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Keywords
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Transcriptional regulation of Caenorhabditis elegans FOXO/DAF-16 modulates lifespan

Ankita Bansal1†, Eun-Soo Kwon1,2†, Darryl Conte Jr3,6, Haibo Liu1, Michael J Gilchrist4, Lesley T MacNeil5 and Heidi A Tissenbaum1,6*

Abstract

**Background:** Insulin/IGF-1 signaling plays a central role in longevity across phylogeny. In *C. elegans*, the forkhead box O (FOXO) transcription factor, DAF-16, is the primary target of insulin/IGF-1 signaling, and multiple isoforms of DAF-16 (a, b, and d/f) modulate lifespan, metabolism, dauer formation, and stress resistance. Thus far, across phylogeny modulation of mammalian FOXOs and DAF-16 have focused on post-translational regulation with little focus on transcriptional regulation. In *C. elegans*, we have previously shown that DAF-16d/f cooperates with DAF-16a to promote longevity. In this study, we generated transgenic strains expressing near-endogenous levels of either *daf-16a* or *daf-16d/f*, and examined temporal expression of the isoforms to further define how these isoforms contribute to lifespan regulation.

**Results:** Here, we show that DAF-16a is sensitive both to changes in gene dosage and to alterations in the level of insulin/IGF-1 signaling. Interestingly, we find that as worms age, the intestinal expression of *daf-16d/f* but not *daf-16a* is dramatically upregulated at the level of transcription. Preventing this transcriptional upregulation shortens lifespan, indicating that transcriptional regulation of *daf-16d/f* promotes longevity. In an RNAi screen of transcriptional regulators, we identify elt-2 (GATA transcription factor) and swsn-1 (core subunit of SWI/SNF complex) as key modulators of *daf-16d/f* gene expression. ELT-2 and another GATA factor, ELT-4, promote longevity via both DAF-16a and DAF-16d/f while the components of SWI/SNF complex promote longevity specifically via DAF-16d/f.

**Conclusions:** Our findings indicate that transcriptional control of *C. elegans* FOXO/daf-16 is an essential regulatory event. Considering the conservation of FOXO across species, our findings identify a new layer of FOXO regulation as a potential determinant of mammalian longevity and age-related diseases such as cancer and diabetes.

**Keywords:** Longevity, DAF-16/FOXO, C. elegans, Transcription, Aging, Isoforms

**Background**

The evolutionarily conserved insulin/IGF-1 signaling (IIS) pathway regulates lifespan from worms to mammals and in *C. elegans* also modulates dauer formation, stress response, and metabolism [1-4]. The *C. elegans* IIS pathway consists of an insulin/IGF-1 receptor (DAF-2) [5], a PI 3-kinase (AGE-1/AAP-1) [6,7] serine/threonine kinases (PDK-1, AKT-1, and AKT-2) [8,9], and a Forkhead Box O (FOXO) transcription factor (DAF-16) [10,11]. IIS ultimately results in the AKT-dependent phosphorylation of DAF-16, thereby preventing DAF-16 from entering the nucleus to regulate its target genes [3,4,12]. Therefore, the *C. elegans* FOXO ortholog, DAF-16 is the primary downstream target of the IIS pathway [10,11].

In contrast to *C. elegans*, mammals have three closely related FOXO proteins (FOXO1, FOXO3, and FOXO4) that share a high degree of homology, overlapping expression patterns, and common target genes [13,14]. These FOXOs appear to have discrete functions as each mutant displays a distinct phenotype: *Foxo1* null mice die as embryos due to defects in angiogenesis [15], *Foxo3* mutants are viable but show age-dependent female sterility [15,16], and *Foxo4* null mutants are viable with no detectable phenotype [15]. Interestingly, conditional somatic deletion of all three *Foxo* loci results in a
cancer-prone phenotype that includes age-progressive thymic cancers and hemangiomias, indicating that Foxo1, Foxo3, and Foxo4 are redundant tumor suppressor genes specifically involved in endothelial growth suppression [17]. Recently, a more distantly related FOXO family member, FOXO6, was shown to regulate gluconeogenesis [18]. Thus, four mammalian FOXO homologs play distinct and overlapping roles in multiple biological processes.

DAF-16, the only *C. elegans* FOXO ortholog, expresses multiple isoforms (Figure 1). Initial studies on DAF-16 identified three different transcripts, daf-16a1, daf-16a2, and daf-16b. daf-16a1 and daf-16a2 share the same promoter and 11 exons, but alternative splicing at exon 3 results in an insertion of two additional amino acids in the DAF-16a1 protein compared to DAF-16a2 [19,20]. daf-16b encodes a shorter isoform, expressed from a different promoter and results in a protein that includes the same C-terminal 319 amino acids as the DAF-16a1/a2 isoforms. Recently, we identified a third DAF-16 isoform, daf-16d/f, which is driven by a distinct promoter and initiates approximately 10 kb upstream of daf-16a1/a2. daf-16d/f is encoded by 14 exons, 10 of which are shared with DAF-16a1/a2 and DAF-16b. Studies using isofrom-specific RNAi and transgenes have revealed cooperative as well as specific functions for these three DAF-16 isoforms (DAF-16a, DAF-16b, and DAF-16d/f) in the regulation of lifespan, dauer formation, fat storage, and stress resistance [21]. For lifespan regulation, however, we found that only DAF-16a and DAF-16d/f are involved [21]. The relative contribution of each of these two isoforms and regulation of each isoform remains unknown.

