu-11* RNAi: 0 μM (m = 14.1, M = 20, n = 74); 500 μM (m = 14.9, M = 20, n = 72, p = 0.100 [ns], 5.7%). (E) *abu-2* RNAi: 0 μM (m = 16.0, M = 24 n = 75); 500 μM (m = 19.1, M = 24, n = 80, p < 0.0001, 19.4%). (F) *abu-5* RNAi: 0 μM (m = 16.1, M = 22, n = 82); 500 μM (m = 17.4, M = 24, n = 78, p = 0.0015, 8.1%). Life span assays were performed once.

trachromasomal arrays to overexpress ABU-11. Wild-type worms were coinjected with plasmid pABU11 containing *abu-11* under its endogenous promoter, and pRF4 containing the dominant transformation marker *rol-6* (*su1006*). Six independently derived lines of worms that stably transmitted their arrays were isolated. Lines with the *rol-6* marker alone were used as control. *abu-11* transgenic animals were assayed for life span along with *rol-6* control worms (Figure 5E). All six lines of *abu-11* transgenic animals demonstrated significant extension of life span relative to *rol-6(geEx106)* control; mean life span extension ranged from 9% to 28%. Transgenic roller worms from each of the transformed lines showed an increased expression of *abu-11* transcript relative to control worms as determined by Northern analysis (Figure 5F). Moreover, a good correlation between the level of *abu-11* expression and life span extension was observed; lines *geEx109–111* with high levels of *abu-11* expression displayed longer life spans than the more moderately expressing lines *geEx102–104*.

Discussion

In this study, we have analyzed the role of resveratrol in extending the life span of *C. elegans* and demonstrated

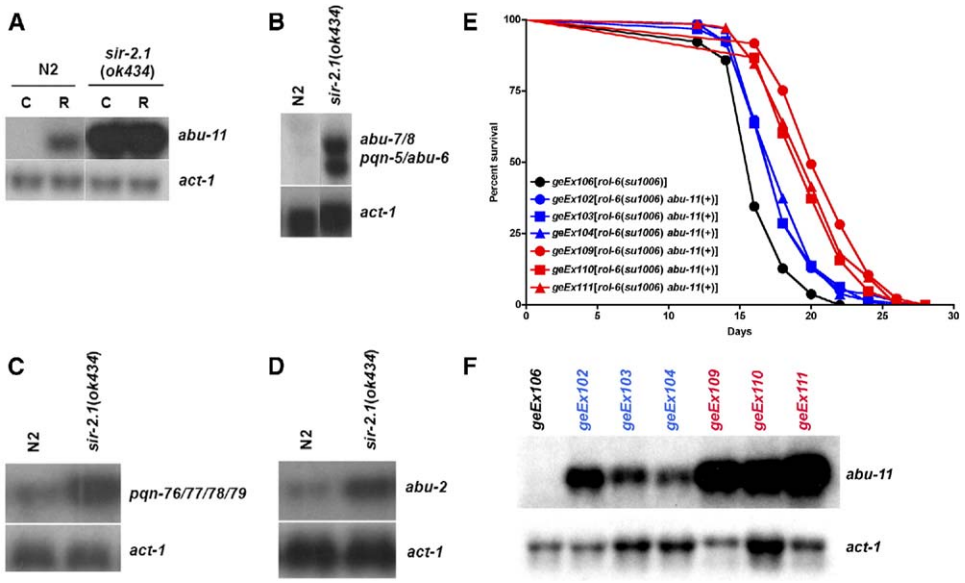

Figure 5. *sir-2.1* Negatively Regulates *abu*/*pqn* Gene Expression, and Overexpression of *abu-11* Extends Life Span

(A–D) Total RNA isolated from wild-type and LG231 *sir-2.1*(*ok434*) mutant worms were subjected to Northern analysis. Northern blots were hybridized with probes to detect levels of (A) *abu-11*, (B) *abu-7/8* and *pqn-5*/*abu-6*, (C) *pqn-76/77/78/79*, (D) *abu-2*, and (A–D) *act-1* transcripts. *pqn-76*, *pqn-77*, *pqn-78*, and *pqn-79* mRNA are 99.5% identical and therefore cannot be differentiated from one another with the probe utilized. *abu-7* and *abu-8* have 93.4% mRNA sequence identity, and *abu-6* and *pqn-5* are 98.3% identical; while the four transcripts hybridize with a common probe, the two sets can be distinguished based on size difference.

