C. elegans SIRT6/7 Homolog SIR-2.4 Promotes DAF-16 Relocalization and Function during Stress

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation

Published Version
doi:10.1371/journal.pgen.1002948

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:10524339

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
C. elegans SIRT6/7 Homolog SIR-2.4 Promotes DAF-16 Relocalization and Function during Stress

Wei-Chung Chiang1,*, Daniel X. Tishkoff2*, Bo Yang2*, Joshua Wilson-Grady3, Xiaokun Yu4, Travis Mazer4, Mark Eckersdorff2*, Steven P. Gygi3, David B. Lombard2,5, Ao-Lin Hsu1,4,5

1 Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, Michigan, United States of America, 2 Department of Pathology, University of Michigan, Ann Arbor, Michigan, United States of America, 3 Department of Cell Biology, Harvard Medical School, Boston, Massachusetts, United States of America, 4 Department of Internal Medicine, Division of Geriatric Medicine, University of Michigan, Ann Arbor, Michigan, United States of America, 5 Institute of Gerontology and the Geriatrics Center, University of Michigan, Ann Arbor, Michigan, United States of America

Abstract

FoxO transcription factors and sirtuin family deacetylases regulate diverse biological processes, including stress responses and longevity. Here we show that the Caenorhabditis elegans sirtuin SIR-2.4—homolog of mammalian SIRT6 and SIRT7 proteins—promotes DAF-16-dependent transcription and stress-induced DAF-16 nuclear localization. SIR-2.4 is required for resistance to multiple stressors: heat shock, oxidative insult, and proteotoxicity. By contrast, SIR-2.4 is largely dispensable for DAF-16 nuclear localization and function in response to reduced insulin/IGF-1-like signaling. Although acetylation is known to regulate localization and activity of mammalian FoxO proteins, this modification has not been previously described on DAF-16. We find that DAF-16 is hyperacetylated in sir-2.4 mutants. Conversely, DAF-16 is hypoacetylated by the acetyltransferase CBP-1, and DAF-16 is hypoacetylated and constitutively nuclear in response to cbp-1 inhibition. Surprisingly, a SIR-2.4 catalytic mutant efficiently rescues the DAF-16 localization defect in sir-2.4 nulls. Acetylation of DAF-16 by CBP-1 in vitro is inhibited by either wild-type or mutant SIR-2.4, suggesting that SIR-2.4 regulates DAF-16 acetylation indirectly, by preventing CBP-1-mediated acetylation under stress conditions. Taken together, our results identify SIR-2.4 as a critical regulator of DAF-16 specifically in the context of stress responses. Furthermore, they reveal a novel role for acetylation, modulated by the antagonistic activities of CBP-1 and SIR-2.4, in modulating DAF-16 localization and function.

Introduction

Elucidation of mechanisms regulating stress resistance and longevity has been aided tremendously by the use of invertebrate models. FoxO transcription factors regulate multiple biological processes in many organisms [1]. In C. elegans and Drosophila, increased FoxO activity promotes longevity, fat storage, and stress resistance [2]. Mammals possess four FoxO homologs, with partially redundant and distinct functions: FoxO1, FoxO3A, FoxO4, and FoxO6 [1]. These proteins regulate apoptosis, cell cycle arrest, oxidative defense, DNA repair, metabolism, differentiation, stem cell function, and tumor suppression in a cell type- and context-specific manner [1].

FoxO activity is tightly controlled, and subcellular localization is a principal mechanism of FoxO regulation [3]. In this context, insulin/IGF-1-like signaling (IIS) is the major influence on FoxO function. IIS leads to FoxO phosphorylation and cytoplasmic segregation in a complex with 14-3-3 chaperone proteins. Conversely, stress stimuli promote nuclear translocation of FoxO proteins by multiple mechanisms, including activation of stress kinases that modify FoxO proteins on residues distinct from those phosphorylated in IIS [2]. In response to oxidative insult, mammalian FoxO proteins are acetylated [4,5,6,7], mono-ubiquitylated [8], and phosphorylated [9,10,11,12]. Overall, these post-translational modifications function as a “FoxO code”, providing a means by which FoxO activity is finely regulated in response to various stimuli to promote altered metabolism, stress responses, or cell death [3].

The sirtuins are an evolutionarily conserved protein family impacting many biological processes, including longevity, stress responses, metabolism, and cancer [13]. Sirtuins modify target proteins by means of their NAD+-dependent lysine deacetylase and ADP-ribosyltransferase activities. Lysine acetylation has emerged as a post-translational modification with a key role in modulating protein function, akin to phosphorylation [14]. Mammals possess seven sirtuins, SIRT1–SIRT7. The C. elegans genome encodes four sirtuins, SIR-2.1 through SIR-2.4, corresponding to mammalian SIRT1 (SIR-2.1), SIRT4 (SIR-2.2 and SIR-2.3), and SIRT6/7 (SIR-2.4) [15]. SIR-2.1 has been
Sensing and responding appropriately to environmental insults is a challenge facing all organisms. In the roundworm *C. elegans*, the FoxO protein DAF-16 moves to the nucleus in response to stress, where it regulates gene expression and plays a key role in ensuring organismal survival. In this manuscript, we characterize SIR-2.4 as a novel factor that promotes DAF-16 function during stress. SIR-2.4 is a member of a family of proteins called sirtuins, some of which promote increased lifespan in model organisms. Worms lacking SIR-2.4 show impaired DAF-16 nuclear recruitment, DAF-16-dependent gene expression, and survival in response to a variety of stressors. SIR-2.4 regulates DAF-16 by indirectly affecting levels of a modification called acetylation on DAF-16. Overall, our work has revealed SIR-2.4 to be a key new factor in stress resistance and DAF-16 regulation in *C. elegans*. Future studies will address whether mammalian SIR-2.4 homologs SIRT6 and SIRT7 act similarly towards mammalian FoxO proteins.