To date, much has been learned about the post-translational regulation of FOXO/DAF-16. For example, FOXO/DAF-16 is directly phosphorylated by kinases including AKT-1, AKT-2, SGK-1, Jun-N-terminal kinase (JNK/JNK-1) [22-24], Ste20-like protein kinase (MST1/CST-1) [25], and AMP-activated protein kinase (AMPK/AAK-1) [26,27], and FOXO/DAF-16 interacts with a Serine/threonine-protein phosphatase 4 regulatory subunit, SMK-1 [28]. FOXO/DAF-16 also interacts with 14-3-3 proteins, which modulate the ability of FOXO/DAF-16 to interact with cofactors, including the sirtuin family (SIRT1/SIR-2.1) of NAD-dependent deacetylases [3,4,29,30].

These interactions and direct post-translational modifications function to modulate the activity of FOXO/DAF-16 under different conditions [3,4].

Despite the growing body of knowledge centered on the post-translational regulation of FOXO/DAF-16, little is known about the transcriptional regulation of daf-16 in *C. elegans*. To begin to address these issues, we examined the expression of the daf-16a and daf-16d/f isoforms throughout development and adulthood. We found that daf-16d/f expression is dramatically increased at the level of transcription during the young adult stage, and this upregulation of daf-16d/f expression is required for longevity. Furthermore, using an RNAi screen we identified two transcriptional regulators of daf-16d/f expression: elt-2, encoding an essential GATA transcription factor required for intestinal development [31,32], and swsn-1, encoding a core component of the SWI/SNF chromatin remodeling complex [33,34]. ELT-2 and another GATA factor, ELT-4, are required for the expression of both daf-16a and daf-16d/f and for the ability of both daf-16a and daf-16d/f to promote longevity. By contrast, SWSN-1 and other SWI/SNF components are required for the upregulation of daf-16d/f expression in the intestine of young adults, but not daf-16a expression. Consistent with this finding, components of the SWI/SNF complex promote longevity via daf-16d/f but not daf-16a. Taken together, our findings reveal that transcriptional regulation of daf-16d/f is an important regulatory event in lifespan determination.

**Results and discussion**

**daf-16 has multiple isoforms**

*daf-16* functions as a central regulator of multiple biological processes including lifespan, development, fat storage, and stress resistance [1-4]. The *C. elegans* genome resource, Wormbase (http://www.wormbase.org), predicts eight putative isoforms of *daf-16*, with each isoform designated by a lowercase letter following the cosmid name for *daf-16* (R13H8.1a-h). To verify expression of the *daf-16* isoforms, we first analyzed the Gurdon Institute EST database (nimr.mrc.ac.uk/online/worm-fl-db.html) [35,36]. As shown in Additional file 1: Table S1, ESTs were identified that map to *daf-16f* (R13H8.1f), *daf-16d* (R13H8.1d), *daf-16a*

![Figure 1 Structure of daf-16 isoforms](image-url)
Effects of altering DAF-16a dosage on lifespan

Dissecting the relative contributions of DAF-16d/f and DAF-16a in the regulation of longevity has been challenging due to incomplete knockdown by isoform-specific RNAi [21] and the lack of isoform-specific mutants. This is further complicated by the existence of a dosage-dependent effect of DAF-16a and DAF-16d/f transgenes [21]. To expand our analysis of the DAF-16 dosage effect on lifespan, we first generated a new low-copy DAF-16a transgene by microparticle bombardment, which we refer to as daf-16a::gfp\textsuperscript{HT}. In addition, we chose three commonly used high-copy DAF-16a transgenes, referred to as daf-16a::gfp\textsuperscript{HT} derived from strain CF1407 [20], daf-16a::gfp\textsuperscript{TJ} from TJ356 [39], and daf-16a::gfp\textsuperscript{GR} from RX86 [40]. For all the analyses that follow, we first placed each daf-16 transgene into the same genetic background which is a null allele of the endogenous daf-16 locus [daf-16(mgDf50)].

To compare the levels of daf-16a in the different transgenic strains, we measured the level of daf-16a expression from each transgene in the daf-16(mgDf50); daf-2(e1370) background and compared it to the endogenous level of daf-16a in daf-2(e1370) mutants (Figure 2A, Additional file 1: Table S2). The level of daf-16a expressed from the newly generated daf-16a::gfp\textsuperscript{HT} transgenic strain was significantly lower than that of the other transgenic strains, indicating that the addition of the tag alone did not affect its expression.