(E) Survival curves of six independently derived *abu-11* extrachromosomal array lines (*geEx102–104* and *geEx109–111*). LG263 *geEx106*, containing an array of the *rol-6*(*su1006*) marker alone, was used as control. LG263 *geEx106* (m = 16.6, M = 22, n = 78), LG259 *geEx102* (m = 18.2, M = 28, n = 77, p = 0.0002, 9.6%), LG260 *geEx103* (m = 18.1, M = 26, n = 94, p < 0.0001, 9.0%), LG261 *geEx104* (m = 18.3, M = 28, n = 81, p < 0.0001, 10.2%), LG271 *geEx109* (m = 21.1, M = 28, n = 85, p < 0.0001, 27.7%), LG272 *geEx110* (m = 20.1, M = 28, n = 83, p < 0.0001, 21.1%), LG273 *geEx111* (m = 20.3, M = 26, n = 72, p < 0.0001, 22.9%). Wild-type worms (not shown) had life spans similar (m = 16.4, M = 22, n = 90, p = 0.890 [ns]) to LG263. p values and percentage change in mean are relative to LG263 *geEx106*. Life span assays were performed twice with similar results.

(F) Total RNA isolated from transgenic (roller) worms from the six lines (*geEx102–104* and *geEx109–111*) of *abu-11* transgenic animals and their respective control (*geEx106*) were probed to detect *abu-11* and *act-1* transcript levels by Northern analysis.

induction of a set of genes previously shown to be involved in ER stress (Urano et al., 2002). These genes are also regulated by the *C. elegans* ortholog of Sir2, *sir-2.1* (see below). Previous work (Tissenbaum and Guarente, 2001) and experiments reported here show that extension of life span by increasing the *sir-2.1* gene dosage requires the forkhead transcription factor DAF-16, a critical component of the insulin/IGF signaling pathway. However, the roles of *sir-2.1* and resveratrol in the ER stress pathway can be separated genetically from the insulin/IGF pathway by virtue of the fact that extension of life span by resveratrol, while dependent upon *sir-2.1*, is completely independent of *daf-16*. Our findings provide a possible link between ER homeostasis and life span, and they show that this pathway is regulated by *sir-2.1* and resveratrol.

Our findings contrast with the recent report that resveratrol reduced poly-glutamine cytotoxicity in *C. elegans* in a manner dependent on *both sir-2.1* and *daf-16* (Parker et al., 2005). The poly-glutamine-related toxicity noted in this study likely occurs in the nucleus and cytoplasm, and it implicates a *sir-2.1* activity that functions through the insulin/IGF pathway. It is interesting to note that the longevity induced by resveratrol may not relate to damage control in the nucleus or cytoplasm, but rather to stress response in the ER.

Resveratrol Induces a Longevity Pathway Involving Putative ER Stress Proteins

Comparative analysis of microarray expression profiles revealed that resveratrol treatment of wild-type and short-lived *daf-16* strains significantly induced many members of the large *C. elegans pqn* gene family that encode prion-like Q/N-rich domain-bearing proteins (Michelitsch and Weissman, 2000). Among the *pqn* genes induced by resveratrol, a subset, renamed *abu* for activated in blocked unfolded protein response, is also induced by the ER stress compound tunicamycin, an inhibitor of ER protein glycosylation (Urano et al., 2002). This induction is manifest in strains in which the canonical unfolded protein response (UPR) pathway is disabled. Most members of the PQN/ABU family found induced by resveratrol bear sequence hallmarks of ER proteins (Table 1). The *pqn* genes may respond to a particular kind of ER stress or may serve as a backup to the UPR.

Functional studies also imply that ABU proteins function in parallel with the UPR pathway. The UPR pathway is regulated by XBP-1 (X-box binding protein), an ER stress-responsive transcription factor that activates many UPR-specific genes, including the ER chaperone BiP (*hsp-4*), protein disulfide isomerases, and proteins involved in the ER-associated protein degradation

pathway (Harding et al., 2002; Ma and Hendershot, 2002; Schroder and Kaufman, 2005). Inactivation of *abu-1* by RNAi slightly decreases the survival of wild-type worms and is associated with the accumulation of misfolded proteins in the ER in certain genetic backgrounds (Urano et al., 2002). Moreover, RNAi of *abu-1* substantially decreases the survival of *xbp-1* mutant worms when treated with cadmium, an ER stressor (Urano et al., 2002). The synthetic effect of inactivating *abu-1* and *xbp-1* suggests that the *abu* genes and the genes controlled by *xbp-1* are in parallel pathways regulating ER stress. These two pathways apparently interact, because *abu-1* RNAi also induces BiP expression, and this induction requires *xbp-1* (Urano et al., 2002).

