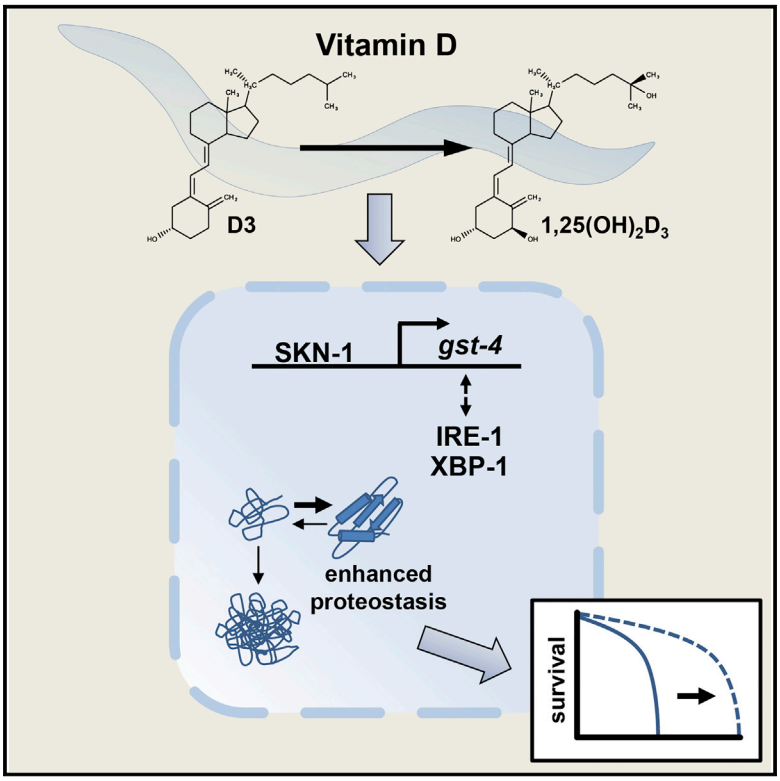
Report

Cell Reports

Vitamin D Promotes Protein Homeostasis and Longevity via the Stress Response Pathway Genes *skn-1*, *ire-1*, and *xbp-1*

Graphical Abstract



Highlights

- Vitamin D metabolism is conserved between nematodes and mammals
- Vitamin D prevents the age-dependent accumulation of SDS-insoluble proteins
- Vitamin D enhances lifespan and protein homeostasis via IRE-1, XBP-1, and SKN-1

Authors

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In Brief

Maintenance of protein homeostasis is crucial to cellular health and contributes significantly to the lifespan of organisms. Mark et al. demonstrate that vitamin D supplementation promotes protein homeostasis and slows aging in the nematode, *C. elegans*. These findings identify a mechanism by which vitamin D influences aging.

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Report

Vitamin D Promotes Protein Homeostasis and Longevity via the Stress Response Pathway Genes *skn-1*, *ire-1*, and *xbp-1*

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SUMMARY

Vitamin D has multiple roles, including the regulation of bone and calcium homeostasis. Deficiency of 25-hydroxyvitamin D, the major circulating form of vitamin D, is associated with an increased risk of age-related chronic diseases, including Alzheimer's disease, Parkinson's disease, cognitive impairment, and cancer. In this study, we utilized *Caenorhabditis elegans* to examine the mechanism by which vitamin D influences aging. We found that vitamin-D3-induced lifespan extension requires the stress response pathway genes *skn-1*, *ire-1*, and *xbp-1*. Vitamin D3 (D3) induced expression of SKN-1 target genes but not canonical targets of XBP-1. D3 suppressed an important molecular pathology of aging, that of widespread protein insolubility, and prevented toxicity caused by human β-amyloid. Our observation that D3 improves protein homeostasis and slows aging highlights the importance of maintaining appropriate vitamin D serum levels and may explain why such a wide variety of human age-related diseases are associated with vitamin D deficiency.

INTRODUCTION

Our understanding of the role of vitamin D has grown significantly over the last several years with evidence that low levels of vitamin D can have a profound effect on human health (Hossein-nezhad and Holick, 2013). Following the discovery of the vitamin D receptor (VDR), which is expressed in a wide range of tissues, the role of vitamin D in the prevention and treatment of chronic diseases has become an important area of study (Holick, 1992; Kalueff and Tuohimaa, 2007). Vitamin D deficiency has been linked to various health problems, including cognitive decline, depression, cardiovascular disease, hypertension, type 2 diabetes, and cancer (Butler et al., 2011; Chan, 2011; Holick, 2003; Ingraham et al., 2008; Ito et al., 2011; Liu et al., 2013). During aging, the risk for vitamin D deficiency significantly increases due to reduced nutritional intake of vitamin D, increased adiposity, and decreased cutaneous synthesis of vitamin D. This has led to considerable debate regarding vitamin D supplementation in the elderly and whether deficiencies in vitamin D represent an indicator of ill health or increases one's susceptibility to chronic disease (Kupferschmidt, 2012).

Vitamin D is a member of the superfamily of secosteroid hormones. There are two major forms of vitamin D, vitamin D2 (ergocalciferol; D2), which is produced by the UV radiation of ergosterol, and vitamin D3 (cholecalciferol; D3), which is a photoproduct produced in the skin from 7-dehydrocholesterol (7DHC) (Smith and Holick, 1987). The vitamin D photoproduct is biologically inert, requiring two separate hydroxylation steps by cytochrome P450 enzymes to produce the biologically active form of vitamin D, 1,25-dihydroxyvitamin D (1,25-$(OH)_2D_3$) (Figure 1A). As the concentration of 1,25-$(OH)_2D_3$ increases, VDRs throughout the body become activated, resulting in extensive alterations in gene expression and numerous physiological alterations.

C. elegans is an excellent model for longevity studies and investigating aspects of chronic disease pathology. Many of the classical signaling pathways and transcription factors that modulate stress response and aging have been identified in the nematode. Enhancing the activity of the FOXO transcription factor DAF-16, which functions in the insulin/IGF-1 signaling pathway, significantly increases lifespan (Kenyon, 2005). Additionally, the activity of the heat shock transcription factor, HSF-1, and the Nrf2-like xenobiotic and oxidative stress-response factor, SKN-1, also affect normal aging in the worm (Tullet et al., 2008). These stress response transcription factors up- or downregulate a diverse range of target genes.

Protein homeostasis plays an important role in aging and age-related disease. Normal aging in *C. elegans* is associated with a loss in protein homeostasis and an accumulation of insoluble protein (David et al., 2010; Reis-Rodrigues et al., 2012; Walther et al., 2015). Neurological diseases including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) share common cellular and molecular features including protein aggregation and inclusion body formation. Neurotoxic aggregated forms of endogenous proteins, such as amyloid-β (AD), α-synuclein (PD), huntingtin (HD), and TAR DNA-binding protein 43 kDa (TDP-43; ALS) underlie the pathogenesis of these diseases.