Figure 2 Effects of altering DAF-16a dosage on lifespan. (A) Expression levels of daf-16a mRNA in various daf-16(mgDf50); daf-2(e1370); daf-16a::gfp strains were compared to that of daf-2(e1370) worms. The graph is plotted on a log2 scale. Error bars represent standard deviation (S.D.) from two independent repeats. Statistical values are given in Additional file 1: Table S2. (B) Lifespan of daf-16(mgDf50) worms carrying various daf-16a::gfp transgenes as well as low-copy daf-16d/f::gfp\textsuperscript{HT}. Three high-copy daf-16a transgenic worms live longer than wild type. daf-16a::gfp\textsuperscript{HT} worms lived shorter than wild-type, while the lifespan of the daf-16d/f::gfp\textsuperscript{HT} strain is comparable to wild type. (C) Lifespan of daf-16(mgDf50); daf-2(e1370) worms carrying various daf-16::gfp transgenes. daf-16a::gfp\textsuperscript{HT} worms lived shorter than the daf-2(e1370) mutants. daf-16(mgDf50); daf-2(e1370); daf-16a::gfp\textsuperscript{HT} worms had the shortest lifespan among the high-copy daf-16a transgenic strains, daf-16d/f::gfp\textsuperscript{HT} transgene alone fully rescued the lifespan extension of daf-2(e1370) mutants. One lifespan experiment is shown from a total of three repeats; each with similar results. All lifespan data are shown in Additional file 1: Table S3.
approximately two-fold higher than the endogenous \textit{daf-16a} in the \textit{daf-2(e1370)} mutant, while the commonly used \textit{daf-16a} transgenes were expressed at 10- to 400-fold higher levels than endogenous \textit{daf-16a}. These results show the following order for levels of the \textit{daf-16a} transcript: endogenous \textit{daf-16a} < \textit{HT} < \textit{CF} < \textit{T} < \textit{GR}. Consistent with the mRNA expression data, the level of \textit{DAF-16a} protein from the transgenic strains also increases (Additional file 1: Figure S1). The GFP intensity in the four transgenic strains correlated with the level of expression of \textit{DAF-16a} (data not shown).

Next, we determined the nuclear:cytosolic ratio of \textit{DAF-16a} in the transgenic strains expressing different levels of \textit{DAF-16a} (Additional file 1: Figure S2) in \textit{daf-2 (e1370)} background. \textit{DAF-16a} was almost entirely in the nucleus when expressed at low levels from the \textit{daf-16a::gfp^{HT}} transgene. By contrast, \textit{DAF-16a} was mainly cytosolic in the \textit{daf-2(e1370); daf-16a^{GR} strain}, which shows the highest level of \textit{DAF-16a} expression, while the \textit{daf-2 (e1370); daf-16a::gfp^{CF} and daf-2(e1370); daf-16a::gfp^{T}} strains showed an intermediate nuclear localization of \textit{DAF-16a}. Importantly, since these different transgenic strains were generated with different methods and may be integrated at different locations, this indicates that it is the level of mRNA and protein of \textit{DAF-16a} that determines its regulatory ability.

We reasoned that if a \textit{daf-16a} transgene is expressed at close to endogenous levels, the \textit{daf-16a} transgenic animals should not live longer than wild-type animals expressing endogenous levels of all three \textit{DAF-16} isoforms. Interestingly, our low-copy \textit{daf-16a::gfp^{HT}} transgenic worms lived shorter than wild-type (Figure 2B, Additional file 1: Table S3), suggesting that low level expression of \textit{daf-16a} alone cannot replace loss of the endogenous \textit{daf-16} for lifespan regulation. However, the three high-copy \textit{daf-16a} transgenic strains lived longer than wild type (Figure 2B, Additional file 1: Table S3). Therefore, these data suggest that the level of \textit{DAF-16a} expressed from the \textit{daf-16a::gfp^{CF}}, \textit{daf-16a::gfp^{T}}, and \textit{daf-16a::gfp^{GR}} transgenes exceeds the inhibitory capacity of the IIS pathway. This gene dosage effect was even more pronounced when each \textit{daf-16a} transgene was analyzed in the \textit{daf-16(mgDf50); daf-2(e1370)} mutant background, we expected that the \textit{daf-16d/f::gfp^{HT}} transgene would be sufficient to fully complement the lifespan of a \textit{daf-16(mgDf50); daf-2(e1368)} double mutant. Yet, to our surprise, \textit{daf-16(mgDf50); daf-2(e1368); daf-16d/f::gfp^{HT}} transgenic worms lived significantly shorter than the \textit{daf-2(e1368)} single mutant (Figure 3A, Additional file 1: Figure S4, Additional file 1: Table S3, Additional file 1: Table S4). In contrast, the \textit{daf-16a::gfp^{HT}} transgene rescued lifespan to similar levels in both \textit{daf-2(e1368)} and \textit{daf-2(e1370)} mutant backgrounds (Additional file 1: Figure S4, Additional file 1: Table S3, Additional file 1: Table S4).

