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Aging Cell. Author manuscript; available in PMC 2009 July 14.

Published in final edited form as:

Aging Cell. 2008 June ; 7(3): 394–404. doi:10.1111/j.1474-9726.2008.00385.x.

Dietary restriction suppresses proteotoxicity and enhances longevity by an *hsf-1*-dependent mechanism in *C. elegans*

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SUMMARY

Dietary restriction increases life span and slows the onset of age-associated disease in organisms from yeast to mammals. In humans, several age-related diseases are associated with aberrant protein folding or aggregation, including neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's diseases. We report here that dietary restriction dramatically suppresses age-associated paralysis in three nematode models of proteotoxicity. Similar to its longevity-enhancing properties, dietary restriction protects against proteotoxicity by a mechanism distinct from reduced insulin/IGF-1-like signaling. Instead, the heat shock transcription factor, *hsf-1*, is required for enhanced thermotolerance, suppression of proteotoxicity, and life span extension by dietary restriction. These findings demonstrate that dietary restriction confers a general protective effect against proteotoxicity and promotes longevity by a mechanism involving *hsf-1*.

Keywords

C. elegans; dietary restriction; proteotoxicity; longevity; hsf1; proteotoxicity

INTRODUCTION

Dietary restriction is known to increase life span and retard a variety of age-associated pathologies in rodents and invertebrate organisms (Weindruch & Walford 1988; Masoro 2005). Among the diseases beneficially impacted by DR in mice are cancer, diabetes, and cardiovascular disease. In addition, DR has been shown to improve outcome in mouse models of neurodegeneration (Martin *et al.* 2006). The mechanism by which DR confers these benefits remains unknown, but is of high interest.

DR can be studied in the nematode *Caenorhabditis elegans* using either environmental or genetic approaches. In the laboratory, *C. elegans* are typically maintained on a nutrient agar nematode growth medium (Youngman *et al.* 1992) and provided *E. coli* OP50 as their dietary food source. Under these standard growth conditions, life span extension from bacterial food restriction is maximized when animals are maintained on nutrient-agar plates without bacterial food after early adulthood (BD, bacterial food deprivation; also referred to previously as dietary restriction through food deprivation or dietary deprivation) (Kaeberlein *et al.* 2006; Lee *et al.* 2006). While BD is similar in concept to an alternative method for DR

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in *C. elegans*, axenic growth (Vanfleteren & Braeckman 1999), BD is instituted post-developmentally and utilizes standard *C. elegans* growth conditions.

An alternative method of DR in *C. elegans* is the use of mutations that reduce food intake, such as loss of function alleles of *eat-2. eat-2* mutants have defects in pharyngeal pumping (Avery 1993) and an adult life span 20–40% longer than wild type N2 animals (Lakowski & Hekimi 1998). Life span extension from BD is non-additive with the long-lived *eat-2(ad465)* allele (Kaeberlein *et al.* 2006; Lee *et al.* 2006), indicating that BD and mutation of *eat-2* are likely to promote longevity via similar or overlapping mechanisms.

Although the mechanism by which DR increases longevity in *C. elegans* is unknown, DR is thought to act through a pathway that is genetically distinct from insulin/IGF-1-like signaling (IIS). Mutations that decrease IIS, such as loss of function alleles of insulin-like receptor *daf-2* (Kenyon *et al.* 1993; Kimura *et al.* 1997) or the PI3-kinase *age-1* (Friedman & Johnson 1988a; Friedman & Johnson 1988b; Morris *et al.* 1996), increase longevity via a mechanism that is dependent on the FOXO-family transcription factor *daf-16* (Lin *et al.* 1997; Ogg *et al.* 1997). In contrast, life span extension from DR (whether accomplished by BD, axenic growth, or mutation of *eat-2*), does not require *daf-16* and is additive with mutation of *daf-2* (Lakowski & Hekimi 1998; Houthoofd *et al.* 2002; Kaeberlein *et al.* 2006; Lee *et al.* 2006). More recently, two transcription factors, *skn-1* and *pha-4*, have been shown to be necessary for life span extension from DR (Bishop & Guarente 2007; Panowski *et al.* 2007); however, it remains unclear how these factors respond to DR and what the relevant downstream targets for life span extension might be.