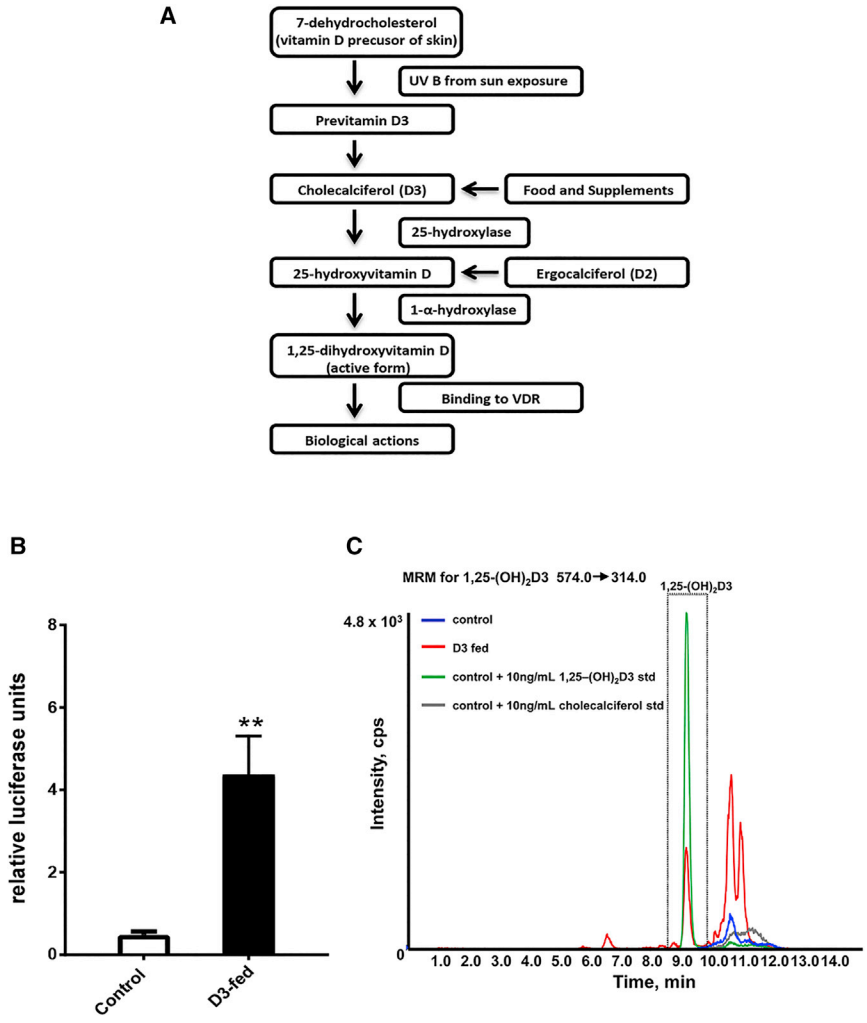

Figure 1. *C. elegans*-Fed D3 Are Capable of Synthesizing the Biologically Active Form of D3, 1,25-$(OH)_2D_3$, and Lipid Extracts Derived from D3-Fed Worms Can Activate Human Vitamin D Receptor Transcriptional Activity

(A) Diagram of the human vitamin D metabolic pathway.

(B) Lipid extracts derived from D3-fed worms activated human VDR transcriptional activity as evidenced by increased luciferase activity compared to control-treated worms. Data are presented as relative luciferase units. Error bars indicate mean + SEM (**$p < 0.05$, unpaired t test, $n = 3$).

(C) Liquid chromatography/mass spectrometry (LC-MS) extracted ion (MRM of m/z 574→314) chromatogram of detected 1,25-$(OH)_2D_3$ from lipid extracts of wild-type (N2) worms synchronously grown until the second day of adulthood on either control or D3 (100 μM) NGM plates. The D3-fed lipid extracts revealed a signal identical to the 1,25-$(OH)_2D_3$ standard, indicated by the boxed green signal. There was no 1,25-$(OH)_2D_3$ detected in the control lipid extracts.

Analogous to their effects on longevity, DAF-16, HSF-1, and SKN-1 all contribute to maintenance of protein homeostasis in *C. elegans* (Alavez et al., 2011; Dostal et al., 2010). In *C. elegans*, DAF-16 and HSF-1 both regulate the formation of age-induced polyglutamine-repeat protein aggregates, similar to those found in HD (Hsu et al., 2003). Deficiency in either DAF-16 or HSF-1 correlates with premature accumulation of age-associated insoluble protein (Walther et al., 2015). SKN-1 has also been shown to be required for the maintenance of protein homeostasis (Alavez et al., 2011). Additionally, SKN-1 activity is associated with another mechanism previously shown to be important in lifespan extension, the endoplasmic reticulum unfolded protein response (ER-UPR) (Glover-Cutter et al., 2013), which is induced in response to proteotoxic stress in the ER to suppress the accumulation of unfolded or misfolded proteins (Zhang and Kaufman, 2006). In *C. elegans*, not only does SKN-1 play a prominent role in the transcriptional regulation of the ER-UPR, but specific ER-UPR regulators are also, in turn, important for SKN-1 target gene expression (Glover-Cutter et al., 2013). Consistent with the hypothesis that impaired protein homeostasis can drive aging, we and others have shown that normal aging is associated with insoluble protein accumulation, and genes encoding these insoluble proteins are enriched for those that determine lifespan (David et al., 2010; Reis-Rodrigues et al., 2012).

Vitamin D has been shown to extend lifespan in *C. elegans* (Messing et al., 2013). Moreover, short-term treatment with vitamin D reduces amyloid-β (Aβ) peptide aggregation and improves cognition in mouse models of AD (Durk et al., 2014). These observations prompted us to investigate whether vitamin D promotes widespread cellular protein homeostasis and consequently influences aging. We found that D3 feeding suppressed the toxicity induced by human β-amyloid ($A\beta_{3-42}$) aggregation and rescued paralysis of worms expressing a metastable perlecan protein. Critically, we found that vitamin D3 treatment slowed proteome-wide, age-related protein insolubility. We examined the mechanism by which vitamin D influences protein homeostasis and longevity and found that the beneficial effects of vitamin D3 require the stress response pathway genes SKN-1, IRE-1, and XBP-1. The role for this secosteroid hormone in suppressing age-related proteotoxic stress provides an explanation for the observed elevated risk for neurological disease associated with human vitamin D deficiency.