To further test the effect of \textit{DAF-16d/f} in the \textit{daf-2 (e1370)} and \textit{daf-2(e1368)} mutant backgrounds, we examined dauer formation as an additional output of \textit{DAF-16} activity. At the restrictive temperature of 25°C, both \textit{daf-2 (e1370)} and \textit{daf-2(e1368)} form 100% dauers whereas \textit{daf-16 (mgDf50); daf-2(e1370)} and \textit{daf-16(mgDf50); daf-2(e1368)} double mutants do not form any dauers. However, once again, the \textit{DAF-16d/f} transgene had different effects in the two \textit{daf-2} alleles: \textit{daf-16(mgDf50); daf-2(e1368); daf-16d/f::gfp^{HT}} transgenic worms formed approximately
39% dauers, whereas daf-16(mgDf50); daf-2(e1368) worms formed approximately 98% dauers at 25°C (Additional file 1: Figure S5A). At the semi-permissive temperature of 20°C, a similar trend was observed. This is in contrast to daf-16a transgenic worms where dauer formation was comparable in both daf-2 mutant backgrounds (Figure 3B, Additional file 1: Figure S5).

Examining the DAF-16 nuclear/cytosolic localization, DAF-16d/f was enriched in the nucleus in the daf-2(e1368) background, but more cytosolic in the daf-2(e1368) background (Figure 3C,D). In contrast, DAF-16a was primarily enriched in the nucleus in both daf-2(e1368) and daf-2(e1370) backgrounds (Figure 3C,D). Thus, the DAF-16 localization data correlated with both functional outputs - lifespan and dauer formation. Whereas DAF-16a is activated to a similar level in both alleles, DAF-16d/f is less active in the daf-2(e1368) mutant than in the daf-2(e1370) mutant. Therefore, these data show that DAF-16a and DAF-16d/f exhibit different thresholds for inhibition by the upstream IIS pathway.

Transcription of daf-16d/f is upregulated during aging
Although the spatial expression patterns of daf-16 have been extensively investigated [19,20,39], the temporal expression of daf-16 has not been studied. We monitored the expression of the daf-16 isoform:gfpl0 strains throughout development and for the first few days of adulthood. Interestingly, as shown in Additional file 1: Figure S6, the GFP intensity of the daf-16d/f::gfp transgenic worms dramatically increased in the adult stage compared to larval stages.

Next, we asked if the increase in GFP intensity in adult daf-16d/f::gfp transgenic worms correlates with the level of the endogenous transcript. Using qRT-PCR, we found that each of the daf-16 isoforms was expressed at a constant level throughout larval development. However, as worms aged, the daf-16d/f transcript dramatically increased in both wild-type and long-lived daf-2(e1370) mutants, while the daf-16a transcript increased only slightly (Figure 4A,B, Additional file 1: Figure S7-S9). To further confirm this result, we tested the upregulation of DAF-16a vs DAF-16d/f at the protein level. Since it is not possible to distinguish differences in endogenous levels of DAF-16a and DAF-16d/f protein, we were limited to examining changes in DAF-16 protein levels in strains that only bear one DAF-16 isoform (daf-2;daf-16;daf-16a::gfpl0 or daf-2;daf-16;daf-16d/f::gfp). Using these transgenic strains, consistent with our mRNA data, the age-dependent
increase in the levels of DAF-16d/f was also observed at the protein level (Figure 4C,D).

The qRT-PCR assay is a read-out of the steady-state level of mRNA, but the DAF-16::GFP fusion fluorescence is the product of both transcriptional and post-transcriptional regulation. Therefore, to measure the transcriptional promoter activity of each daf-16 isoform, we generated daf-16 promoter::GFP fusions (Pdaf-16::gfp). The promoter of each daf-16 isoform was placed upstream of the GFP open reading frame fused to the unc-54 3′UTR, which is known to be absent of temporal regulatory elements [42]. We reasoned that if increased transcription is responsible for the elevated mRNA level in young adults, then the GFP intensity should become brighter in the promoter::gfp transgenic worms as the worms age. In the Pdaf-16a::gfp worms, the GFP signal throughout the body increased slightly with age. In contrast, in Pdaf-16d/f::gfp worms, GFP expression increased dramatically throughout the intestine (data not shown Figure 4E). Therefore, across multiple independent experiments we found that transcription of daf-16, particularly daf-16d/f, was regulated in an age-dependent manner.

daf-16d/f transcript level in the early adult determines lifespan

daf-16 expression in the intestine is critical for lifespan regulation in C. elegans [21,43], and our temporal and
spatial expression data indicate that \textit{daf}-16\textit{d/f} mRNA is dramatically upregulated in the intestine as worms age. To determine the importance of \textit{DAF}-16 upregulation in aging worms, we used RNAi to maintain \textit{daf}-16\textit{d/f} expression in the adult stage at a level comparable to that in the L4 stage, thereby preventing the upregulation of \textit{DAF}-16\textit{d/f} during aging. To achieve the correct knockdown level, we prepared serial-dilutions of \textit{DAF}-16\textit{d/f} during aging. To achieve the correct knock-

expression of endogenous \textit{daf}-16, as well as \textit{daf}-16\textit{(mgDf50)}; \textit{daf}-2\textit{(e1370)}; \textit{daf}-16\textit{a::gfp} and \textit{daf}-16\textit{(mgDf50)}; \textit{daf}-2\textit{(e1370)}; \textit{daf}-16\textit{d/f::gfp} transgenic worms (Figure 6A, C, D, Additional file 1: Table S6). Relative to the control RNAi, \textit{elt}-2 RNAi did not shorten the lifespan of a \textit{daf}-16\textit{(mgDf50)}; \textit{daf}-2\textit{(e1370)} mutant (Figure 6B), suggesting that \textit{elt}-2 RNAi shortens lifespan by reducing intestinal \textit{daf}-16 gene expression and not by a \textit{daf}-16-independent pathway.