We have used *C. elegans* to explore the effect of DR on disease processes associated with protein misfolding or aggregation (proteotoxicity). Several transgenic models of proteotoxicity have been developed and characterized in worms. These models show pleiotropic phenotypes (Link 2001; Brignull *et al.* 2007), including defective posterior mechanosensation (Parker *et al.* 2001; Parker *et al.* 2005), impaired ubiquitin-proteasomal function (Khan *et al.* 2006), decreased nose touch response (Faber *et al.* 1999), or age-associated paralysis (Link 1995; Satyal *et al.* 2000; Morley *et al.* 2002; Link *et al.* 2006). Here we report that both genetic and environmental models of DR are potent suppressors of proteotoxicity and we identify a novel role for the heat shock transcription factor, *hsf-1*, as a key mediator of DR in *C. elegans*

RESULTS

DR confers protection against polyglutamine proteotoxicity

We first examined the effects of DR on proteotoxicity using a nematode model of polyglutamine disease. Huntington's disease and several other neurodegenerative disorders are caused by polyglutamine tract expansions, though the mechanism of disease progression remains unknown (Bonini & La Spada 2005). For our studies, we utilized a nematode model of Huntington's disease in which a tract of 35 consecutive glutamine residues is fused to YFP (Q_{35}YFP) and expressed in the body wall muscles (Morley *et al.* 2002). This tract length has been shown to be at the threshold for age-related toxicity in *C. elegans* (Morley *et al.* 2002). As previously reported (Morley *et al.* 2002), we observed an age-dependent paralysis phenotype in Q_{35}YFP animals fed a control diet of abundant UV-killed *E. coli* OP50 (Figure 1A). The life span of these animals was relatively normal (Supplemental Figure 1A), suggesting that the proteotoxicity caused by the Q_{35}YFP protein, while sufficient to cause paralysis, does not necessarily limit longevity. When placed on a BD regimen at the 2nd day of adulthood, the survival of Q_{35}YFP animals was enhanced in a manner similar to wild-type controls (Supplemental Figure 1A). Strikingly, Q_{35}YFP animals

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on BD were resistant to proteotoxicity ($p = 6.2 \times 10^{-12}$) and remained largely paralysis free (Figure 2A; Supplemental video files).

To determine whether BD confers general protection against muscular defects independently of the Q_{35}YFP transgene, we examined the effects of BD in two mutant backgrounds that display and age-associate defect in movement similar to that of Q_{35}YFP: *unc-52(e444)* and *unc-22(e66)* (Moerman & Baillie 1979; Waterston *et al.* 1980; Rogalski *et al.* 1995). In both cases, BD failed to improve movement or suppress paralysis, relative to control fed isogenic animals (Figure 1B; Supplemental video files). Thus, we conclude that the suppression of paralysis by BD is unlikely to be the result of a general improvement in muscle function or increase in movement.

We have previously reported that BD increases life span to a similar extent whether initiated early in adulthood (day 2) or post-reproductively (day 10) (Kaeberlein *et al.* 2006). Accordingly, we wished to determine whether suppression of polyglutamine toxicity is observed by initiating BD at ages later than 2 days of adulthood. Consistent with the effect of BD on longevity, BD initiated at day 3, 5, or 7 of adulthood significantly reduced age-associated paralysis in Q_{35}YFP animals (Figure 1C; $p = 8.3 \times 10^{-12}$ day 3, 7.6×10^{-14} day 5, 5.1×10^{-5} day 7). BD was not sufficient to reverse paralysis, however. Out of more than 50 individual paralyzed animals examined, none regained movement when transferred from a control fed diet to BD. These observations can be explained by a model in which BD confers protection against polyglutamine toxicity until a threshold level of toxicity results in paralysis, after which BD is unable to reverse the accumulated cellular damage.

The formation of insoluble aggregates is a hallmark of human polyglutamine diseases such as Huntington's disease (Borrell-Pages *et al.* 2006). Whether aggregates are causal in disease progression remains controversial, however. To address this question, we quantified the abundance of aggregates in aged animals fed either a control diet or maintained on BD from the 2nd day of adulthood. Although paralysis was dramatically reduced in 14 day old BD animals relative to control fed animals, there was no significant difference in either the number or size of Q_{35}YFP-aggregates between the two groups (Figure 1D; See also Supplemental Videos and Supplemental Figure 2). Consistent with this, the relative amount of Q_{35}YFP protein, when normalized to total protein or to actin (0.96 ± 0.1), was not significantly different in BD animals relative to control fed animals at day 9 of adulthood (Supplemental Figure 2C). Thus, BD appears to protect against proteotoxicity without significantly altering the age-associated accumulation of polyglutamine aggregates.