RESULTS

C. elegans Can Metabolize Vitamin D3 to 1,25-Dihydroxyvitamin D3

To test the suitability of *C. elegans* as a model for investigating vitamin D mechanisms, we asked whether worms fed vitamin D3 have the ability to produce the bioactive form of vitamin D3,

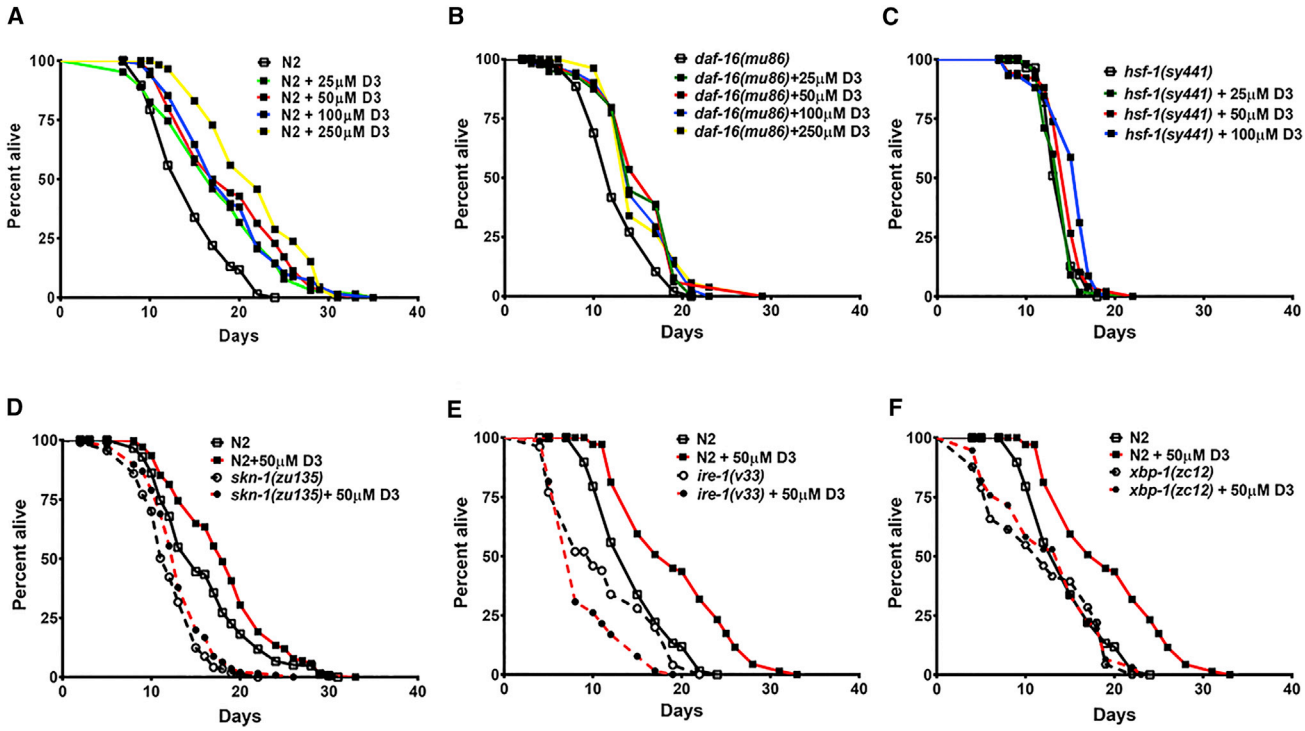

Figure 2. Vitamin D3 Requires *skn-1*, *ire-1*, and *xbp-1* Stress Response Genes for Lifespan Extension

(A) Kaplan-Meier survival curves of N2 hermaphrodite worms exposed to increasing concentrations of D3 from day 1 of adulthood ($p < 0.0001$; log-rank test).

(B) D3 (25–250 μM) extended the lifespan of CF1038 [*daf-16(mu86)*] worms, which lack functional DAF-16 protein, when treated from day 1 of adulthood at 20°C ($p < 0.0001$, log-rank test).

(C) D3 (25–100 μM) feeding resulted in marginal lifespan extension in PS3551 [*hsf-1(sy441)*] ($p = 0.0090$, log-rank test).

(D) D3 (50 μM) treatment resulted in little or no lifespan extension of the EU31 [*skn-1(zu135) IV/nT1 [unc-?(n754) let-?]*] worms ($p = 0.002$, log-rank test).

(E) D3 (50 μM) treatment shortens the lifespan in RE666 [*ire-1(v33)*] mutant worms ($p < 0.0001$, log-rank test).

(F) D3 (50 μM) treatment does not extend lifespan in SJ17 [*xbp-1(zc12); zcIs4*] mutant worms ($p = 0.142$, log-rank test).

1,25-dihydroxyvitamin D3 (1,25-$(OH)_2D_3$), which is required for VDR activity. We grew large populations of synchronously aging N2 wild-type hermaphrodite worms at 25°C on either vitamin-D3- or control-treated nematode growth media (NGM) plates and prepared lipid extracts on the second day of adulthood (5-day-old worms). We tested the worm lipid extracts for biological activity in a one-hybrid human cell-based VDR activity assay and found that lipid extracts made from D3-fed worms showed enhanced human VDR transcriptional activity, as evidenced by increased luciferase activity, compared to control-treated worms (Figure 1B). Addition of vitamin D3 alone to the VDR expressing cells had no effect on VDR transcriptional activity (data not shown). This demonstrated that *C. elegans* worms are able to metabolize vitamin D3 into a ligand that activates human VDR. To test whether worms metabolized vitamin D3 to the known active ligand, 1,25-$(OH)_2D_3$, lipid extracts made from vitamin D3-fed worms were subjected to liquid chromatography/mass spectroscopy (LC-MS). A signal identical to the 1,25-$(OH)_2D_3$ standard was present in the D3-fed lipid extracts, but not in extracts from control-treated worms (Figure 1C). Quantification of the amount of 1,25-$(OH)_2D_3$ in the lipid extracts derived from D3-fed worms revealed approximately 5.95E-03 pg/worm. By comparison, in humans, plasma 1,25-$(OH)_2D_3$ levels range from 10 to 70 pg/mL (Bikle et al., 1984). Since *C. elegans* are grown on a live *Escherichia coli* (*E. coli*) food source, we tested whether exposure of *E. coli* to D3 would result in 1,25-$(OH)_2D_3$ production but found that the bacteria alone did not make this active form of vitamin D (data not shown). Collectively these data demonstrated that *C. elegans* are capable of synthesizing 1,25-$(OH)_2D_3$, and that lipid extracts derived from these worms can activate human VDR, confirming that this critical component of vitamin D metabolism is conserved between nematodes and mammals.