**Results**

SIR-2.4 is required for efficient stress-induced DAF-16 nuclear localization

Mammalian SIRT6 and SIRT7 proteins both promote genotoxic stress resistance [38,39]. We therefore tested a potential role for SIR-2.4 in stress resistance and DAF-16 regulation. We generated an RNAi construct encoding nucleotides 1–467 of the *SIR-2.4* open reading frame in the RNAi vector L4440. *sir-2.4* knockdown (KD) resulted in no obvious defects under basal conditions. However, *sir-2.4* RNAi severely impaired stress-induced DAF-16 nuclear localization (Figure 1A). *sir-2.4* RNAi inhibited DAF-16 nuclear translocation in response to either heat shock or oxidative insult by ~50% shortly after stress induction (Figure 1B). At later timepoints, DAF-16 did translocate to the nucleus in *sir-2.4* KD worms (see below and data not shown).

*sir-2.4* deletion is reported to confer lethality/sterility (National Bioresource Project, Japan). However, during the course of analyzing the effects of *sir-2.4* RNAi, we obtained a viable strain with a deletion removing all but the initial 9 amino acids of the *SIR-2.4* open reading frame (kind gift of H.R. Horvitz; see Materials and Methods section for a complete description of this strain). As with *sir-2.4* RNAi, *sir-2.4* KO animals showed no apparent defects under unstressed conditions. However, like *sir-2.4* KD worms, *sir-2.4* knockouts (KOs) showed significantly delayed stress-induced DAF-16 nuclear translocation in response to oxidative stress (Figure 1C; *p*<0.001 by Poisson regression analysis) and heat shock (Figure 1D; *p*<0.001). We conclude that SIR-2.4 is dispensable for viability and fertility, but plays a crucial role in directing DAF-16 to the nucleus in response to stress, particularly at early time points following stress induction.

It has been reported that SIR-2.1 and 14-3-3 proteins act in concert to activate DAF-16 [21,26]. To examine whether SIR-2.1 plays a role in directing DAF-16 to the nucleus in response to stress, we assessed the effect of *sir-2.1* KD on DAF-16 nuclear localization. *sir-2.1* KD alone had little impact on DAF-16 nuclear recruitment in response to either oxidative insult (Figure 1C; *p*<0.72) or heat stress (Figure 1D; *p*<0.44), indicating that SIR-2.1 does not play a major role in stress-induced DAF-16 nuclear localization, consistent with published data [17]. Moreover, KD of *sir-2.1* in the context of *sir-2.4* mutation did not produce any statistically significant additional delay in DAF-16 nuclear recruitment versus *sir-2.4* KO alone (Figure 1C; *p*<0.89 and *p*<0.13 for oxidative and heat stress, respectively). We conclude that SIR-2.4, but not SIR-2.1, plays a major role in promoting rapid nuclear recruitment of DAF-16 in response to oxidative stress or heat shock. These results imply that SIR-2.1 and SIR-2.4 act in distinct pathways to influence DAF-16 functions. While stress-induced subcellular translocation of DAF-16 was affected in all cell types by *sir-2.4* inhibition, we did note that *sir-2.4* KD and KO had a bigger impact in head hypodermis cells than in intestinal cells (data not shown). SIR-2.4 was very weakly expressed in most cell types, but showed much stronger expression in a subset of head and tail neurons as well as in a subset of somatic gonadal cells (Figure S1). We have not yet formally assessed the tissue requirements for SIR-2.4 function in the regulation of DAF-16 localization, though our functional data suggest a cell autonomous mechanism (see below).

SIR-2.4 promotes DAF-16–dependent transcription under basal and stress conditions

DAF-16 carries out its functions by transcriptional regulation of a large number of target genes [40,41,42]. The role of SIR-2.4 in DAF-16-dependent gene expression was tested in the context of six well-known DAF-16 targets. We confirmed the published role of DAF-16 in regulating expression of all six of these genes (Figure S2A). Under both basal and stress conditions, *sir-2.4* RNAi led to decreased mRNA levels of three genes positively regulated by DAF-16 (*SOD-3*, *HSP-16.1*, and *DOD-3*). (Figure 2, top row).
Figure 1. **SIR-2.4, but not SIR-2.1, is required for stress-induced DAF-16 nuclear localization.** TJ356 animals carrying an integrated daf-16::gfp array were fed either vector control or sir-2.4 RNAi bacteria for at least one generation before being subjected to heat-shock or oxidative stress. (A) Images of TJ356 animals grown on control or sir-2.4 RNAi bacteria after 15 min heat-shock. (B) Quantification of DAF-16::GFP nuclear localization. (C) Time-course analysis of DAF-16 nuclear localization in response to H$_2$O$_2$. (D) Time-course analysis of DAF-16 nuclear localization in response to heat shock.
Expression of these DAF-16 targets was similarly attenuated in the sir-2.4 KO strain (Figure S2B). Conversely, expression of three genes negatively regulated by DAF-16 was greatly increased by sir-2.4 RNAi (DOD-24, C32H11.4, and INS-7) (Figure 2, bottom row). We conclude that SIR-2.4 promotes DAF-16 transcriptional function under both basal and oxidative stress conditions.