We analyzed several additional GATA transcription factors for a role in \textit{daf}-16 transcriptional regulation and longevity, including \textit{elt}-4 and \textit{elt}-7, which are expressed in the intestine [44], and \textit{elt}-3, \textit{elt}-5, and \textit{elt}-6, which were previously implicated in lifespan regulation [48]. Only \textit{elt}-4 RNAi reduced the expression of the \textit{Pdaf}-16\textit{d/f::gfp} transcriptional reporter (Additional file 1: Figure S13), though less dramatically than \textit{elt}-2 RNAi. Furthermore, only \textit{elt}-4 RNAi reduced the lifespan of both \textit{daf}-16\textit{(mgDf50)}; \textit{daf}-2\textit{(e1370)}; \textit{daf}-16\textit{a::gfp} and \textit{daf}-16\textit{(mgDf50)}; \textit{daf}-2\textit{(e1370)}; \textit{daf}-16\textit{d/f::gfp} transgenic worms (Figure 6E–H, Additional file 1: Table S6).

Neither \textit{Pdaf}-16\textit{d/f::gfp} expression nor lifespan was affected by \textit{elt}-7, \textit{elt}-3, \textit{elt}-5, or \textit{elt}-6 RNAi (Additional file 1: Figure S13 and Additional file 1: Table S6). These data are consistent with a recent study [49] suggesting that the previously reported role of \textit{ELT}-3 in lifespan regulation [48] should be re-evaluated. Our findings indicate that the \textit{ELT}-2 and \textit{ELT}-4 GATA factors promote longevity by regulating intestinal \textit{daf}-16 expression.

\textbf{SWI/SNF determines lifespan by regulating \textit{daf}-16\textit{d/f} expression}

The second positive clone we obtained from our screen was \textit{swsn-1}, which encodes a core component of the highly conserved SWI/SNF nucleosome-remodeling complex [50]. In \textit{C. elegans}, SWI/SNF is required for asymmetric cell division [51] and differentiation [52]. Prior to the L4 stage, \textit{Pdaf}-16\textit{d/f::gfp} expression was unaffected by \textit{swsn-1} RNAi (Figure 5A,B). Expression of endogenous \textit{daf}-16\textit{d/f} was also reduced in animals exposed to \textit{swsn-1} RNAi (Figure 5C). Interestingly, \textit{swsn-1} RNAi did not significantly change the expression of \textit{Pdaf}-16\textit{a::gfp} (Additional file 1: Figure S11C), suggesting that \textit{SWSN}-1 regulates the temporal and spatial expression of \textit{daf}-16\textit{d/f}, specifically. RNAi targeting \textit{swf-5} and \textit{swsn-2.1}, core and accessory subunits of SWI/SNF, respectively, also reduced the temporal upregulation of \textit{daf}-16\textit{d/f} expression with age (Additional file 1: Figure S12) but did not change \textit{daf}-16\textit{a} expression, indicating that the SWI/SNF complex specifically controls \textit{daf}-16\textit{d/f} expression.
We next asked if SWI/SNF plays a role in lifespan regulation. Both *swsn-1* and *snfc-5* are essential genes [51] and hatched larvae grown on *swsn-1* or *snfc-5* RNAi develop into sick adults with pleiotropic defects [51,53]. To avoid these complications in lifespan analysis, we used post-developmental (L4) RNAi to silence *swsn-1*. However, *Pdaf-16d/f::gfp* expression was largely unaffected by post-developmental *swsn-1* RNAi (data not shown) and we observed only a marginal reduction of lifespan in *daf-16* (*mgDf50*); *daf-2(e1370); daf-16d/f::gfpHT* transgenic worms (Figure 6D, Additional file 1: Table S6), suggesting that post-developmental (L4) RNAi does not efficiently silence *swsn-1*. Worms exposed to *swsn-2.1* RNAi from hatching, however, were much healthier than animals grown on *swsn-1* or *snfc-5* RNAi, which allowed us to assess the effect of SWI/SNF on lifespan. As shown in Figure 6H and