Vitamin D3-Induced Lifespan Extension Requires SKN-1, IRE-1, and XBP-1

We confirmed that vitamin D3 extended *C. elegans* lifespan (Messing et al., 2013). Feeding vitamin D3 throughout adulthood extended lifespan in a dose-dependent manner, and was not toxic even at the highest concentration (250 μM) tested (Figure 2A; Table S1). Given that normal aging is modulated by a network of transcription factors (Hsu et al., 2003; Tullet et al., 2008), we tested whether members of this network were required for the beneficial effects of vitamin D3 on lifespan in *C. elegans*. First, we tested the requirement of DAF-16 in D3-induced lifespan extension. We found that D3 feeding extended the lifespan of *daf-16(mu86)* worms, which lack functional DAF-16 protein (Figure 2B; Table S1). Additionally, D3 treatment did not alter

the subcellular localization of a DAF-16::GFP fusion protein (TJ356 strain; data not shown). These data suggest that the effect of D3 feeding on lifespan extension is independent of DAF-16. In addition, we found that vitamin-D3-induced lifespan extension did not require DAF-12 (Figures S1A and S1B; Table S1), the proposed ortholog of VDR in *C. elegans* (Antebi et al., 2000; Mangelsdorf et al., 1995). Furthermore, in a cell-based luciferase reporter assay, 1,25-$(OH)_2D_3$ did not increase DAF-12 transcriptional activity (data not shown). Taken together, we conclude that vitamin-D3-induced lifespan extension is independent of DAF-12. We next tested the requirement of the HSF-1 in D3-induced lifespan extension. D3 treatment resulted in marginal or no lifespan extension in *hsf-1(sy441)* mutant worms (Figure 2C; Table S1), suggesting that the effect of D3 on lifespan may partially require the participation of HSF-1-regulated genes. Last, we examined the effect of SKN-1 in D3-induced lifespan extension. We observed no lifespan extension by D3 for *skn-1(zu135)* mutant worms, demonstrating that SKN-1 is required for the effects of D3 feeding (Figure 2D; Table S1).

SKN-1 activity is associated with another mechanism important in lifespan extension, the endoplasmic reticulum unfolded protein response (ER-UPR) (Glover-Cutter et al., 2013). Specifically, SKN-1 regulates transcription of the entire core of the ER-UPR and many downstream ER stress defense genes. Moreover, ER stress influences the levels of *skn-1* mRNA and SKN-1 protein (Glover-Cutter et al., 2013). Proteotoxic stress triggers the ER-UPR by activating the stress sensors ribonuclease inositol requiring protein-1 (IRE-1), PERK kinase homolog (PEK-1), and activating transcription factor-6 (ATF-6) (Calfon et al., 2002; Shen et al., 2001, 2005). Activation of each sensor produces a transcription factor that activates genes to increase the protein-folding capacity in the ER. Of the three stress responsive ER-UPR pathways, IRE-1 is the most conserved. Upon activation of the UPR, IRE1-dependent splicing of a small intron from the *xbp-1* mRNA leads to synthesis of XBP-1 transcription factor, which, in turn, induces expression of *hsp-4* and other ER-UPR-associated genes. Given the requirement for SKN-1 in D3-mediated lifespan extension and that SKN-1 and the ER-UPR form a regulatory network, we tested the dependency of each ER-UPR pathway for the lifespan response to D3 feeding. We found that the D3-induced increase on survival was dependent on IRE-1/XBP-1 signaling. Worms carrying the loss-of-function allele, *ire-1(v33),* showed significantly reduced lifespan with D3 feeding compared to vehicle-treated worms (Figure 2E; Table S1). Lifespan of worms maintaining the loss-of-function allele, *xbp-1(zc12)*, showed no significant change with D3 feeding compared to vehicle-treated worms (Figure 2F; Table S1). Interestingly, *ire-1(v33)* mutant worms exhibited a shortened lifespan upon D3 feeding. In contrast, D3 feeding significantly increased lifespan in worms maintaining loss-of-function alleles for *pek-1* and *atf-6* (Table S1; Figure S1C). Collectively, these data specifically implicate the stress response genes SKN-1, IRE-1, and XBP-1 in vitamin-D3-induced lifespan extension.

Vitamin D3 Induces SKN-1, but Not HSF-1, nor ER-UPR Gene Targets

Given that the effect of D3 feeding on lifespan is dependent on a genetic network, we next surveyed the downstream target genes of HSF-1, SKN-1, and IRE-1/XBP-1. We examined the effect of D3 feeding on the expression of an HSF-1 target gene encoding a molecular chaperone, using the transgenic transcriptional reporter strain, *phsp-16.2::GFP*. D3 treatment had no effect on the expression of this transcriptional reporter (Figures S2A and S2C). We examined other molecular chaperones not under direct regulation by HSF-1, *hsp-6* (mitochondrial chaperone) and *hsp-4* (ER chaperone). Using the transcriptional reporter stains *phsp-6::GFP* and *phsp-4::GFP*, we found that vitamin D3 feeding only increased the levels of *hsp-4* expression (Figures S2A, S2B, and S2D). HSP-4 is a direct target of the IRE-1/XBP-1 pathway, and it is upregulated in response to ER stress. Surprisingly, we failed to observe a significant D3-associated upregulation of *hsp-4* mRNA levels in wild-type N2 worms as assessed by RNA sequencing (RNA-seq) and quantitative real-time PCR at various time points (data not shown). These results indicate that lifespan extension from D3 requires IRE-1/XBP-1 but does not appear to result in robust constitutive induction of the downstream XBP-1 target gene, *hsp-4*.

We then tested the effects of D3 treatment on the expression of a target of SKN-1 using a transgenic transcriptional reporter strain *pgst-4::GFP*. GST-4 (glutathione transferase-4) is involved in the phase II oxidative stress response and its expression reports on SKN-1 activity. D3 feeding significantly upregulated *pgst-4::GFP* compared to control-treated worms (Figure 3A) in a SKN-1-dependent manner (Figure 3B). We confirmed this result by quantitative real-time PCR analysis of endogenous *gst-4* mRNA levels (Figure 3C).

To gain a more detailed picture of the genomic response to vitamin D3, we undertook a genome-wide analysis of altered mRNA abundance. Specifically, synchronous populations of D3 fed and control L4 stage hermaphrodite worms were processed for RNA-sequencing. We observed 253 significantly upregulated and 78 significantly downregulated genes in response to vitamin D3 treatment (data not shown). Gene ontology (GO) analysis of this dataset revealed several clusters of genes with functional properties that are consistent with previously reported microarray studies of 1,25-$(OH)_2D_3$-regulated genes (Heikkinen et al., 2011; Hossein-nezhad et al., 2013). These included a significant enrichment of genes associated with apoptosis, immune functions, response to stimulus, transport, cellular component organization, development, and metabolism.

Given the dependency of HSF-1, SKN-1, and IRE-1/XBP-1 in D3-induced lifespan extension, we further examined our RNA-seq dataset to determine whether expression of target genes of any of these transcription factors might be perturbed by vitamin D3 treatment. First, we examined whether our RNA-seq dataset was enriched for heat shock proteins (HSPs), since HSF-1 has been shown to be a major transcriptional regulator of these genes. We observed no significant enrichment for HSPs in the transcriptional effects of D3 feeding. These data are consistent with our previous finding that D3 feeding had no effect on the molecular chaperone transcriptional reporter strain, *phsp-16.2::GFP*. We next examined SKN-1 gene targets from a previously reported array that examined differential expression between *skn-1* knockdown and control worms, profiled at L4 larval stage (Oliveira et al., 2009). Comparison of our dataset with the subset of genes found to be downregulated in *skn-1*