**SIR-2.4 is required for normal stress resistance**

DAF-16 is a key regulator of stress responses in *C. elegans* [43]. We therefore tested the impact of SIR-2.4 on stress resistance. sir-2.4 KO and sir-2.4 KD worms were hypersensitive to heat shock (Figure 3A, Table S1) and oxidative insult (Figure 3B, Table S1). Simultaneous inhibition of both sir-2.4 and daf-16 increased stress sensitivity to a similar extent as observed in daf-16 single mutants, and the degree of hypersensitivity conferred by either single KO/KD alone was similar (Figure 3A-3B, Figure S2C-S2D), suggesting that SIR-2.4 and DAF-16 modulate stress resistance via a common pathway. Conversely, overexpression of SIR-2.4 did not produce increased stress resistance (Figure S3A-S3B; Table S1); hence SIR-2.4 levels are not limiting for DAF-16 regulation and stress resistance.

Expression of fluorescently tagged polyglutamine (polyQ) repeat-containing proteins in *C. elegans* body wall muscle causes paralysis that is antagonized by DAF-16 [44,45], a model for proteotoxicity occurring in human neurodegenerative diseases such as Huntington’s disease. The role of SIR-2.4 in proteotoxicity resistance was assessed. Worms expressing 35 glutamine residues conjugated to YFP in body wall muscle (*unc-54p::Q35::YFP*) were accumulation in response to heat-shock (35°C for 15 min.) or oxidative stress (1.5 mM H2O2 for 1 hr). Worms were scored for the presence or absence of GFP accumulation within the intestinal nuclei (n=120 or greater for all treatments). An animal was scored as having nuclear GFP if one or more intestinal nuclei contained DAF-16-GFP. (C–D) Time course analysis of DAF-16::GFP nuclear accumulation in response to stress. TJ356 or EQ200 [sir-2.4(n5137); daf-16::gfp] animals grown on either control or sir-2.1 RNAi bacteria were subjected to (C) heat-shock (35°C) or (D) oxidative stress (1.5 mM H2O2). Worms were scored for GFP accumulation within the head hypodermic nuclei at day 1 of adulthood (n=30–50) every 5–30 min.

doi:10.1371/journal.pgen.1002948.g001

**Figure 2. SIR-2.4 is required for optimal DAF-16–dependent gene expression.** Wild-type N2 animals fed on either vector control or sir-2.4 RNAi bacteria from the time of hatching were exposed to 10 mM H2O2 for 80 min. Relative mRNA levels of SOD-3, HSP-16.1, DOD-3, DOD-24, C32H11.4, and INS-7 were measured by quantitative RT-PCR and the means of three different sample sets are shown. Relative mRNA levels were normalized against ACT-1 (beta-actin). Error bars: ± STD. Statistical significance as determined by two-tailed t-test is shown in the table below; significant differences are represented in black font.

doi:10.1371/journal.pgen.1002948.g002
grown on daf-2 RNAi, daf-16 RNAi, sir-2.4 RNAi, or control bacteria. daf-2 RNAi delayed, and both daf-16 and sir-2.4 RNAi accelerated, the onset of Q35::YFP-induced paralysis (Figure 3C). Thus, like DAF-16, SIR-2.4 is required for resistance to multiple stressors, including proteotoxic injury.

SIR-2.4 is largely dispensable for DAF-16 function in response to reduced IIS

Reduced IIS promotes DAF-16 nuclear localization and functions independently of exogenous stress. We performed several assays to determine whether SIR-2.4 regulates DAF-16 in the context of IIS. Many manipulations that reduce IIS increase lifespan in C. elegans. However, neither sir-2.4 RNAi nor SIR-2.4 overexpression affected the lifespan of wild-type worms (Figure 4A and Figure S3C, Table S2), nor did sir-2.4 RNAi suppress increased longevity of daf-2 (e1370) insulin/IGF-I-like receptor mutants (Figure 4A, Table S2), sir-2.4 deletion or sir-2.4 RNAi minimally impacted DAF-16 nuclear translocation induced by reduced IIS (Figure 4B and Figure S2E), in contrast to its potent impact on stress-induced DAF-16 relocation. Moreover, sir-2.4 RNAi only slightly impaired dauer formation, a process antagonized by IIS (Figure 4C). We conclude that the effects of SIR-2.4 on DAF-16 are largely independent of IIS, and are most functionally significant in the context of stress.