**Figure 5** ELT-2 and SWSN-1 are required for *daf-16* gene expression. (A) *Pdaf-16d/f::gfp* expression grown on control, elt-2 RNAi or *swsn-1* RNAi bacteria. Left and middle panels worms grown on RNAi from hatching, right panel worms grown on RNAi from L4. Red arrows indicate the head region of the worms. (B) Close-up of worms shown in Panel A Left and middle columns with additional GFP RNAi control. *Pdaf-16d/f::gfp* expression in the head and anterior intestine of worms grown on control, *gfp*, *elt-2*, or *swsn-1* RNAi bacteria from hatching. (C) Quantitative RT-PCR analysis of endogenous *daf-16d/f* in L4 larvae and Days 1, 2, and 5 adult *daf-2(e1370)* worms. For *elt-2*, worms were grown on RNAi bacteria from L4 stage and for *swsn-1* were grown on RNAi bacteria from hatching. The expression of *daf-16d/f* is reduced by *elt-2* or *swsn-1* RNAi treatment. Additional details are shown in Additional file 1: Figure S13.
Additional file 1: Table S6, swsn-2.1 RNAi shortened the lifespan of daf-16(mgDf50); daf-2(e1370); daf-16d/f::gfpHT worms, but only modestly reduced the lifespan of daf-2(e1370) or daf-16(mgDf50); daf-2(e1370); daf-16a::gfpHT animals (Figure 6E,G, Additional file 1: Table S6). Interestingly, a very recent paper found that SWI/SNF interacts with DAF-16 [54]. These studies revealed that a SWI/SNF-DAF-16 complex co-localizes at the promoters of DAF-16 direct targets and activates genes important for lifespan, dauer formation, and stress resistance [54]. Therefore, these results support a biochemical interaction of DAF-16 and SWI/SNF. Taken together, our data are consistent with a model in which the SWI/SNF complex directly interacts with DAF-16 to specifically promote longevity by regulating daf-16d/f expression.

Conclusion
In C. elegans, the single FOXO family member, DAF-16 regulates lifespan, metabolism, development, and stress resistance by generating multiple isoforms including DAF-16/f, DAF-16a, and DAF-16b [21]. Comparing the relative abundances of the different isoforms revealed that daf-16d/f is the most abundant isoform in early adulthood and that transcription of daf-16d/f mRNA is dramatically increased in the intestine as animals age. These findings are in agreement with previous studies showing that DAF-16 expression in the intestine is important for lifespan regulation and that lifespan is determined in adulthood [43,55]. Therefore, the temporal control of daf-16d/f transcription in the intestine is a critical determinant of longevity.
Importantly, temporal regulation of FOXO expression is conserved in mammals. In rats, FOXO3 and FOXO4 transcript are undetectable in very young animals but increase as animals age in the duodenum [56], and human FOXO1 mRNA is significantly enriched in muscle samples from old individuals [57]. It will be particularly interesting to determine whether the age-dependent increase in mammalian FOXO expression are regulated at the level of transcription, as we have shown for daf-16d/f in C. elegans.

In worms, flies, and mammals, FOXO is the primary target of the IIS pathway [2,58]. In C. elegans, the DAF-2 IIS receptor has been extensively studied by genetic analysis. The daf-2(e1370) allele harbors a mutation in the tyrosine kinase domain, whereas daf-2(e1368) has a mutation in the ligand-binding domain [5]. These daf-2 alleles show phenotypic differences, with daf-2(e1370) displaying longer lifespan and stronger dauer arrest compared to daf-2(1368) [41,58], indicating that the IIS pathway is more active in daf-2(e1368) than in daf-2(e1370). Our lifespan, dauer, and DAF-16 nuclear localization data in these daf-2 mutants revealed that daf-16d/f and daf-16a respond differently to changes in the levels of IIS. In both mutant backgrounds, DAF-16a is predominantly nuclear indicating that DAF-16a is activated to a similar level in both daf-2 mutants. However, DAF-16d/f is nuclear only in the daf-2(e1370) background and therefore appears to be more active in this background. This suggests that a small decrease in IIS activates DAF-16a, whereas large decreases in IIS will activate DAF-16d/f and could add another layer of regulation by the different DAF-16 isoforms.

Using an RNAi screen, we identified the GATA factor ELT-2 and the SWI/SNF subunit SWSN-1 as factors required for daf-16d/f expression. Our functional analysis reveals that the intestinal GATA transcription factors, ELT-2 and ELT-4, regulate daf-16 expression and, in turn, longevity. Our findings are consistent with the work of McGhee et al. [32], who identified multiple GATA binding sites upstream of both daf-16a and daf-16d/f and proposed that intestinal GATA factors, particularly ELT-2, might regulate the expression of daf-16. Moreover, the promoters of many DAF-16 targets also have GATA binding sites [32,48,59], suggesting that ELT-2/ELT-4 may cooperate with DAF-16 to regulate longevity genes in the intestine.

Recent studies suggest that epigenetic control of gene expression is important for the aging process [60]. In C. elegans, IIS-dependent and IIS-independent epigenetic modifications have been linked to longevity [61-64]. Our study shows that SWI/SNF regulates lifespan by promoting the age-dependent activity of a FOXO gene. Importantly, components of the SWI/SNF complex are required for the age-dependent upregulation of daf-16d/f but not daf-16a. SWSN-2.1 is homologous to mammalian BAF60, a subunit known to interact with a number of transcriptional activators [65,66]. BAF60 has three variants (a, b, c) [66] and BAF60c has been shown to interact with the GATA4 transcription factor to promote differentiation in early mouse heart development [67]. We envision a similar role in stimulation of daf-16d/f transcription in the adult intestine. For example, ELT-2/ELT-4 GATA or other transcription factor(s) together with SWSN-2.1/BAF60 may interact with the daf-16d/f promoter during development. At the young adult stage, an intestine-specific developmental cue may stimulate the SWSN-2.1-dependent recruitment of the core SWI/SNF machinery to remodel the daf-16d/f promoter and activate daf-16d/f transcription.