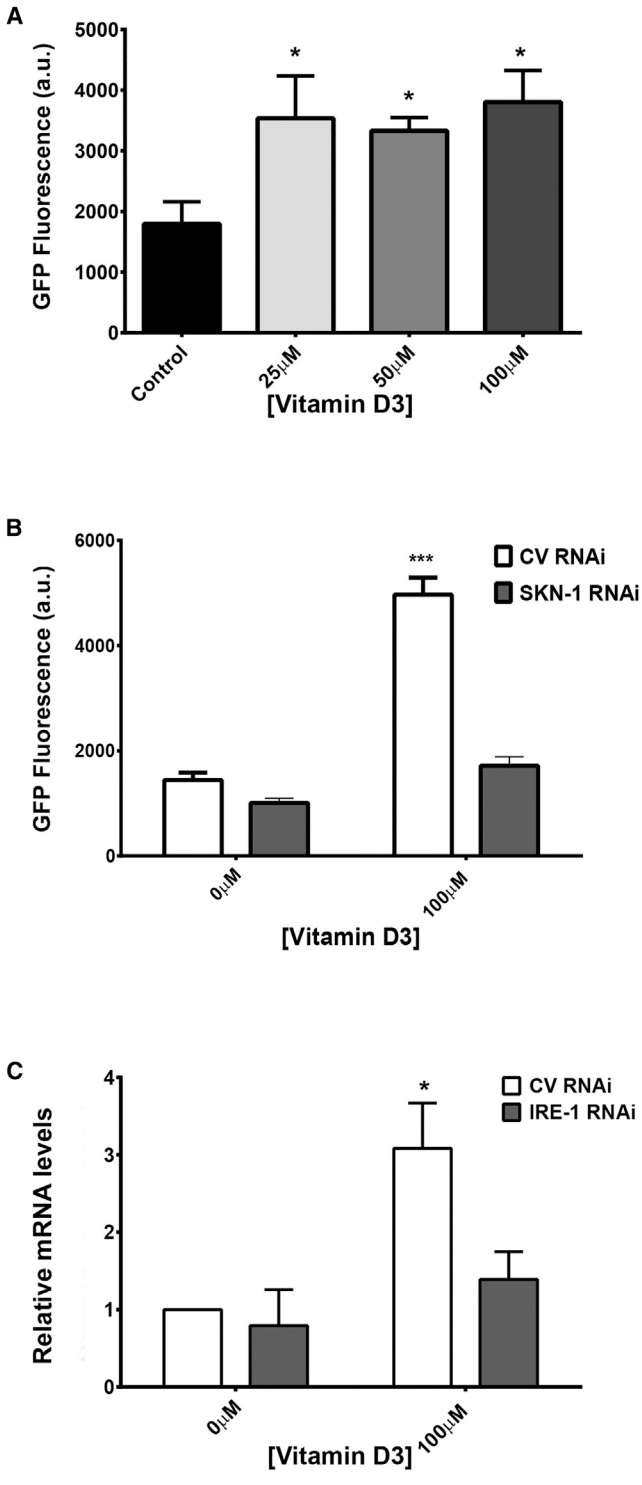

Figure 3. Vitamin-D3-Induced Activation of the SKN-1 Target Gene *gst-4* Is IRE-1 Dependent

(A) Quantification of a reporter strain (CL2166) containing *pgst-4::GFP* following D3 feeding (25–100 μM) from L1 larval stage and scored at L4 larval stage at 15°C. Data are represented as GFP Fluorescence (arbitrary units, a.u.).

(B) Reducing SKN-1 by *skn-1* RNAi prevents the D3-induced increase in *pgst-4::GFP* fluorescence.

(C) Relative *gst-4* mRNA levels (mean + SEM) in N2 worms fed D3 (100 μM). *ire-1* RNAi prevents the D3-induced increase in *gst-4* mRNA levels. Data are presented as relative mRNA levels. (*$p < 0.05$, unpaired t test, $n = 3$).

knockdown animals revealed a striking enrichment for genes expressed in response to D3 feeding (empirical $p = 10^{-6}$; Table S2). Genes negatively regulated by SKN-1 were not significantly perturbed by vitamin D (empirical $p = 0.48$).

Since SKN-1 activates the transcription of genes encoding phase II detoxification enzymes in response to oxidative stress (An and Blackwell, 2003) and vitamin D induces SKN-1 gene targets, we next evaluated whether D3 induced oxidative stress. To test this, we measured reactive oxygen species (ROS) levels, using the superoxide ROS indicator dihydroethidium (DHE), in synchronously aged N2 worms grown from eggs on either vitamin-D3- or control-treated NGM plates. We found that DHE-derived fluorescent ethidium levels were unchanged between D3-treated and vehicle-treated worms. In contrast, N2 worms treated with paraquat (PQ), a known oxidative stress inducer, had significantly increased ROS levels compared to both vitamin-D3- or control-treated worms (data not shown). We asked whether vitamin-D3-treated worms differed in their resistance to PQ. Treatment with either vitamin D3 or vehicle control during development and subsequent exposure to PQ resulted in no difference in survival between D3 and control worms. These data indicate that vitamin D does not induce oxidative stress nor oxidative stress resistance.

We next considered whether ER-UPR gene targets could be affected by vitamin D feeding. To test this, we used three previously reported definitions of the ER-UPR pathway: genes annotated in the ER-UPR according to the Gene Ontology consortium (http://amigo.geneontology.org/amigo); genes dependent on *ire-1* and/or *xbp-1* for response to the UPR inducer tunicamycin (Shen et al., 2005); and genes dependent on *pek-1* and/or *atf-6* for tunicamycin response. In contrast to our findings with SKN-1, in each cohort, and in a cohort defined as their union, we observed no significant enrichment for the transcriptional effects of D3 feeding (empirical $p = 0.17$, 0.99, 0.71, and 0.78, respectively), consistent with our single-gene analyses of ER-UPR targets (data not shown). Given the IRE-1/XBP-1 and SKN-1 dependency for D3 lifespan extension, we further examined the crosstalk between these pathways. Interestingly, we found that reduction of IRE-1 by RNAi suppressed the D3-induced increase in *gst-4* mRNA levels (Figure 3C). In contrast, reduction of XBP-1 by RNAi resulted in elevated *gst-4* mRNA levels in D3-treated worms. Although IRE-1 and XBP-1 together regulate transcription of most inducible ER-UPR genes, there is evidence that IRE-1 may have additional distinct functions independent of XBP-1 (Hollien and Weissman, 2006; Shen et al., 2005; Urano et al., 2000; Yoneda et al., 2001), and that *xbp-1*(RNAi) increases *gst-4* mRNA abundance in the absence of vitamin D treatment (Glover-Cutter et al., 2013).