Regulation of *pqn/abu* Genes by *sir-2.1* and Resveratrol

Is the induction of *pqn* genes the mechanism by which resveratrol extends life span in *C. elegans*? RNAi of *abu-5* partially reduced resveratrol-mediated life span extension, and RNAi of *abu-11* fully abolished this effect in both wild-type and *daf-16* mutant backgrounds (Figure 4D and Figure S2). Moreover, overexpression of *abu-11* in transgenic worms extended their life span in a dosage-dependent manner (Figure 5E). These findings indicate that induction of ABU proteins is an essential part of the mechanism by which life span is extended by resveratrol. In agreement with the microarray data, *abu-11* is transcriptionally induced after resveratrol treatment, as determined by Northern and RT-PCR analyses (Figure 3). Our findings suggest that ER stress during aging may limit nematode life span and that ER stress genes are longevity determinants in *C. elegans*.

Given that resveratrol has been reported to activate Sir2 deacetylases and loss of *sir-2.1* or *abu-11* each abolished the effect of resveratrol on life span, we first reasoned that SIR-2.1 must be a positive regulator of the *abu* genes. We were surprised to find that, in fact, the opposite is the case: expression of *abu-11* and several other *pqn/abu* family members is actually much higher in *sir-2.1* mutant animals. These findings suggested that SIR-2.1 is a repressor of *pqn* genes. Moreover, the fact that worms overexpressing *sir-2.1* repress basal expression of many *pqn/abu* genes further demonstrates that *sir-2.1* is a transcriptional repressor of this gene family.

It seems curious that *sir-2.1* mutants that have high levels of *pqn* gene expression actually display a short life span. One possible explanation is that *sir-2.1* has at least one other function, in addition to regulating the insulin/IGF pathway and *pqn* genes, that is required for long life. Alternatively, highly elevated expression of multiple *pqn* family genes, caused by loss of sir-2.1 function, may be detrimental to life span. If this were the case, then setting optimum levels of expression of these genes may be critical to having beneficial effects on life span.

How Does Resveratrol Induce *pqn/abu* Genes?

Given that SIR-2.1 is a repressor of *pqn/abu* genes, the induction of these genes by resveratrol is surprising, because this compound has been reported to increase the catalytic activity of Sir2 proteins (Howitz et al., 2003). Resveratrol instead appears to inhibit SIR-2.1 activity with respect to *pqn/abu* gene regulation. It may appear that resveratrol indirectly induces the ER stress genes, for example, by creating a higher level of cellular stress. However, the genes induced by resveratrol do not evince a broad induction of stress response genes. A Venn diagram comparing genes induced by resveratrol to those induced by heat shock, oxidative stress, or ethanol stress treatments illustrates the complete lack of overlap between resveratrol-induced genes and genes induced by these other cellular stressors (Figure 6A). Also, resveratrol does not appear to generate broad stress in the ER, since it does not induce canonical UPR pathway genes such as *xbp-1*, *hsp-4*, *sel-1*, and *pdi-1* (Table S1) as other ER stress agents do (Urano et al., 2002).

Nevertheless, there is a substantial overlap between resveratrol-induced genes and genes induced by tunicamycin in worms lacking the UPR (Figure 6A), suggesting a more specific mechanism of activation of these ER stress genes by resveratrol. We suspect that resveratrol does act directly on SIR-2.1, but not as an activator of the catalytic activity of the protein. Rather, resveratrol may bind to Sir2 proteins and exert unpredictable effects on Sir2 activity. In *C. elegans*, resveratrol inhibits SIR-2.1 activity with respect to *pqn/abu* genes, but it must not inhibit SIR-2.1 in the insulin/IGF pathway, since it augments the life-extending effect of overexpressed SIR-2.1. By binding to Sir2 proteins, resveratrol may change their affinity for substrate proteins, potentially increasing, reducing, or having no effect, depending on the specific Sir2-substrate protein interaction. This would explain how resveratrol could exert substrate-specific activities in vitro (Borra et al., 2005; Kaeberlein et al., 2005) and have differential affects on different pathways in *C. elegans*.