SIR-2.4 regulates DAF-16 acetylation and localization independently of its catalytic function

In mammals, SIRT1 and other sirtuins deacetylate FoxO proteins to promote stress responses and other processes [4,6,30,31,32,33,36]. Although DAF-16 interacts with the acetyltransferases CBP and p300 [46], acetylation of DAF-16 itself has never been demonstrated. Given our data linking SIR-2.4 to stress resistance and DAF-16 localization and function, we tested whether DAF-16 was acetylated, and whether SIR-2.4 might play a role in regulating levels of this modification. Indeed, we were able to detect DAF-16 acetylation in C. elegans, and levels of this modification were elevated in sir-2.4 KO worms (Figure 5A) or KD worms (Figure S4A). Moreover, reciprocal co-immunoprecipitation studies revealed that SIR-2.4 binds to DAF-16 in mammalian cells (Figure 5B), suggesting that SIR-2.4 might interact with DAF-16 to deacetylate this protein. To test this hypothesis, we performed in vitro deacetylation assay with purified SIR-2.4 proteins and pre-acetylated DAF-16 as substrates. However, in multiple experiments we were unable to detect significant deacetylation of DAF-16 by SIR-2.4 in vitro, despite efficiently deacetylating histones with mammalian SIRT1 and SIRT6 in parallel under identical conditions (data not shown).

Given this result, the requirement for SIR-2.4 catalytic function in DAF-16 localization was directly tested in worms. As shown above, sir-2.4 KO worms showed impaired DAF-16 nuclear localization in response to stress. This defect was rescued by transgenic expression of wild-type SIR-2.4 (Figure 5C). Curiously, expression of the SIR-2.4 N124A mutant, bearing a mutation at highly conserved residues predicted based on homology to greatly diminish catalytic function [47], restored DAF-16 relocalization as efficiently as wild-type SIR-2.4. Unfortunately, co-overexpression of DAF-16 together with chromosomally integrated SIR-2.4 caused worms to be very sick and produce few progeny, precluding a direct assessment of DAF-16 acetylation in rescued animals (data not shown). We conclude that SIR-2.4 regulates DAF-16 acetylation and localization independently of its catalytic activity.
potentially by preventing the action of an acetyltransferase on DAF-16 and/or recruiting another deacetylase.

Potential cooperativity between SIR-2.1 and SIR-2.4 with respect to regulation of DAF-16 acetylation was assessed (Figure S4B). As before, sir-2.4 KO caused a large increase in DAF-16 acetylation. Interestingly sir-2.1 RNAi caused a modest but detectable increase in DAF-16 acetylation. In sir-2.1 KD; sir-2.4 KO animals, levels of DAF-16 acetylation resembled those in the sir-2.4 KO. Thus, SIR-2.1 does impact DAF-16 acetylation, perhaps by directly deacetylating DAF-16, as has been shown for its homolog mammalian SIRT1. However, in contrast to SIR-2.4, the impact of SIR-2.1 on DAF-16 acetylation is quantitatively insufficient to promote significant DAF-16 nuclear localization (our results and [17]). Alternatively, it is possible that SIR-2.1 and SIR-2.4 modulate acetylation on different DAF-16 lysine residue that regulate divergent aspects of DAF-16 function.

SIR-2.4 blocks CBP1-dependent DAF-16 acetylation

Acetylation regulates of FoxO proteins in a context-specific manner [3]. To identify the enzyme that acetylates DAF-16 in vivo, five C. elegans acetyltransferases – CBP-1 (p300/CBP), PCAF-1 (p300/CBP-associated factor), MYS-1, MYS-2 and MYS-3 (MYST histone acetyltransferases) – were examined for potential impacts on nuclear translocation and acetylation of DAF-16. RNAi KD of cbp-1 induced dramatic, virtually complete DAF-16 nuclear translocation under basal, unstressed conditions (Figure 6A). In contrast, inhibition of pcaf-1 or simultaneous KD of all three MYST acetyltransferases had no effect on DAF-16 localization (data not shown). This effect of cbp-1 RNAi on DAF-16 nuclear localization was largely epistatic to the impact of sir-2.4 KO (Figure 6A). These results are consistent with a recent report showing that mammalian CBP promotes cytoplasmic localization of FoxO3A [48]. Consistent with a role for CBP-1 in acetylating DAF-16 in vivo, we found that DAF-16 is hypo-acetylated in cbp-1 KD animals (Figure 6B). To identify CBP-1 target sites on DAF-16, we performed mass spectrometry analysis on recombinant DAF-16 treated with CBP-1 in vitro (Figure 6C). We identified four CBP-1 dependent acetylation sites on DAF-16: K248, K253, K375 and K379 (Table S3). Together, these findings suggest that DAF-16 is acetylated by CBP-1 in vivo to promote its nuclear exclusion under basal conditions.

It has been reported that cbp-1 RNAi confers stress sensitivity and reduced lifespan [49]. We confirmed that cbp-1 RNAi caused hypersensitivity to heat shock (Figure S5A) and oxidative insult (Figure S5B). Moreover, as previously reported, cbp-1 RNAi caused worms to be very short-lived (Figure S5C). We conclude that the constitutive nuclear localization of DAF-16 occurring in the context of cbp-1 inhibition is inadequate to promote stress resistance or increased longevity, perhaps due to loss of cbp-1 acetylation of other key targets necessary for health and normal
lifespan, potentially in many different cell types. Consistent with this hypothesis, CBP-1 is widely expressed in most or all somatic cells of the worms [50,51,52].