Vitamin D3 Reduces Age-Dependent Insoluble Protein Accumulation

Given the connection between lifespan and protein homeostasis, and the known role for IRE-1/XBP-1 and SKN-1 in both

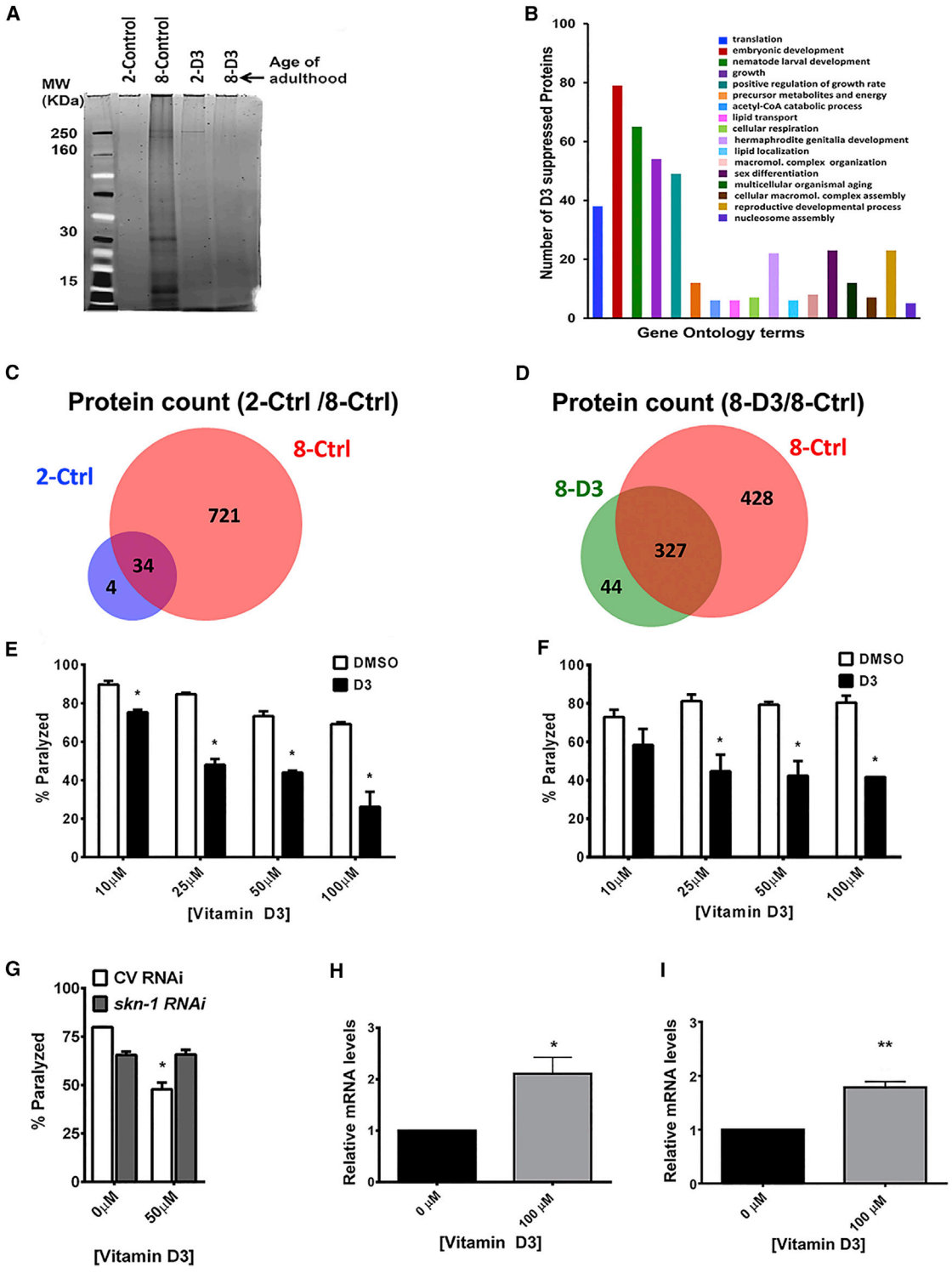

Figure 4. Vitamin D3 Feeding Prevents the Accumulation of Insoluble Proteins in Aged *C. elegans* and Slows Protein Aggregation-Associated Paralysis

(A) SDS-PAGE of the SDS-insoluble fraction of cellular proteins from 2- and 8-day adult TJ1060 worms grown at 25°C. D3 (100 μM) prevents the accumulation of SDS-insoluble proteins in aged worms.

(B) Gene ontology (GO) analysis of proteins observed in the insoluble fraction of old worms that were suppressed by D3 treatment (as determined by quantitative mass spectrometry/MS1 filtering). Classification is as assigned by Klusters of Orthologous Groups (KOG).

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protein homeostasis and longevity, we pursued the hypothesis that vitamin D3 treatment might control lifespan via improving protein homeostasis. Using an unbiased biochemical and proteomic approach, we tested whether vitamin D3 modulated protein homeostasis by analyzing the age-dependent accumulation of SDS-insoluble proteins. We grew large populations of synchronously aged, sterile TJ1060 *C. elegans* and collected worms at day 2 and day 8 of adulthood at 25°C. Worm protein extracts were prepared as described in Experimental Procedures. Purified SDS-insoluble proteins were re-solubilized, subjected to in-solution tryptic digestion, and analyzed by tandem mass spectrometry on a TripleTOF 5600 (Tables S3A–S3H). We found that in aged worms, D3 treatment significantly decreased the number of detectable and identified SDS-insoluble proteins compared to control samples (Figures 4A–4D; Table S3). We next applied a quantitative approach to compare the relative levels of peptides (and thus proteins) between the D3- and control-treated samples, using a label-free quantitative proteomics method referred to as "Skyline MS1 Filtering" (Rardin et al., 2013; Schilling et al., 2012). We determined that D3 feeding significantly reduced the abundance of most proteins detected in aged samples (Figure S3); we observed a 2- to 13-fold reduction of these proteins (Tables S3I–S3M; Figure S3). GO analysis revealed that the SDS-insoluble fraction in control older worms contained a significant enrichment of proteins associated with ribosomes, translation, mitochondrial function, energy metabolism, growth, and development (Figure 4B). D3 treatment reduced the formation of insoluble proteins across a wide range of predicted functions and cellular compartments. Previous work found that reducing expression of several genes encoding proteins suppressed by D3 treatment in aged worms by RNAi resulted in significant lifespan extension (Table S4) (Reis-Rodrigues et al., 2012). Together, this supports the hypothesis that decreasing protein insolubility can prolong lifespan.

SKN-1, IRE-1, and XBP-1 Are Required for the Beneficial Effects of Vitamin D3 on Protein Homeostasis

We further investigated whether vitamin D could suppress toxicity associated with expression of the human neurotoxic peptide, amyloid beta. We employed a well-characterized model of human proteotoxic disease, the strain CL4176, which expresses an aggregation prone amyloid-β peptide ($A\beta_{3-42}$) (Drake et al., 2003; McColl et al., 2009). When shifted from a permissive temperature (15°C) to a restrictive temperature (25°C), worms expressing this peptide accumulate Aβ aggregates and become paralyzed. D3 feeding decreased the proportion of paralyzed CL4176 worms in a dose-dependent manner (Figure 4E). To further probe the suppression of Aβ-associated paralysis, we examined the effect of several vitamin D metabolites, many of which can be converted to active vitamin D ligand in humans. We found that all vitamin D metabolites downstream of 7DHC suppressed Aβ-induced paralysis (Figure S4A). We then utilized a protein folding "sensor" strain, HE250, which carries a mutation in the endogenous gene *unc-52* resulting in the expression of a metastable muscle specific protein, UNC-52 (perlecan). At 25°C, the UNC-52 mutant protein exhibits altered structure and subsequently causes paralysis (Zengel and Epstein, 1980). D3 suppressed the paralysis of this mutant (Figure 4F), demonstrating that D3 prevents a detrimental physiological outcome of proteostatic loss.