DR is a general suppressor of proteotoxicity

In order to determine whether the suppression of proteotoxicity conferred by BD would be specific to polyglutamine toxicity, we next examined a transgenic model of Alzheimer's disease. A 42 amino acid amyloid beta peptide ($A\beta_{42}$) is expressed in body wall muscle cells of these animals under control of the *unc-54* promoter (Link 1995). The $A\beta_{42}$ peptide is a cleavage product of amyloid precursor protein, implicated in Alzheimer's disease (Koo *et al.* 1999). Similar to the Q_{35}YFP animals, BD was sufficient to largely suppress age-associated paralysis caused by $A\beta_{42}$-induced proteotoxicity (Figure 2A; $p = 1.4 \times 10^{-7}$). BD also increased both median and maximum survival of $A\beta_{42}$ animals (Supplemental Figure 1B; $p = 8.7 \times 10^{-4}$). In multiple independent experiments we observed that BD caused a notable increase in age-independent early mortality. This may indicate that amyloid beta toxicity (but not polyglutamine toxicity) causes frailty in response to food restriction among a percentage of the population. Interestingly, the subset of animals that died early also tended to show early paralysis, while the survivors remained largely paralysis free. Further studies will be necessary to address the mechanism behind this observation.

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Given that BD was effective at suppressing proteotoxicity in two different nematode models of human disease, we wished to further characterize the generality of this phenomenon. Recently, an aggregation prone form of GFP (GFP-degron) was described that causes progressive paralysis when expressed transgenically in *C. elegans* from a *myo-3* promoter (Link *et al.* 2006). As observed in the Q_{35}YFP and $A\beta_{42}$ models, BD significantly suppressed paralysis in animals expressing the GFP-degron peptide (Figure 2B; $p = 1.0 \times 10^{-14}$). We also assayed wether the levels of aggregated GFP:degron was decreased in worms subjected to BD. As with the Q_{35}YFP animals, age-matched GFP:degron animals did not display a notable loss of the aggregated protein by microscopy (Figure 2C).

Genetic models of DR suppress proteotoxicity

Although BD is unquestionably a form of food (dietary) restriction, it remains possible that BD could have different or additional properties that are not shared by other methods of DR described in the nematode. To address this possibility, we examined whether decreased *eat-2* function could suppress proteotoxicity. RNAi inhibition of *eat-2* resulted in a significant decrease in paralysis in both the Q_{35}YFP (Figure 3A; $p = 1.0 \times 10^{-3}$) and $A\beta_{42}$ (Figure 3B; $p = 1.0 \times 10^{-5}$) models. Suppression of proteotoxicity by inhibition of *eat-2* did not appear to be as effective as BD, however. This is similar to the relative effects of BD and mutation of *eat-2* on life span (Kaeberlein *et al.* 2006; Lee *et al.* 2006), and may suggest that BD represents a level of DR more optimal for longevity and suppression of proteotoxicity. Alternatively, BD may have additional properties that influence aging and proteotoxicity other than those shared by both BD and mutation of *eat-2*.

We next examined genetic models of DR identified from a genome-wide RNAi screen for increased life span (Hansen *et al.* 2005). RNAi knock-down of *rab-10*, *sams-1*, or *drr-2* increases life span through a *daf-16*-independent mechanism, but fails to further increase the life span of *eat-2(ad1116)* animals (Hansen *et al.* 2005). Each of these genes are transcriptionally down-regulated in *eat-2* mutants, suggesting that they function downstream of food consumption to modulate life span (Hansen *et al.* 2005). Paralysis was dramatically reduced in Q_{35}YFP animals in response to RNAi inhibition of *rab-10* (Fig 3C; $p = 1.3 \times 10^{-11}$), *sams-1* (Fig 3D; $p = 1.3 \times 10^{-14}$) or *drr-2* (Fig 3D; $p < 1 \times 10^{-15}$). Suppression of proteotoxicity in these three genetic models of DR was comparable in magnitude to BD. Therefore, we conclude that both genetic and environmental models of DR confer a substantial protective effect against proteotoxicity in *C. elegans*.