The DEK protein binds histones to prevent p300- and PCAF-mediated histone acetylation [53]. To test whether SIR-2.4 might exert its effect on DAF-16 through a similar mechanism, we examined the effect of the presence of SIR-2.4 on CBP-mediated DAF-16 acetylation in vitro. Purified DAF-16 and CBP were incubated with or without SIR-2.4. The presence of SIR-2.4 blocked CBP-mediated acetylation of DAF-16 (Figure 6C). A similar result was also obtained when DAF-16 and CBP were incubated with the SIR-2.4 N124A catalytic mutant (Figure 6C). We conclude that SIR-2.4 inhibits CBP-mediated DAF-16 acetylation likely through protein-protein interaction, independent of deacetylase function (Figure 7).

**Discussion**

We have shown that the sirtuin SIR-2.4 plays a crucial role in promoting DAF-16 activity and stress resistance. In response to multiple forms of stress, SIR-2.4 promotes DAF-16 nuclear recruitment and function, and ultimately organismal survival. SIR-2.4 is also required for optimal DAF-16 deacetylation and transcriptional activity under basal, unstressed conditions. In contrast, SIR-2.4 does not greatly impact DAF-16 nuclear localization, dauer formation, or longevity due to reduced IIS. We find that SIR-2.4 associates with DAF-16 and negatively regulates DAF-16 acetylation by interfering with the ability of CBP-1 to acetylate DAF-16 under stress conditions. Although mammalian FoxO proteins are acetylated in a functionally significant manner [3], to our knowledge this is the first demonstration that this modification is conserved in *C. elegans*. These data suggest that deacetylation likely plays two distinct roles in modulating DAF-16 function, in promoting optimal DAF-16 transcriptional activity under both basal and stress conditions, and in promoting DAF-16 nuclear recruitment following stress. We propose a model in which SIR-2.4 does not interact with DAF-16 under unstressed conditions. This allows CBP-1 to acetylate DAF-16 and sequester DAF-16 in the cytosol. In response to stress, SIR-2.4 binds to DAF-16 to block CBP-1-dependent acetylation and...
promote DAF-16 nuclear translocation and gene expression (Figure 7).

There are several important issues raised by our results. First, our data suggest that deacetylation promotes DAF-16 nuclear localization and function in response to stress, but is largely dispensable in the context of reduced IIS, suggesting that regulation of this modification may be involved in adjusting DAF-16 activity in the face of different organismal requirements. Our mass spectrometry analysis has identified four acetylation sites on DAF-16 (Table S3). Notably, acetylation at one of these (K248), is also present at homologous lysine residues in FoxO1 (K262) and FoxO3A (K259) [4,5,45,56]. Future studies mutating these acetylation sites individually and in combinations will be required to identify specific residues involved in regulating DAF-16 nuclear recruitment and transcriptional activity.

Our results provide further evidence that multiple sirtuins regulate FoxO proteins by diverse mechanisms to achieve different functional outcomes. In mammals, several sirtuins directly deacetylate FoxO proteins [4,6,30,31,32,33,36]. In worms, SIR-2.1 promotes DAF-16 function to increase stress resistance via 14-3-3 proteins [26]. We find SIR-2.4 suppresses DAF-16 acetylation and is critical in the organismal response to a variety of stressors. Whereas SIR-2.4 is required for optimal DAF-16 nuclear recruitment in response to stress, SIR-2.1 is dispensable for this process (Figure 1C-1D) [17]. It has been reported that SIR1 promotes survival of cerebellar granule neurons in response to low extracellular potassium levels in a catalytic activity-independent fashion [57]. Thus, sirtuins may regulate stress responses through mechanisms other than direct covalent substrate modification. Consistent with this idea, we found that SIR-2.4 regulates DAF-16 function independent of its catalytic function, by preventing CBP-1-mediated DAF-16 acetylation. We note that SIR6 was recently shown to be capable of binding NAD⁺ and undergoing a conformational change in the absence of substrate; based on this finding, it has been speculated that SIR6 might act as an NAD⁺ metabolite sensor, independently of catalytic activity [58]. This hypothesis is consistent with our data regarding the SIR6 homolog SIR-2.4. Chromatin modifying complexes frequently involve multiple enzymes with apparently redundant biochemical activities, including multiple histone deacetylases; analogously SIR-2.4 likely associate with other deacetylases to regulate DAF-16. SIR-2.1 associates with DAF-16, although SIR-2.1 was not shown to deacetylate DAF-16 to the same extent as SIR-2.4 [21,26]. DAF-16 deacetylases could in principle be SIR-2.1 or other worm sirtuins, or non-sirtuin deacetylases. However, the fact that SIR-2.1 does not play a major role in stress-induced DAF-16 nuclear recruitment strongly suggests that SIR-2.1 is not likely the major DAF-16 deacetylase.