Consistent with our lifespan experiments, we found that reduction of *ire-1*, *xbp-1*, or *skn-1* by RNAi prevented D3-induced suppression of paralysis in the perlecan HE250 strain (Figures 4G, S4B, and S4C). In contrast, reduction of *pek-1* and *atf-6* expression by RNAi had no effect on D3-induced suppression of paralysis (Figure S1D). Since vitamin D-induced suppression of paralysis is SKN-1 dependent, we measured *gst-4* mRNA expression prior to the onset of paralysis in the protein misfolding strains. We found that D3 significantly increased *gst-4* mRNA levels in both HE250 and CL4176 strains (Figures 4H and 4I).

DISCUSSION

Numerous hormonal and intracellular signaling pathways are conserved between nematodes and mammals. We have demonstrated an apparent conservation of metabolism and action of the hormone vitamin D in *C. elegans*. D3-fed worms can synthesize physiological levels of bioactive 1,25-$(OH)_2D_3$. Unlike mammals, where cholesterol is the major synthesized sterol, the major sterol found endogenously in *C. elegans* is the provitamin D, 7DHC (Chitwood et al., 1983; Lee et al., 2005). Thus worms have the necessary steroid hormone precursor to synthesize 1,25-$(OH)_2D_3$. Populations of *C. elegans* species dwell on rotting fruits (Félix et al., 2013) where they likely have access to sunlight sufficient to enable the conversion of 7DHC to D3. While it remains to be seen whether *C. elegans* wild strains utilize vitamin D in a natural setting, the conserved metabolism we observe suggests that this organism may be a good model to study the effects of vitamin D on aging and age-related pathologies.

Our results demonstrate that dietary D3 reduced the age-dependent formation of insoluble proteins across a wide range

(C and D) Mass spectrometry quantification of unique proteins in (C) young (day 2) and aged (day 8) control-treated worms, and (D) comparison between proteins identified in aged (day 8) D3-treated and aged control worms.

(E) Exposing worms to D3 (10–100 μM) suppresses the paralysis phenotype associated with protein aggregation in CL4176 expressing $A\beta_{(3-42)}$ in the muscle after 34 hr at 25°C. Data are presented as percentage paralyzed. DMSO bars represent amount of solvent used for D3 feeding (*$p < 0.05$, unpaired t test; $n > 50$ hermaphrodites).

(F) D3 treatment (10–100 μM) rescues the paralysis in the strain HE250 *[unc-52(e669su250)]* after 42 hr at 25°C. Data are presented as percentage paralyzed. DMSO bars represent amount of solvent used for D3 feeding (*$p < 0.05$, unpaired t test; $n > 50$ hermaphrodites).

(G) Reducing SKN-1 by *skn-1* RNAi prevents the D3-induced suppression of paralysis in the temperature-sensitive strain HE250. Data are presented as percentage paralyzed. DMSO bars represent the equivalent amount of solvent used for D3 treatment (*$p < 0.05$ Multiple t tests comparison, Holm-Sidak method, alpha = 5.0%).

(H and I) Relative *gst-4* mRNA levels (mean + SEM) in HE250 (H) and CL4176 (I) worms fed D3 (100 μM) (*$p < 0.05$, **$p < 0.001$; unpaired t test). Error bars indicate mean + SEM and represent the average of three to four independent experiments, 30–40 worms per group, per experiment.

of predicted functions and cellular compartments. D3 feeding also extends lifespan consistent with the hypothesis that protein insolubility is a factor that determines the rate of aging. The dependency on SKN-1 and the upregulation of SKN-1 gene targets by D3 treatment further suggests that SKN-1 functions to promote protein homeostasis during normal aging. SKN-1 regulates a wide range of stress responses and detoxification factors and is central to a healthy extended lifespan (Blackwell et al., 2015). For example, loss of SKN-1 leads to sensitively to oxidative stress (An and Blackwell, 2003). Oxidative stress can lead to irreversible oxidation, nitration, and carbonylation of proteins, which impairs degradation, and enhances aggregation (Poon et al., 2006; Squier, 2001). A recent study showed that vitamin D3 deficiency induces mild oxidative stress in the rat muscle, as observed by increased protein carbonyls and altered antioxidant enzyme activities. Conversely, supplementation with vitamin D3 corrected all of these oxidative stress defects (Bhat and Ismail, 2015). In addition to its role as an regulator of antioxidant functions, SKN-1 plays an important part in maintaining protein homeostasis (Li et al., 2011). SKN-1 maintains protein homeostasis by regulating proteasome subunit gene expression and activity in response to perturbations in either protein synthesis or degradation. Furthermore, the connection between SKN-1 and the ER-UPR indicates cooperativity between these pathways to promote protein homeostasis (Glover-Cutter et al., 2013). Our results further these findings, demonstrating that the beneficial effects of vitamin D3 on lifespan and protein homeostasis are dependent upon the cooperative actions of this stress response network.

The role of SKN-1 in the regulation of detoxification genes is also likely to contribute to the benefits of vitamin D supplementation. Our RNA-seq data revealed that D3 feeding resulted in upregulation of several phase I (cytochrome P450 and short-chain dehydrogenase/reductase), phase II (UGT-UDP-glucuronosyltransferase and gluthathione S transferases) genes, and ATP-binding cassette (ABC) transporters. Collectively, these five gene classes act together in drug metabolism and excretion. Previously, it was reported that long-lived *C. elegans* dauer larvae and *daf-2* mutants shared a significant enrichment for several classes of detoxification genes (McElwee et al., 2004), supporting the theory that aging occurs as a result of internal molecular damage, which gives rise to a wide range of toxic lipophilic compounds. This theory postulates that induction of detoxification genes reduce levels of these toxic species that limit lifespan. Our findings suggest that vitamin D3 has a broad effect on systemic detoxification, which, in turn, could reduce toxic compounds and promote longevity. Thus, further investigation into the biochemical and cellular processes these detoxifying genes might be influencing will be important in understanding the beneficial actions of vitamin D.

In this study, we have shown that vitamin D promotes protein homeostasis and slows aging via IRE-1, XBP-1, and SKN-1 functions. Our results demonstrate that dietary supplementation of *C. elegans* with D3 results in endogenous 1,25-$(OH)_2D_3$ production at a physiologically relevant range and has profound effects on lifespan and protein homeostasis. This is an interesting observation when considered alongside the fact that there is a decline in efficient vitamin D production with age in humans. While the benefits of dietary supplementation in humans are highly controversial (de Paula and Rosen, 2012), there are considerable epidemiological data correlating vitamin D deficiency to multiple diseases. However, causality has not been clearly established, with the possibility that low vitamin D levels are a marker of ill health (Rosen and Manson, 2010). Our results suggest that, in *C. elegans*, an absence of vitamin D in the diet accelerates age-related loss-of-protein homeostasis and shortens lifespan. Supporting this idea, the vitamin D receptor knockout mouse exhibits some premature aging phenotypes (Keisala et al., 2009), although mouse models of hypervitaminosis D also appear to prematurely age (Tuohimaa, 2009). If vitamin D generally affects aging in mammals, it will be of interest to establish whether Nrf2-regulated gene networks have a role to play (Nakai et al., 2014).