BD suppresses proteotoxicity through a mechanism distinct from IIS

IIS has been previously found to modulate resistance to proteotoxicity in both $A\beta_{42}$ and Q_{35}YFP animals (Hsu *et al.* 2003; Cohen *et al.* 2006). We therefore wished to determine the relationship, if any, between DR and IIS in these models. As previously reported for $A\beta_{42}$ animals (Cohen *et al.* 2006), we observed that RNAi knock-down of the FOXO-family transcription factor, *daf-16*, accelerated paralysis in Q_{35}YFP animals (Figure 4A; $p = 8.0 \times 10^{-3}$), while RNAi knock-down of the insulin-like receptor, *daf-2*, delayed paralysis (Figure 4B; $p = 7.6 \times 10^{-7}$). For these experiments, animals were maintained on RNAi bacteria from egg until the second day of adulthood, then transferred to either empty vector RNAi bacteria or BD. Interestingly, RNAi knock-down of *daf-16* did not block the effectiveness of BD (Figure 4A; $p = 4.5 \times 10^{-9}$). When combined with RNAi knock-down of *daf-2*, BD conferred an even greater suppression of paralysis than was observed in BD animals fed empty vector RNAi (Figure 4B; $p = 1.0 \times 10^{-14}$). Identical epistasis interactions were observed between IIS and BD in $A\beta_{42}$ animals (Supplemental Figure 3). These data recapitulate the genetic relationship between DR and IIS with respect to longevity: DR can increase life span by a mechanism that is independent of *daf-16* and additive with mutation of *daf-2* (Lakowski & Hekimi 1998; Houthoofd *et al.* 2003; Kaeberlein *et al.* 2006; Lee *et*

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al. 2006). Thus, BD and reduced IIS appear to modulate both longevity and proteotoxicity through distinct genetic pathways.

DR protects against proteotoxicity through an *hsf-1* dependent mechanism

The heat shock transcription factor, *hsf-1*, regulates expression of many different heat-inducible target genes such as small heat shock proteins, and has been implicated in modulating both longevity (Garigan *et al.* 2002; Hsu *et al.* 2003; Morley & Morimoto 2004) and proteotoxicity (Hsu *et al.* 2003; Cohen *et al.* 2006). In order to determine whether *hsf-1* is required for phenotypes associated with DR, we subjected *hsf-1(sy441)* animals to BD and monitored thermotolerance and longevity (Kaeberlein *et al.* 2006; Lee *et al.* 2006). The *hsf-1(sy441)* allele contains a point mutation in the *hsf-1* transactivation domain that prevents induction of *hsf-1* target genes in response to heat stress (Hajdu-Cronin *et al.* 2004). Unlike the case in N2 animals, BD failed to result in enhanced thermotolerance in *hsf-1(sy441)* animals (Figure 5A). The effect of BD on the longevity of *hsf-1(sy441)* animals was complex: BD failed to significantly increase median life span ($p = 0.34$); however, maximum life span appeared to be increased (Figure 5B). The inability of BD to increase the median life span of *hsf-1(sy441)* animals is notably different than the increase in both median and maximum life span of *daf-16(mu86)* animals in response to BD (Figure 5B; $p = 3.9 \times 10^{-19}$).

There are two possibilities to explain the observation that *hsf-1* mutation prevents median, but not maximum, life span extension from BD. First, it may indicate that life span extension from BD involves two different mechanisms, one of which acts through *hsf-1* and one of which is *hsf-1*-independent. Alternatively, the residual *hsf-1* function present in *hsf-1(sy441)* animals may be sufficient to partially mediate BD-associated life span extension. Consistent with this latter possibility, RNAi knock-down of *hsf-1* completely prevented both median and maximum life span extension by BD (Figure 5C). Thus, mutation of the *hsf-1* transactivation domain prevents induction of thermotolerance and median life span extension in response to BD, and RNAi knock-down of *hsf-1* completely abrogates median and maximum life span extension by BD.

Finally, we asked whether RNAi knock-down of *hsf-1* would affect suppression of proteotoxicity by BD. Q_{35}YFP animals were maintained on *hsf-1* RNAi from egg until the 2nd day of adulthood then transferred to either empty vector bacteria or BD. The onset of paralysis was accelerated when *hsf-1* function was reduced by RNAi, relative to animals maintained on empty vector RNAi from egg (Figure 6A; $p = 1.1 \times 10^{-11}$). Strikingly, BD failed to significantly suppress paralysis in Q_{35}YFP animals where *hsf-1* was inhibited ($p = 0.5$). RNAi knock-down of *hsf-1* had a similar effect on paralysis in $A\beta_{42}$ animals (Fig 6b; $p = 0.1$). Thus, we conclude that *hsf-1* activity is required both for life span extension and for the general suppression of proteotoxicity associated with BD.

DISCUSSION

Relationship between DR, IIS, and *hsf-1*

DR and reduced IIS result in several shared phenotypes, including enhanced thermotolerance, enhanced resistance to proteotoxicity, and increased longevity. In the case of reduced IIS, each of these phenotypes is known to be dependent on both *hsf-1* and *daf-16*, which function to co-regulate the expression of a subset of IIS-responsive genes (Hsu *et al.* 2003; Murphy *et al.* 2003). In contrast, the effects of DR require *hsf-1*, but *daf-16* is expendable. A model consistent with these observations is one in which BD promotes the activity of a transcription factor which functions in a manner similar to *daf-16* to regulate target genes co-operatively with *hsf-1* (Figure 7A). In this model, BD would not act solely

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by inducing hsf-1 activity or expression, but hsf-1 would function together with another transcription factor. Thus, hsf-1 activity would be necessary, but not sufficient, to recapitulate many of the phenotypes associated with BD, consistent with the observation that *daf-16* is required for life span extension from overexpression of *hsf-1* in animals fed a normal bacterial diet (Hsu *et al.* 2003; Morley & Morimoto 2004).