Conclusions

Our findings identify a family of putative ER stress response proteins as positive determinants of life span in *C. elegans*, thereby implicating unfolded proteins in the ER in normal aging. These genes are regulated by the *Sir2* ortholog *sir-2.1*. Resveratrol activates these genes to extend life span by inhibiting the repressive activity of *sir-2.1*. Since *sir-2.1* also functions independently to modulate life span via the insulin/IGF pathway, this sirtuin sits astride multiple pathways that influence life span by different mechanisms (Figure 6B).

Experimental Procedures

Chemicals

Ampicillin (Ap), Tetracycline (Tc), Isopropyl β-D-thiogalactopyranoside (ITPG), 5-Fluorodeoxyuridine (FUdR), and Dimethylsulfoxide (DMSO) were utilized at final concentrations of 100 μg/ml, 15 μg/ml, 1 mM, 12.5 μg/ml, and 2%, respectively. Resveratrol (Sigma; St. Louis, MO) was dissolved in ethanol at a concentration of 50 mg/ml and was stored at −20°C.

Media and Strains

Standard nematode growth medium (NGM) (Brenner, 1974) seeded with *E. coli* OP50 (Brenner, 1974) was used for *C. elegans* propagation at 20°C. Growth of worms for RNA isolation was done on NGM

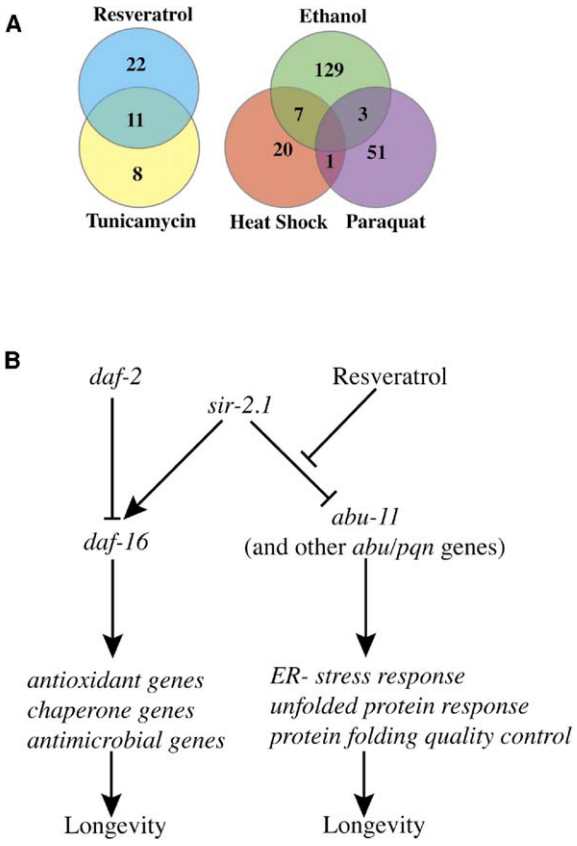

Figure 6. Resveratrol Extends *C. elegans* Life Span by Inducing ER Stress Response Genes

(A) Venn diagram illustrating the overlap between *C. elegans* genes induced by resveratrol and those induced by various stress treatments. Included are genes induced by resveratrol treatment of wild-type worms (mean $\log_2$-fold difference > 1.5, $p < 0.05$), tunicamycin treatment of the UPR-deficient *xbp-1* mutant strain (Urano et al., 2002), paraquat treatment of *fer-15* animals (mean $\log_2$-fold difference > 1.5, $p < 0.001$ [one-way ANOVA], M. Jiang and S. Kim unpublished results), ethanol treatment of wild-type worms (Kwon et al., 2004), and heat shock of wild-type worms (GuhaThakurta et al., 2002). A list of the overlapping genes can be found in Table S3. (B) *C. elegans sir-2.1* is involved in at least two different pathways that regulate nematode life span. In the first, SIR-2.1 acts via DAF-16, leading to the activation of DAF-16-responsive genes. In a separate pathway, *sir-2.1* acts to repress the expression of *abu-11* and a number of other *pqn* family genes believed to be involved in regulating protein folding within the endoplasmic reticulum. Resveratrol evidently acts to inhibit SIR-2.1, leading to derepression of *abu-11* and other *abu/pqn* genes, which potentially results in a heightened level of protein folding surveillance within the ER that, in turn, ultimately increases the life span of the animal.

media containing 2.5 g/l yeast extract and 20 g/l agar. Resveratrol-containing media was supplemented with DMSO. Resveratrol-containing media without DMSO failed to show any substantial life span extension, suggesting that solubility in DMSO is necessary for drug efficacy (data not shown). Life span assays were performed in the presence of FUdR. FUdR and DMSO were found to have no impact on life span at the utilized concentrations.