FoxO proteins have been shown to interact with acetyltransferases CBP and p300 in mammalian cells [46], although it was previously unknown whether these proteins directly acetylate DAF-16 in worms. In this study, we showed that inactivation of cbp-1 by RNAi results in constitutive nuclear localization of DAF-16, and that CBP-1 can directly acetylate DAF-16 on multiple residues in vitro (Figure 6C, Table S3). However, cbp-1 KD only modestly reduces the level of DAF-16 acetylation in vivo (Figure 6B). Since the pan-acetyl-lysine antibody we used in these experiments is not able to discriminate among DAF-16 species acetylated at different sites, or singly versus multiply acetylated DAF-16 species, it is possible that CBP-1 is not the only acetyltransferase that acetylates DAF-16 in vivo. Thus the effect of cbp-1 KD on DAF-16 acetylation may be partially obscured by CBP-1-independent acetylation of DAF-16. Nevertheless, CBP-1 has a major impact on DAF-16 localization (Figure 6A).

Finally, it is currently unclear whether SIR-2.4 regulates DAF-16 in a cell-autonomous matter, or whether SIR-2.4 is required in only a subset of cells to carry out this function. The tissue expression pattern of endogenous SIR-2.4 is ambiguous. A SIR-2.4::GFP translational fusion protein shows weak expression in most cell types, but much stronger expression in a subset of head and tail neurons as well as in a subset of somatic gonad cells (Figure S1). Thus, SIR-2.4 could conceivably regulate DAF-16 in...
neurons to influence the systemic hormonal milieu – e.g., by reducing INS-7 expression – thereby impacting DAF-16 localization and function throughout the animal. However, our observation that DAF-16 is hyperacetylated in extracts from whole worms, together with our biochemical data, supports the hypothesis that SIR-2.4 regulates DAF-16 acetylation in most tissues of the animal cell autonomously. Overall, SIR-2.4 is a novel regulator of DAF-16 localization and function in response to stress in *C. elegans*.

**Materials and Methods**

**Strains**

CF1041: *daf-2(e1370)III, TJ356: zIs356 [daf-16::zfp + rol-6], AM140: mIs122[unc-54p-Q53::GFP], MT198069: sir-2.4(n5173)I, EQ 137: iqEx4 [sin-2.4p:sir-2.4::zfp + rol-6], EQ 158: iqEx50 [sin-2.4p:sir-2.4 + myo-3p::zfp], EQ 200: sir-2.4(n5173); zIs356 [daf-16::zfp + rol-6], EQ 205: sir-2.4(n5173); zIs356 [daf-16::zfp + rol-6]; iqEx50 [sin-2.4p:sir-2.4A + myo-3p::zfp], EQ 211: sir-2.4(n5173); zIs356 [daf-16::zfp + rol-6]; iqEx60 [sin-2.4p:sir-2.4 + myo-3p::zfp].

All strains used were maintained and handled as described previously [59]. TJ356, AM140 and CF1041 were obtained from the Caenorhabditis Genetic Center. For the generation of transgenic animals, plasmid DNA mixes were injected into the gonad of young adult hermaphrodite animals, using the standard method described previously [60]. F1 progeny were selected on the basis of the rollover phenotype. Individual F2 progenies were isolated to establish independent lines. For the generation of the EQ137 (SIR-2.4::GFP overexpressor) strain, plasmid DNA containing a mixture of 100 ng/ml of sin-2.4p:sir-2.4::zfp and 50 ng/ml of pRF4 (rol-6) constructs was injected into N2 animals. For the generation of EQ158 (native SIR-2.4 overexpressor), plasmid DNA containing a mixture of 30 ng/ml of native SIR-2.4 driven by its own promoter and 50 ng/ml of coinjection marker myo-3p::zfp was injected into N2 animals. For the generation of EQ200, plasmid DNA containing a mixture of 30 ng/ml of sin-2.4p::sir-2.4 and 80 ng/ml of myo-3p::zfp constructs was injected into TJ356 animals. For the generation of EQ203, plasmid DNA containing a mixture of 30 ng/ml of sin-2.4p::sir-2.4A and 80 ng/ml of myo-3p::zfp constructs was injected into TJ356 animals.

**RNA–interference (RNAi) experiments**

HT115 bacteria transformed with RNAi vectors (L4440) expressing dsRNA of the genes indicated were grown at 37°C in LB with 10 μg/mL tetracycline and 50 μg/mL carbenicillin, to establish independent lines. For the generation of the EQ137 (SIR-2.4::GFP overexpressor) strain, plasmid DNA containing a mixture of 100 ng/ml of sin-2.4p:sir-2.4::zfp and 50 ng/ml of pRF4 (rol-6) constructs was injected into N2 animals. For the generation of EQ158 (native SIR-2.4 overexpressor), plasmid DNA containing a mixture of 30 ng/ml of native SIR-2.4 driven by its own promoter and 50 ng/ml of coinjection marker myo-3p::zfp was injected into N2 animals. For the generation of EQ211, plasmid DNA containing a mixture of 30 ng/ml of sin-2.4p::sir-2.4 and 80 ng/ml of myo-3p::zfp constructs was injected into TJ356 animals. For the generation of EQ203, plasmid DNA containing a mixture of 30 ng/ml of sin-2.4p::sir-2.4A and 80 ng/ml of myo-3p::zfp constructs was injected into TJ356 animals.