Recently, the *daf-16* homolog *pha-4* was found to be required for life span extension in *eat-2* mutants (Panowski *et al.* 2007). Expression of *pha-4* is normally low during adulthood, but is increased in *eat-2* mutants (Panowski *et al.* 2007). Unlike *hsf-1* (Hsu *et al.* 2003), however, overexpression of *pha-4* is not sufficient to increase life span in control fed animals (Panowski *et al.* 2007). Thus, it could be that in well-fed animals *hsf-1* activity is limiting for longevity, but in response to nutrient deprivation both *hsf-1* and *pha-4* are activated and promote longevity. Since *skn-1* is also required for life span extension from DR (Bishop & Guarente 2007), it may be the case that *hsf-1* and *skn-1* act to co-regulate genes important for DR or that *skn-1* acts upstream of *hsf-1* and *pha-4* in response to DR. Similar to the case for *pha-4*, increased expression of *skn-1* is not sufficient to increase the life span of control fed animals (Bishop & Guarente 2007). *Skn-1* is important for regulating gene expression in response to oxidative stress (An & Blackwell 2003; Inoue *et al.* 2005), but no role for *pha-4* or *skn-1* in modulating the response to proteotoxic stress has been described.

An alternative, but not mutually exclusive, possibility is that BD and *hsf-1* function in parallel to promote longevity by improving protein homeostasis (Figure 7B). This hypothesis is attractive, since *hsf-1* is known to regulate expression of chaperones, and BD could contribute to protein homeostasis by enhancing protein degradation or by modulating mRNA translation (or both). In *C. elegans*, starvation causes rapid turnover of muscle proteins and induces autophagy (Fostel *et al.* 2003; Jia & Levine 2007), suggesting that turnover of damaged proteins is likely to be enhanced by BD. This model also explains that apparent correlation between longevity and resistance to proteotoxicity. For example, a recent RNAi screen identified several genes that play a role in both longevity determination and the formation of polyglutamine aggregates (Curran & Ruvkun 2007). We have also observed that RNAi of several previously reported longevity factors (Dillin *et al.* 2002; Lee *et al.* 2003; Hamilton *et al.* 2005; Hansen *et al.* 2005; Hansen *et al.* 2007; Pan *et al.* 2007) also suppresses paralysis in Q_{35}YFP and $A\beta_{42}$ animals (KAS, CD, DC and MK, unpublished data). Further studies will be necessary to clarify the precise relationship between hsf-1 and DR, and the importance of protein homeostasis as a determining factor for longevity.

Conclusion

The data presented here demonstrate a novel and previously unsuspected role for *hsf-1* as a key mediator of DR. This finding is noteworthy, as *hsf-1* orthologs are highly conserved from yeast to humans, as are many of the known *hsf-1* targets. Although it remains unclear whether *hsf-1* is activated by DR in mammals, DR enhances Hsf1 binding activity in aging rats (Heydari *et al.* 1996), indicating that the link between DR and *hsf-1* activity may be conserved. Activation of *hsf-1* or specific *hsf-1* target genes may prove therapeutic toward proteotoxic insults leading to neurodegeneration and other age-associated pathologies in people and may also mimic the longevity enhancing effects of DR.

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EXPERIMENTAL PROCEDURES

Strains and genetics

Standard procedures for *C. elegans* strain maintenance and manipulation were used. Experiments were conducted at 20°C unless otherwise noted in the text. Nematode strains were obtained from the *Caenorhabditis* Genetics Center, or the laboratories of Dr. Richard Morimoto (Northwestern University, Chicago, IL), Dr. Chris Link (University of Colorado, Boulder, CO) or Dr. Jim Thomas (University of Washington, Seattle, WA). The strains used in this study are listed in Supplemental Table 1.