We have shown here and elsewhere [21] that daf-16a and daf-16d/f are sensitive to changes in gene dosage. For daf-16d/f, mRNA and protein are dramatically upregulated with age, whereas daf-16a mRNA only minimally changes with age, and preventing the upregulation of daf-16d/f in adults shortens lifespan. ELT-2 and ELT-4 are likely to be direct regulators of DAF-16. Indeed, multiple GATA binding sites exist upstream of both daf-16a and daf-16d/f contain (data not shown). Based on our finding that SWI/SNF specifically regulates the expression of daf-16d/f but not daf-16a, we propose that SWI/SNF modifies the activity of the daf-16d/f promoter but not the daf-16a promoter. Taken together, and consistent with our findings, we suggest a model where daf-16d/f plays a more prominent role in lifespan regulation than daf-16a (Figure 7).

C. elegans has proved to be an invaluable model system for understanding the regulation and biological functions of FOXO transcription factors. The mechanisms that control FOXO proteins at a post-translational level are remarkably conserved from worms to mammals. Our findings indicate that transcriptional control of daf-16/FOXO is also an essential regulatory event. As there is increasing evidence that FOXOs are required for human longevity [68-73], our findings will advance our understanding of the regulation and function of DAF-16/mammalian FOXO in aging and age-dependent diseases including cancer and diabetes.

Methods

Strain maintenance

All strains were maintained and handled as described [74]. Animals were grown on standard NGM plates at 15°C using standard C. elegans techniques, unless otherwise indicated. Mutants used in this study included, LGI: daf-16(ngD50); LGIII: daf-2(e1368, e1370); unc-119 (ed3). All transgenic worms have an unc-119(ed3) mutation rescued by an unc-119' co-transformation marker. Transgenic strains generated and/or used in this study are listed in Additional file 1: Table S8.
### Strain construction

**i) daf-2(e1368) unc-119(ed3) double mutants**

Both genes map to Chromosome II but are 13 map units apart. *daf-2(e1368)* males were mated to *unc-119* (ed3) hermaphrodites. Approximately 20 F1 progeny were picked onto individual plates and allowed to produce progeny at 25°C. F2 *daf-2(e1368)* dauers were selected from each plate and allowed to recover at 15°C. From the F3 progeny, Unc worms were selected, generating *daf-2(e1368); unc-119(ed3)*.

**ii) daf-16(mgDf50); daf-2(e1368) unc-119(ed3) mutants**

*daf-16(mgDf50); daf-2(e1368)* males were mated to *daf-2(e1368); unc-119(ed3)* hermaphrodites. Approximately 20 F1 progeny were picked onto individual plates and allowed to have progeny at 25°C. From the F2 progeny on each plate, Unc Non-dauer worms were selected and tested for the *daf-16(mgDf50)* mutation by PCR. Primer sequences are listed in Additional file 1: Table S7.

### DNA construction for *daf-16* isoform specific GFP and RNAi

To generate *daf-16* isoform::gfp constructs, each cDNA encoding *daf-16a*, *daf-16d/f* was cloned into the pGEM-T vector. The clones were then verified by sequencing. For the *daf-16a* isoform promoter construct, a *SalI/BamHI* fragment containing 6.0 kb upstream of *daf-16a* was cloned into pPD95.75 (GFP containing plasmid). For the *daf-16d/f* isoform promoter construct, a *SphI/BamHI* fragment containing 4.1 kb upstream of *daf-16d/f* was cloned into pPD95.75 (GFP containing plasmid). We then generated full-length *daf-16a1* cDNAs using mutagenic primers with *BamHI/SmaI* restriction enzyme sites, which were subcloned into pPD95.75 with the upstream promoter fragment. For *daf-16d/f*, since we were unable to amplify the *daf-16d/f* specific cDNA, we generated a construct which could generate both DAF-16d1 and DAF-16d/f by subcloning the 4.1 kb upstream genomic DNA next to the 5' putative start codon of *daf-16d1* cDNA in frame [21].

To allow the constructs to be compatible with ballistic transformation, the *unc-119* gene was introduced into each vector using PCR redirected DNA recombination method [76]. To generate *daf-16* isoform specific RNAi vector, *Nhel/HindIII* fragments covering isoform specific/overlapping cDNA were cloned into L4440 vector [21]. The primers used for the PCR analysis are listed in Additional file 1: Table S7.

### RNA isolation and real-time PCR

**Growing samples**

To compare the expression of *daf-16* isoforms in developmental stages, worms were synchronized by bleaching,
followed by transferring eggs on plates seeded with OP50 bacteria. Worms were grown until the L1, L2, L3, and L4 stage and then harvested. To obtain aged worms, L4 stage worms were transferred to FuDR plates with final concentration of 0.1 mg/mL [77] seeded with OP50 bacteria. On days 1, 2, 5, and 10, worms were harvested and frozen at -80°C. To compare the expression of daf-16α in L4 stage, in Figure 2, worms were synchronized by bleaching, followed by L1 arrest in M9 buffer. The following day, the L1 worms were placed on plates seeded with OP50 bacteria and further incubated at 15°C until worms reached the L4 stage.