**Paralysis assay**

Synchronized eggs from AM140 animals (i.e. transgenic animals expressing Q53::YFP) were seeded on either vector control or the indicated RNAi bacteria. Animals were scored for polyQ-induced paralysis every other day during adulthood. Paralyzed worms were identified as those failing to make forward or backward movement in response to stimulation by plate-tapping and tail-prodding; these worms still exhibited pharyngeal pumping. For the paralysis assay, a Kaplan–Meier survival analysis with a log-rank test was performed. P-value of 0.05 was considered statistically significant.

**Lifespan analysis**

Lifespan experiments were conducted at 20°C as described previously [61,62]. Strains were grown at 20°C for at least two
generations without starvation prior to lifespan analysis. At least 60 worms were used for each experiment. In all experiments, the pre-fertile period of adulthood was used as t = 0 for lifespan analysis. Stata 8 software was used for statistical analysis to determine the means and percentiles. In all cases, P-values were calculated using the log-rank (Mantel-Cox) method.

Assessment of DAF-16 acetylation in worms

∼15,000 synchronized day 1 adult worms grown at 20°C were harvested by washing three times with cold M9 buffer and once with HB-high salt buffer (10 mM HEPES, pH 7.9; 10 mM KCl; 1.5 mM MgCl2, 0.1 mM EDTA; 0.5 mM EGTA; 44 mM Sucrose; 100 mM NaCl; 0.5% Triton X-100). Worm pellets were then resuspended in 3× volume of HB-high salt buffer supplemented with Protease Inhibitor Cocktail Complete (Roche), 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM nicotinamide and 1 μM trichostatin A. Pellets were immediately frozen and stored in liquid nitrogen. Frozen suspensions were thawed, homogenized with a Dounce homogenizer (30 strokes with pestle B), and centrifuged at 14,000 g for 20 minutes. Supernatants were collected and total protein concentrations were quantified by Bradford assay. For immunoprecipitation, 30 μl of anti-acetyl-lysine agarose beads (Immunechem; ICP0388) were added to 1 mg of protein extract with 100 μg/ml of ethidium bromide and incubated with gentle shaking at 4°C overnight. The beads were then washed 3 times with HB-high salt buffer supplemented with 50 mg/ml ABESF, 1 mM sodium orthovanadate, 1 mM nicotinamide and 1 μM trichostatin A. After incubation, beads were pelleted and washed three times in HB-high salt buffer supplemented with 50 mM Tris pH 7.4, 10% glycerol, with Protease Inhibitor Cocktail Complete-EDTA free (Roche) [63], followed by brief sonication. 1 mg of whole cell extract from each cell line was pre-cleared by slow rotation with 50 μl of protein G conjugated agarose beads. For immunoprecipitation, either 25 μl of M2-agarose beads (Sigma) (anti-FLAG IP) or 25 μl of anti-FLAG-agarose beads (Roche) (anti-HA IP) was added to pre-cleared WCE, and IPs were incubated overnight by slow rotation at 4°C. After incubation, beads were pelleted and washed three times in LB. FLAG-tagged proteins were eluted in 80 μl of FLAG elution buffer (150 ng/μl 3XFLAG peptide (Sigma), 10 mM Tris-HCl (pH 8.0) and 150 mM NaCl) at 4°C for 4 hours. 40 μl of eluate was loaded on an SDS-PAGE gel. For HA IPs, 80 μl of Laemmli buffer was added to the anti-FLAG-agarose beads, and beads were boiled at 100°C for 5 minutes; 40 μl was loaded on a gel. DAF16 and SIR2.4 interaction was assessed by immunoblot using anti-DAF-16 (Santa Cruz) or anti-HA antibodies.

In vitro DAF-16 acetylation assay

3×FLAG tagged DAF-16, CBP or SIR-2.4 were each transfected and expressed in 293T cells. These proteins were then purified with Anti-FLAG M2 Affinity Gel (Sigma, A2220). 0.5 μg of purified DAF-16 was incubated with 0.2 μg of purified CBP with or without 0.5 μg of purified SIR-2.4 in the presence of HAT buffer (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 mM dithiothreitol, 10%glycerol) supplemented with 200 μM of acetyl-CoA. The reactions were incubated at 37°C for 3 hours and analyzed by western blot. Acetylated DAF-16 was queried with anti-acetyl-lysine antibody (Immunotech; ICP0380) and total level of DAF-16 were assessed with anti-FLAG antibody (Sigma, #F3165).

Supporting Information

Figure S1 Expression pattern of sir-2.4 in C. elegans. Transgenic lines expressing a SIR-2.4 translational GFP fusion (sir-2.4::Sir-2.4∷gfp) were utilized to analyze the expression pattern of sir-2.4 in C. elegans. Images of (a–b) L3 or (c–e) late L4/young adult stage transgenic animals (EQ137) expressing GFP protein under control of the sir-2.4 promoter. sir-2.4 is highly expressed in a subset of head and tail neurons beginning at early larval stage, indicated by white arrows. High expression of sir-2.4 is also found in spermathecal-uterine valve (sp-ut valve) cells beginning at L4 larval stage, indicated by white arrows. The yellow arrow indicates the nuclear accumulation of SIR-2.4∷GFP fusions in these cells. It is worth noting that very weak expression of sir-2.4 is found ubiquitously in most tissues, although it is difficult to capture in these images. (PDF)