Bacterial Food Deprivation

BD experiments were carried out essentially as described previously (Kaeberlein *et al.* 2006). In brief, adult hermaphrodites were allowed to lay eggs on nematode growth medium (Youngman et al.) containing UV-killed *E. coli* OP50. At L4, worms were transferred to fresh NGM + UV-killed OP50 supplemented with 50 µM 5-fluorodeoxyuridine (FUDR) to prevent further eggs from hatching. Unless otherwise stated, worms were then transferred to experimental media at the 2nd day of adulthood: NGM + UV-killed OP50 + 50µM FUDR (control fed) or NGM + 50µM FUDR (Parker et al.). For the experiment shown in Figure 1C, adult worms were transferred to experimental media on the days indicated in the figure legend. OP50 were UV-killed by irradiation with a Stratagene UV Stratalinker 2400 on maximal energy setting. UV-killing was periodically verified by streaking UV-irradiated bacteria onto LB plates and confirming absence of growth.

RNA interference combined with BD

RNA interference (RNAi) experiments were conducted using feeding protocols according to standard procedures. Unless otherwise stated, RNAi feeding strains were obtained from the Ahringer RNAi library (Kamath *et al.* 2003); library clones were sequenced for verification. RNAi plates consisted of NGM supplemented with 1mM β-D-isothiogalactopyranoside (IPTG) and 25µg/ml carbenicillin. Unless otherwise indicated, worms were raised on RNAi bacteria from egg. At L4, they were then transferred to plates containing freshly-seeded RNAi bacteria plus 50µM FUDR. To combine RNAi with BD, worms were maintained on the indicated RNAi bacteria until the 2nd day of adulthood, at which point they were transferred to experimental media: RNAi plates + 50µM FUDR seeded with empty vector control bacteria (control fed) or RNAi plates + 50µM FUDR (Parker et al.). The *daf-2* RNAi construct used for these studies was provided to us by J. McElwee.

Paralysis and life span quantification

The paralysis of worms expressing any of the indicated transgenes (Q35-YFP, Aβ1-42, or GFP::degron) was determined by visual analysis. Worms were scored as paralyzed if they were unable to make forward progress on the NGM surface in response to both plate-tapping and tail-prodding. If paralyzed, worms were scored as alive if nose-tapping resulted in nose movement. If no nose movement could be seen, worms were scored as dead.

Thermotolerance

Animals were maintained at 20°C on either control fed or BD media as described until the 6th day of adulthood (4 days without food for the BD group). The morning of the 6th day of adulthood, plates (each containing approximately 20 animals) were transferred to 38°C. Viability was determined periodically by removing one plate at a time and assaying for movement in response to nose-prodding.

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Fluorescence microscopy and quantification of Q35YFP aggregates

Movie files were generated using Canon Powershot S31S digital camera connected to the eyepiece of a Zeiss SteREO Lumar.V12 microscope equipped with a broad-range GFP filter. Still GFP images were obtained with a Biorad MRC-1024 LSCM (Hercules, CA). This package included a computer assisted Zeiss inverted microscope and 15mW Krypton/Argon laser (ILT laser**,** Aachen Germany) emitting 3 three excitation lines at 488 nm, 568 nm, and 647nm. The 488 nm line was used to excite the green fluorescent protein. The emitted green fluorescence was filtered through a 522 nm DF 32 band pass filter. Unless otherwise stated, a 10x objective lens was used for fluorescence measurements. Identical settings were used for all worms analyzed (laser power at 1%, gain @1500, Iris@4.0 uM, scan time@488 lines/sec). The worms were paralyzed with 25mM sodium azide and placed on a glass slide under a cover slip to flatten the worm somewhat and optimize viewing. The microscope was focused at the position of maximum fluorescence for each worm. The images were analyzed using Image J 1.341.5.0_07 software [available from NIH website (http://rsb.info.nih.gov/ij/java)] and a Macintosh computer with OS X operating system. The "count objects" command of Image J was used to count the number of fluorescent spots in the images. A constant minimum intensity threshold was set at 75/255 pixels, and a minimum spot size of 3 pixels/spot were used for all worms analyzed.

Quantification of Q35YFP expression by Immunoblot Analysis

Worms were prepared for immunoblot analysis using the control fed and BD protocols described above. At day 9 of adulthood, 1000 worms per group were harvested in 1ml M9 buffer and concentrated by low speed centrifugation. Worms were washed in M9 twice, then resuspended in PBS at a concentration of 1 worm per microliter buffer. They were homogenized in a glass homogenizer and spun at 3000rpm for 3 minutes; the supernatant was collected as "total lysate." Samples were subjected to SDS PAGE analysis followed by immunoblot, according to standard protocols. Q35-YFP was detected with a GFP-specific antibody (Roche Applied Science, Indiana, USA); protein loading was controlled for using a β-actin specific antibody (Abcam, Massachusetts, USA). The relative abundance of Q35YFP protein and actin was determined by quantifying the band intensities after probing with the GFP-specific antibody or the β-actin specific antibody, respectively. When normalized to actin, the relative abundance of Q35YFP in control fed to BD animals was 0.96 ± 0.1, based on three biological replicates (independent experiments and independent blots).