**RNA preparation**

Total RNA was isolated using acidic phenol (Sigma). Briefly, worms were washed off the plates using ice-cold M9 buffer, followed by three additional washes. Next, 0.5 mL of AE buffer (acetic acid, EDTA), 0.1 mL of 10% SDS, and 0.5 mL of phenol were added and the mixture and vortexed vigorously for 1 min followed by incubation at 65°C for 4 min. The RNA was then purified by phenol:chloroform extraction followed by ethanol precipitation. The concentration and the purity of the RNA were determined by measuring the absorbance at 260/280 nm. To further determine the quality of the RNA, both the ribosomal 28 S and 18 S were visually inspected on an agarose gel. For Figure 4A and B, total RNA was purified from approximately 100 worms using Directzol™ RNA MiniPrep kit (Zymo Research), as described by the manufacturer. cDNA was synthesized using total RNA and the SuperScript cDNA synthesis kit (Invitrogen, USA). Gene expression levels were then determined by real-time PCR using the PowerSYBR® Green PCR Master Mix and StepOnePlus Real-Time PCR System (Applied Biosystems, USA). Relative gene expression was compared to actin as an internal control. Primers used are listed in Additional file 1: Table S7.

**Western blots**

All strains were allowed to grow at 15°C. For Additional file 1: Figure S1, approximately 100 L4 stage worms were collected for each of the transgenic strain in 10 to 15 mL of M9 buffer. Worms were then washed once with M9. An equal amount of SDS containing loading buffer was added to the worm pellets and samples were immediately boiled to lyse the worms. Samples were cooled, centrifuged briefly, and the supernatant was loaded onto the gel. For the western blot analyses in Figure 4C and D, equal amounts (10 mL) of worms were used. For each experiment, the lysates were resolved on a 10% polyacrylamide gel by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and probed with antibody against DAF-16 [78] (1:2,500 dilution). The membrane was then reprobed with anti-α-tubulin (1:8,000 dilution).

**Growth assays**

All the strains were grown at 15°C and five L4s were picked onto plates at 15°C. The plates were left undisturbed for 7 days and photos of each of the plates were taken using Nikon Coolpix995 camera to compare the growth of the strains.

**Lifespan assays**

All lifespan analyses were performed at 20°C. Strains were semi-synchronized by allowing gravid adults to lay eggs overnight and then removing the adult worms. Worms were grown for several days until they reached the young adult stage at 15°C. Approximately 150 young adult worms were transferred to five freshly seeded plates containing FuDR to a final concentration of 0.1 mg/mL [77]. Worms were then scored as dead or alive by tapping them with a platinum wire every 2 to 3 days. Worms that died from vulval bursting were censored. Day 1 of the lifespan was when worms were transferred to the FuDR plate. Statistical analyses were done using the standard chi-squared-based log rank test. Lifespans were also verified on non-FuDR NGM plates (Additional file 1: Figure S15).

**DAF-16::GFP analysis**

For measuring the nuclear:cytosolic ratio (Additional file 1: Figure S2 and Figure 3), worms were grown at 15°C until they reached young adult stage. Then, worms were mounted on glass slides in 50 mM sodium azide and visualized using Zeiss AxioScope 2+ microscope. Fluorescent images were taken using OpenLab.3.1.7 software with a Hamamatsu camera. For the quantification, only the region above intestinal cells in the pharynx was considered. Using ImageJ software, the fluorescence was quantified by measuring the pixel intensity in the nuclear versus cytosolic region. The ratio between the respective intensities was then calculated and plotted. This was repeated twice with approximately 14 to 15 worms in each assay.

**RNAi screen**

RNAi knockdown by feeding was performed essentially as described in Timmons et al. [79] using a library of clones representing 892 of the predicted 937 C. elegans transcription factors (MacNeil and Walhout, unpublished). Briefly, 50 μL of an overnight culture of HT115 bacteria carrying RNAi clones was used to inoculate 1 mL LB supplemented with 50 μg/mL ampicillin. Cultures were grown, with shaking, for 6 h at 37°C. Bacteria were pelleted and resuspended in 10% of the original volume. NGM plates containing 5 mM IPTG were seeded with concentrated bacteria and allowed to dry. Synchronized L1 larvae were used to seed prepared RNAi plates. Animals were visually examined for changes in GFP expression during the adult stage.
Availability of supporting data
The datasets supporting the results of this article are included as additional files.

Additional file

Additional file 1: Supplemental Text.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AB contributed to the conception and design, data collection and analysis, manuscript writing, critical revision, and final approval of the manuscript. HC contributed to the conception and design, data collection and analysis, manuscript writing, and final approval of the manuscript. DC contributed to data collection and final approval of the manuscript. HL, AB contributed to the conception and design, data collection and analysis, manuscript writing, and final approval of the manuscript. All authors read and approved the final manuscript.

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