Figure S2 Effects of sir-2.4 RNAi or deletion on gene expression, stress resistance, and IIS-mediated DAF-16 translocation. (A–B) Expression of the genes indicated was measured in worms of the indicated genotypes as in Figure 2. (C) Thermotolerance was assessed in worms of the indicated genotypes as in Figure 3A. (D) Oxidative stress resistance was assessed in worms of the indicated genotypes as in Figure 3B. (E) TJ356 animals were fed with either vector control, daf-2 RNAi, a 1:1 mix of daf-2 and sir-2.4 RNAi, or a 1:1 mix of daf-2 and ykb615e RNAi bacteria from the time of hatching. ykb615e is a gene randomly selected as a negative control in double RNAi experiments. Animals (n = 125 or greater) were scored for DAF-16::GFP nuclear translocation as described in Figure 4B. (PDF)

Figure S3 Minimal impact of SIR-2.4 overexpression on stress resistance or lifespan. Transgenic animals overexpressing (A) a SIR-2.4∷GFP fusion protein or (B) native untagged SIR-2.4 protein and N2 controls were exposed to 35°C heat stress. Viability was then scored at the timepoints indicated. (C) Survival curves of wild-type (N2) animals or transgenic animals overexpressing native SIR-2.4 (EQ158) at 20°C. All statistical details are presented in Tables S1 and S2. (PDF)

Figure S4 sir-2.4 RNAi promotes DAF-16 hyperacetylation. (A) DAF-16 acetylation was assessed in control or sir-2.4 RNAi worms by acetyl-lysine immunoprecipitation followed by GFP immunoblot as described in Figure 5A. (B) DAF-16 acetylation was assessed in sir-2.4 KO, sir-2.1 RNAi, or double loss-of function animals as indicated. (PDF)
**Figure S5**  
*cbp-1* loss of function confers stress sensitivity and shortened lifespan. Mean survival of *cbp-1* RNAi worms in response to heat shock (A) or peroxide stress (B). (C) Lifespan curves of wildtype or *sir-2.4* KO animals in the presence of *cbp-1* or control RNAi bacteria.  
(PDF)

**Table S1** Effects of *sir-2.4* expression on stress resistance. Animals indicated were exposed to heat or oxidative stress. Mean survival ± SEM, in hours, observed in the stress analysis is shown.  

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Stress</th>
<th>Lifespan (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 control</td>
<td>Heat</td>
<td>5.2 ± 1.1</td>
</tr>
<tr>
<td>N2 control</td>
<td>Peroxide</td>
<td>4.8 ± 0.9</td>
</tr>
<tr>
<td>sir-2.4 RNAi</td>
<td>Heat</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>sir-2.4 RNAi</td>
<td>Peroxide</td>
<td>3.5 ± 0.3</td>
</tr>
</tbody>
</table>

* Asterisks indicate the sets of experiments plotted and shown in bacteria. *'* indicates the experiment.  
**Table S2** Effects of *SIR-2.4* on lifespan. Adult mean lifespan ± SEM, in days, observed in lifespan analyses. Lifespan experiments were carried out at 20°C.  

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Lifespan (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 control</td>
<td>25.4 ± 1.0</td>
</tr>
<tr>
<td>sir-2.4 KO</td>
<td>23.5 ± 1.2</td>
</tr>
</tbody>
</table>

* Asterisks indicate the sets of experiments plotted and shown in bacteria. *'* indicates the same mutants grown on vector control of the same experiment.  
**Table S3** Peptides representing unique acetylated sequences from the FOXO transcription factor are shown with additional information. A*"* sign indicates the site of acetylation in the sequence. The sequence location refers to which lysine residue on FOXO the identified acetylation site represents. The mass to charge ratio (m/z) and charge state of the peptide as they were observed in the mass spectrometer are given. The mass error specifies the difference between the observed m/z ratio and the theoretical m/z ratio of a peptide, reported in part per million (ppm). The XCorr is the cross correlation score between the theoretical and observed MS² spectra for the matched peptide.  

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Site Position</th>
<th>Charge State</th>
<th>m/z</th>
<th>ppm</th>
<th>XCorr</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>W-CC DXT BY JW-G XY SPG DBL A-LH</td>
<td>K1-1</td>
<td>2+</td>
<td>858.5</td>
<td>0.1%</td>
<td>0.999</td>
</tr>
</tbody>
</table>

**Acknowledgments**  
The authors thank H. R. Horvitz for providing the MT18068 strain; the Rusk lab for providing thedaf-16 RNAi plasmid; the *Caenorhabditis* Genetic Center for the TJ356, AM140, and CF1041 strains; and the National Biosource Project of Japan (Dr. Mitani) for the *sir-2.4* (tm2817) strain. Scott Fletcher is gratefully acknowledged for helpful discussions and comments on the manuscript. Hsiu-yin Chiang is gratefully acknowledged for statistical analysis. The authors also thank Carol Mousigan for technical assistance.

**Author Contributions**  
Conceived and designed the experiments: A-LH DBL. Performed the experiments: W-CC DXT BY JW-G XY TM ME A-LH. Analyzed the data: W-CC DXT BY JW-G XY SPG DBL A-LH. Wrote the paper: DBL: A-LH.

**References**  