Statistical analysis and replication

A Wilcoxon Rank-Sum test (MATLAB 'ranksum' function; Mann-Whitney U Test) was used to generate *p*-values to determine statistical significance for life span and paralysis assays. This test is a nonparametric comparison of medians between two groups. Each experiment was repeated at least three times with similar results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank the R. Morimoto and C. Link for providing the transgenic *C. elegans* strains used in this study. We would also like to thank J. McElwee and J. Thomas for helpful discussion and shared reagents. Some strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources. This work was conducted while MK was an AFAR Research Grant recipient. This work was also supported by a grant from The Ellison Medical Foundation to MK and BKK. KAS is supported by a post-doctoral fellowship from the Hereditary Disease Foundation. EDS was supported by NIH Training Grant P30 AG013280.

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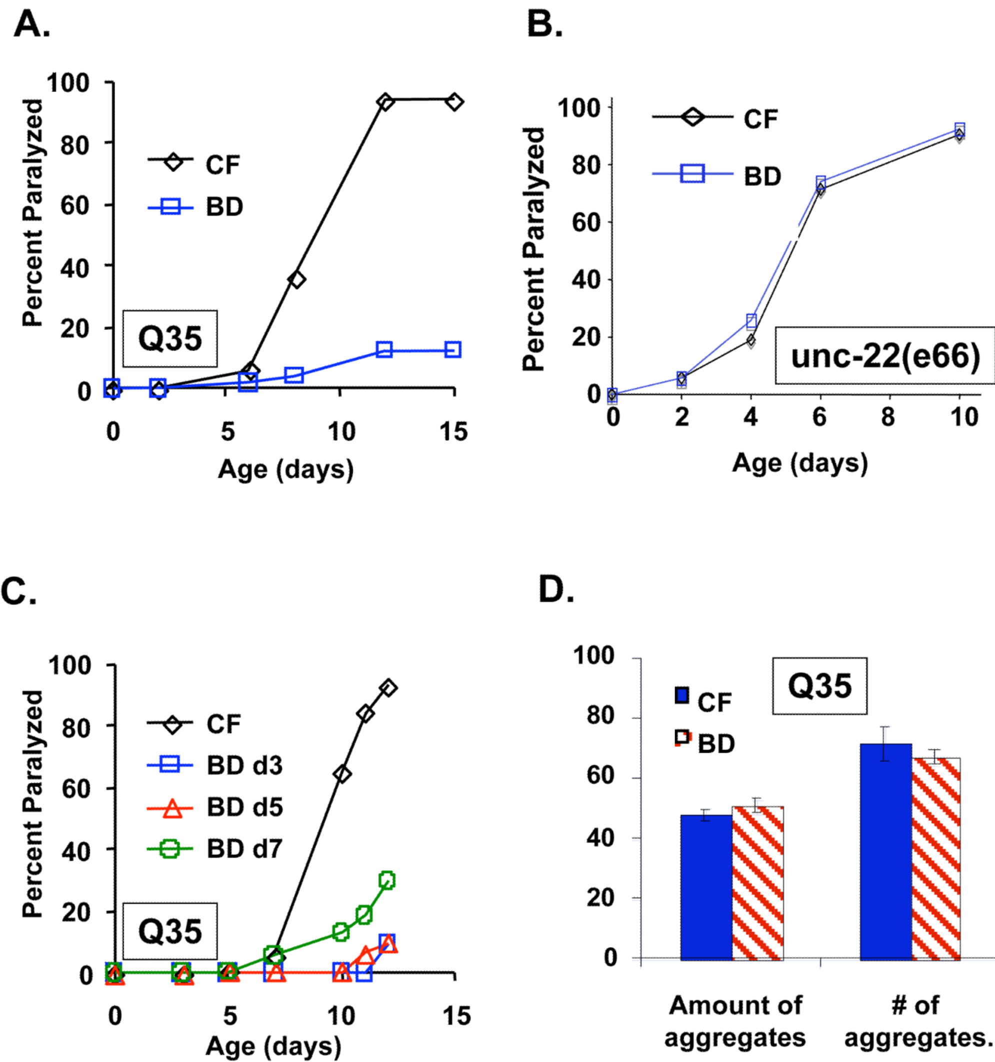

Figure 1. Bacterial food deprivation suppresses proteotoxicity in a nematode model of polyglutamine disease