EXPERIMENTAL PROCEDURES

Strains

Strains were cultured under standard laboratory conditions. All strains used in this study were obtained from the *Caenorhabditis* Genetics Center (CGC) and are detailed in the Supplemental Experimental Procedures.

Lifespan Assays

Lifespan assays were performed as described previously (Lithgow et al., 1995). Nematodes were transferred to fresh compound plates every 3–5 days. All lifespan experiments were performed at 20°C. Lifespan data were analyzed by GraphPad Prism v.7.01, and p values were calculated using the Mantel-Cox log-rank test.

Worm Paralysis Assays

Synchronized populations of HE250 [*unc-52(e669su250)II*] and CL4176 [*dvIs27 [myo::A$\beta_{(3-42)}$-let 3′UTR(pAF29); pRF4 (rol-6(su1006))*] were used in these studies. See Supplemental Experimental Procedures for details on the treatment groups, experimental conditions, and scoring of the paralysis assays.

RNAi Knockdown of Gene Expression

RNAi bacteria strains expressing double-stranded RNA that inactivates specified genes were cultured and used as previously described (Timmons et al., 2001).

Microscopy and Quantification of GFP Fluorescence

See Supplemental Experimental Procedures for details on mounting and imaging of worms expressing GFP.

Lipid Extracts

Lipid extracts were generated by a modification of the method described previously (Gill et al., 2004). See Supplemental Experimental Procedures for details on preparation of worm samples, treatment groups, and experimental conditions.

Liquid Chromatography/Mass Spectrometry

Diels-alder derivatization of 1,25$(OH)_2D_3$ was adapted from previously established methods (Aronov et al., 2008). See Supplemental Experimental Procedures for details on preparation of worm samples, treatment groups, instrument, and experimental conditions.

Plasmid Construction

We obtained the vitamin D receptor (VDR) clone (Id # 30343975) from Open Biosystems. VDR amino acid 141–477 was PCR amplified and cloned into the pBIND vector (Promega) as a BamH1-Xba1 fragment, and the sequence was verified.

Transfection Assays

HEK293T were used in these assays. See Supplemental Experimental Procedures for details on preparation of cells, treatment groups, and experimental

conditions. Luciferase activity was normalized to the GFP values. Results are expressed as mean + SEM for three experiments.

C. elegans Insoluble Protein Extraction

TJ1060 [*spe-9(hc88)I; fer-15(b26)II*] temperature sensitive mutants were grown until gravid adults in synchronous mass cultures (Fabian and Johnson, 1994). See Supplemental Experimental Procedures for details on preparation of worm samples, treatment groups, and instrument and experimental conditions.

Gel Electrophoresis of SDS-Insoluble Protein Samples

See Supplemental Experimental Procedures for details on preparation of nematode samples. The SDS-insoluble protein fraction was then visualized with SYPRO Ruby gel staining.

In Solution Digestion and Mass Spectrometric Analysis of the SDS-Insoluble Protein

See Supplemental Experimental Procedures for details on preparation of *C. elegans* samples, treatment groups, instrument, and experimental conditions.

Bioinformatic Database Searches

Mass spectrometric data were searched using the database search engine ProteinPilot (Shilov et al., 2007) (AB SCIEX Beta 4.5, revision 1656) with the Paragon algorithm (4.5.0.0, 1654). A detailed protocol can be found in Supplemental Experimental Procedures.

Quantitative Skyline MS1 Filtering Analysis

MS1 chromatogram based quantification was performed in Skyline 1.4 an open source software project (https://proteome.gs.washington.edu/software/skyline) as previously described in detail by Schilling et al. (2012). A detailed protocol can be found in Supplemental Experimental Procedures.

Quantitative-Data-Independent Acquisitions, SWATH-MS2 Analysis

Proteomic analysis was generated by SWATH-MS2 analysis (Gillet et al., 2012). A detailed protocol can be found in Supplemental Experimental Procedures.

Functional Analysis: Protein Ontology

The web-based program DAVID v.6.7 (The Database for Annotation, Visualization and Integrated Discovery) was used for functional analysis and protein ontology analysis (Huang et al., 2009). A detailed protocol can be found in Supplemental Experimental Procedures.

Raw Data Accession and Panorama Spectral Libraries

The raw and processed data associated with this manuscript may be downloaded from MassIVE at https://massive.ucsd.edu/ProteoSAFe/datasets.jsp; to access the dataset, use the MassIVE ID number MSV000079132. The spectral viewer can be accessed at https://panoramaweb.org/labkey/project/Schilling/Nature_Lithgow_Celegans_VitaminD3/begin.view?.

RNA Sequencing, Gene Expression Profiling, and Bioinformatic Analysis

RNA was extracted and quantified as described in Supplemental Experimental Procedures. RNA samples were then sent to the University of Minnesota BioMedical Genomics Center for Illumina HiSeq RNA-seq, where RNA-seq (50-bp paired-end sequencing) was carried out on a HiSeq2000 according to the manufacturers protocols (Illumina) after size selecting for an insert averaging ~200 bp. Average quality scores for the completed run across all 12 samples was >30, with an average of greater than 20 million reads per sample. The sequencing reads were then mapped to the worm genome WBcel235 (GenBank ID GCA_000002985.3) for differential gene expression analysis via the "seed and vote" workflow using the package Rsubread (Liao et al., 2014) in bioconductor (Gentleman et al., 2004). For the mapped reads, greater than 95% of sequencing reads in each sample was mapped to the reference worm genome. RNA-seq data have been deposited in the NCBI GEO under accession number GSE86493.

RNA Extraction and Quantitative Real-Time PCR

See Supplemental Experimental Procedures for details on preparation of *C. elegans* RNA samples, treatment groups, and primer information. Data were compiled from three independent experiments, and each experiment was conducted in triplicate.

Statistics

Statistical analysis was performed in GraphPad Prism v.7.01, as detailed in the figure legends.

ACCESSION NUMBERS

The accession number for the RNA-seq data reported in this paper is NCBI GEO: GSE86493.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.086.

AUTHOR CONTRIBUTIONS

K.A.M. and G.J.L. conceived the study. K.A.M., K.J.D., and G.J.L. wrote the manuscript with contributions from co-authors. D.B., B.S., S.D., D.J.S., A.R., B.W.G., and M.L. performed the experiments. K.A.M., K.J.D., D.B., B.S., S.D., A.R., R.B.B., T.R.O., and S.M. analyzed the data.

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