RNAi was performed by feeding worms bacteria expressing gene-specific double-stranded RNA (dsRNA) (Timmons et al., 2001). Bacteria used for RNAi were grown in LB + Ap + Tc liquid media at 37°C to OD_{600} = 0.4 and were induced with IPTG for 4 hr. Five-fold concentrated cultures were spotted onto NGM + Ap + Tc + IPTG + FUdR plate media. Bacteria producing dsRNA triggers for *pqn-78*, *abu-2*, *pqn-91*, *abu-5*, *abu-11*, and *pqn-54* were obtained from J. Ahringer's (Cambridge, United Kingdom) bacterial RNA interference (RNAi) library. RNAi-inducing bacterial strains are *E. coli* HT115 (λDE3 F⁻ *mcrA mcrB* IN[*rrnD-rrnE*]*1 rnc14*::*Tn10*) transformants of vector L4440 containing gene-specific RNAi trigger sequences (Kamath et al., 2003). The control bacterial feeding strain, MV175, is a L4440 transformant of HT115. Due to DNA sequence similarity amongst some *pqn* genes (i.e., *pqn-76/78, pqn-90 /91*), there is the possibility for RNAi of *pqn* genes other than the ones targeted. The genomic sequence used for *abu-11* RNAi, however, does not share sufficient sequence similarity to any other *C. elegans* gene so as to cause nonspecific interference. Northern analysis of total RNA from *sir-2.1* mutant animals fed either control or *abu-11* RNAi confirmed that *abu-11* RNAi fully attenuates expression of the *abu-11* transcript (data not shown). Sequences used for RNAi are available on WormBase (http://www.wormbase.org).

C. elegans wild-type strain N2 (var. Bristol) and GR1307 *daf-16*(*mgDf50*)I were obtained from the *Caenorhabditis elegans* Genetics Center. Construction of strains NL4251 *sir-2.1*(*pk1640::Tc1*)IV, LG231 *sir-2.1*(*ok434*)IV, LG267 *sir-2.1*(*pk1640::Tc1*)IV; *daf-16* (*mgDf50*)I, NL3908 *unc-119*(*dp38*)III *pkIs1641*(*unc-119*[+]), NL3909 *unc-119*(*dp38*)III *pkIs1642*(*unc-119*[+] *sir-2.1*[+]), and LG248 *unc-119*(*dp38*)III *pkIs1642*(*unc-119*[+] *sir-2.1*[+]); *daf-16*(*mgDf50*) will be described in detail elsewhere. NL4251 *sir-2.1*(*pk1640::Tc1*)IV and LG231 *sir-2.1*(*ok434*)IV were derived from progenitor strains NL4250 *mut-7*(*pk204*)III; *sir-2.1*(*pk1640::Tc1*)IV (Van der Linden and Plasterk, 2004) and VC199 *sir-2.1*(*ok434*)IV (from *C. elegans* gene knockout consortium), respectively, following six outcrosses with N2. Strain NL3908 contains a low-copy integrant of plasmid pRP2510 (*unc-119*[+]), used as a cotransformation rescue marker during bombardment transformations. LG259-261 *geEx102–104* and LG271-273 *geEx109–111* are independent isolates of the genotype (pRF4*rol-6*[*su1006*] + pABU11*abu-11*[+]) obtained by coinjection of plasmid pABU11(50 μg/ml) (see below) with pRF4 (50 μg/ml) (Mello et al., 1991) into wild-type worms. pRF4 allows for dominant roller selection of transgenic worms. LG263 *geEx106* (*rol-6* [*su1006*]) was obtained by injection of pRF4 (50 μg/ml) alone. In all cases, F1 roller worms were plated individually, and their broods were scored for transmission of the transgenic array to their progeny. pABU11 contains a 5373 bp PCR-amplified (Expand Long Template PCR System; Roche, Indianapolis, IN) genomic product with endpoints 2274 bp upstream of the first *abu-11* exon and 1071 bp downstream of the last exon cloned into pCR4 (Invitrogen, Carlsbad, CA). Ten independent pABU-11 clones were combined for injection.

Life Spans

All life span assays were performed at 20°C, starting with L4-stage to young adult-stage worms. Animals were monitored every other day, and death was scored by failure to move after being prodded with a platinum wire. To obviate the need for censor, animals that either crawled off the plate or that died from vulval bursting were replaced by age-matched worms from spare plates. Worm replacement never exceeded 5% of the total number in any life span. Log-rank test for comparison of survival curves was performed with Graphpad Prism v4.0a. p values < 0.05 indicate statistically significant differences between tested populations. Unless otherwise stated, life span assays were repeated at least once and showed similar trends in relative life span effect; representative Kaplan-Meier survival curves are shown in the figures.

Microarray, Northern Analysis, RT-PCR

Four independent cultures of each strain to be analyzed by microarray were grown to produce gravid adults. Eggs were isolated by alkaline hypochlorite treatment (Emmons et al., 1979) and were allowed to hatch without food, accumulating as growth-arrested L1 larvae. Synchronized worms were then grown on modified NGM media in the presence or absence of resveratrol at 20°C until the young adult stage. Worms were harvested, frozen, and ground in a mortar before extraction of total RNA by using TRIzol reagent (Invitrogen). Poly(A)⁺ RNA used for cDNA synthesis was purified from total RNA by using the Oligotex mRNA kit (Qiagen; Valencia,

CA). Five micrograms of poly(A)$^+$ RNA was used for cDNA synthesis. cDNA synthesis, genome-wide microarray hybridization, and scanning were performed as previously described (Jiang et al., 2001). cDNA from control and experimental worms were labeled with Cy3-UTP and Cy5-UTP, respectively. The arrays were computer normalized by the default procedure in the Stanford Microarray Database (http://genome-www.stanford.edu/microarray). Normalized Cy5/Cy3 intensities and mean $\log_2$ Cy5/Cy3 for each gene in each set of experiments were calculated as described in Jiang et al. (2001). Student t distribution tests were applied independently to data sets of mean $\log_2$ Cy5/Cy3 values to determine the probability that differences in the hybridization intensity signals between control and experimental animals were due to chance alone. A listing of differentially regulated genes from the microarray hybridizations can be found in Table S1.

Total RNA for Northern analysis was extracted from young adult worms by using TRIzol reagent. Northern analysis of *abu-11* transgenic worms utilized total RNA isolated from hand-picked adult roller worms of each line tested. Northern gels and transfer to Nylon membranes was performed by using the Ambion NorthernMax kit. Membranes containing transferred RNA were UV crosslinked and probed overnight at 42°C. Membranes were washed with 0.2× SSC (30 mM NaCl, 3 mM Na$^+$Citrate [pH 7.0]) + 0.1% SDS solution three times for 20 min at 42°C and exposed to film. Radioincorporated probes were prepared with the Prime-It II (Stratagene) random priming kit. Gel-purified templates for random priming were synthesized by PCR utilizing primers listed in Table S2.

For RT-PCR, equal amounts of RNA (5 μg) for each sample tested were added to 1× reaction buffer, oligo-dT, and SuperScriptII reverse transcriptase (Invitrogen) to generate a cDNA template for PCR as per manufacturer's directions. Primers used for RT-PCR are listed in Table S2. cDNA samples were serially diluted stepwise four times in 10-fold increments, and each was used for 30 cycles of Taq PCR amplification (92°C 20 s, 55°C 20 s, 72°C 1 min). Equal amounts of each reaction were then analyzed on 1.5% agarose gels stained with ethidium bromide.

Supplemental Data

Supplemental Data including microarray data files, primer sequences, and protein sequence alignments are available at http://www.developmentalcell.com/cgi/content/full/9/5/605/DC1/.

Acknowledgments

We thank L. Bordone, S. Lin, and N. Bishop for critical reading of the manuscript; Min Jiang (Stanford University) for her help with microarray hybridization; and Ronald Plasterk (Hubrecht laboratory, University of Utrecht, The Netherlands), Alexander Van der Linden (Brandeis University), Theresa Sternagle, and the *C. elegans* Genetics Center (University of Minnesota) and the *C. elegans* gene knockout consortium (Oklahoma Medical Research Foundation) for providing strains. This work was supported by grants from the Jane Coffin Childs Foundation (to M.V.) and the National Institutes of Health (to L.G.).

Received: May 17, 2005
Revised: August 24, 2005
Accepted: September 28, 2005
Published: October 31, 2005

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