(**A**) Age associated paralysis caused by expression Q_{35}YFP (Q35) is significantly reduced in BD animals relative to control fed (CF) animals ($p=6.2 \times 10^{-12}$). (**B**) The age-associated paralysis of unc-22(e66) mutants is unaltered in BD animals relative to control fed (CF) animals. (**C**) BD significantly reduces polyglutamine-associated paralysis when initiated at day 3 ($p=8.3 \times 10^{-12}$), day 5 ($p = 7.6 \times 10^{-14}$), or day 7 ($p=5.1 \times 10^{-5}$) of adulthood. (**D**) BD does not significantly reduce the formation of polyglutamine aggregates. The total amount of aggregates, as measured by the integral of the fluorescent area per animal or the number of discrete aggregates visible as fluorescent spots, of BD animals relative to CF animals were counted. Error bars are standard error of the mean.

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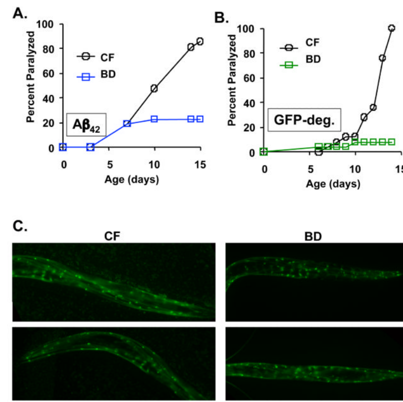

Figure 2. BD suppresses proteotoxicity in a nematode model of Alzheimer's disease

(A) Relative to control fed (CF) animals BD significantly reduces age-associated paralysis in animals expressing $A\beta_{42}$ ($p=1.4 \times 10-7$). (B) BD significantly reduces age-associated paralysis in animals expressing the aggregation prone GFP-degron peptide (GFP-deg), relative to CF animals ($p=1.0 \times 10^{-14}$). (C) By fluorescence microscopy, the steady-state levels of the GFP-degron peptide are not reduced in BD animals as compared to control fed (CF) animals.

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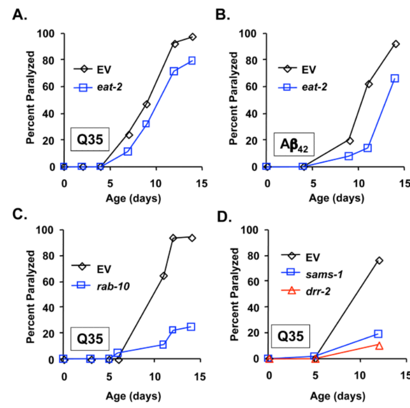

Figure 3. Genetic models of dietary restriction suppress proteotoxicity

(**A**) RNAi knock-down of *eat-2* significantly reduces paralysis in Q35YFP animals, relative to animals maintained on empty vector bacteria ($p=1.0 \times 10^{-3}$). (**B**) RNAi knock-down of *eat-2* significantly reduces paralysis in $A\beta_{42}$ animals, relative to animals maintained on EV bacteria ($p=1.0 \times 10^{-5}$). (**C**) RNAi knock-down of *rab-10* significantly reduces paralysis in Q35YFP animals, relative to animals maintained on empty vector bacteria ($p=1.3 \times 10^{-11}$). (**D**) RNAi knock-down of *sams-1* or *drr-2* significantly reduces paralysis in Q35YFP animals, relative to animals maintained on empty vector bacteria ($p=1.3 \times 10^{-14}$ for *sams-1* and $p < 10^{-15}$ for *drr-2*).

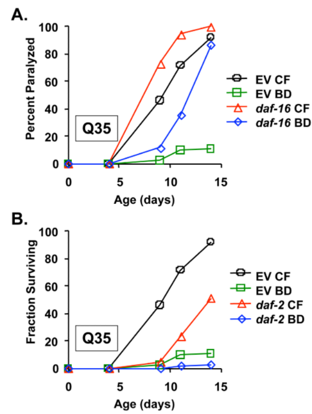

Figure 4. BD suppresses proteotoxicity by a mechanism distinct from insulin/IGF-1 like signaling
(**A**) RNAi inhibition of *daf-16* significantly accelerates paralysis in Q35YFP animals relative to growth on empty vector bacteria (8.0×10^{-3}), but does not prevent suppression of paralysis by BD ($p=4.5 \times 10^{-9}$). (**B**) RNAi inhibition of *daf-2* significantly reduces paralysis in Q35YFP animals relative to growth on EV bacteria ($p=7.6 \times 10^{-7}$). BD further reduces paralysis of *daf-2* RNAi treated animals ($p=1.0 \times 10^{-14}$).

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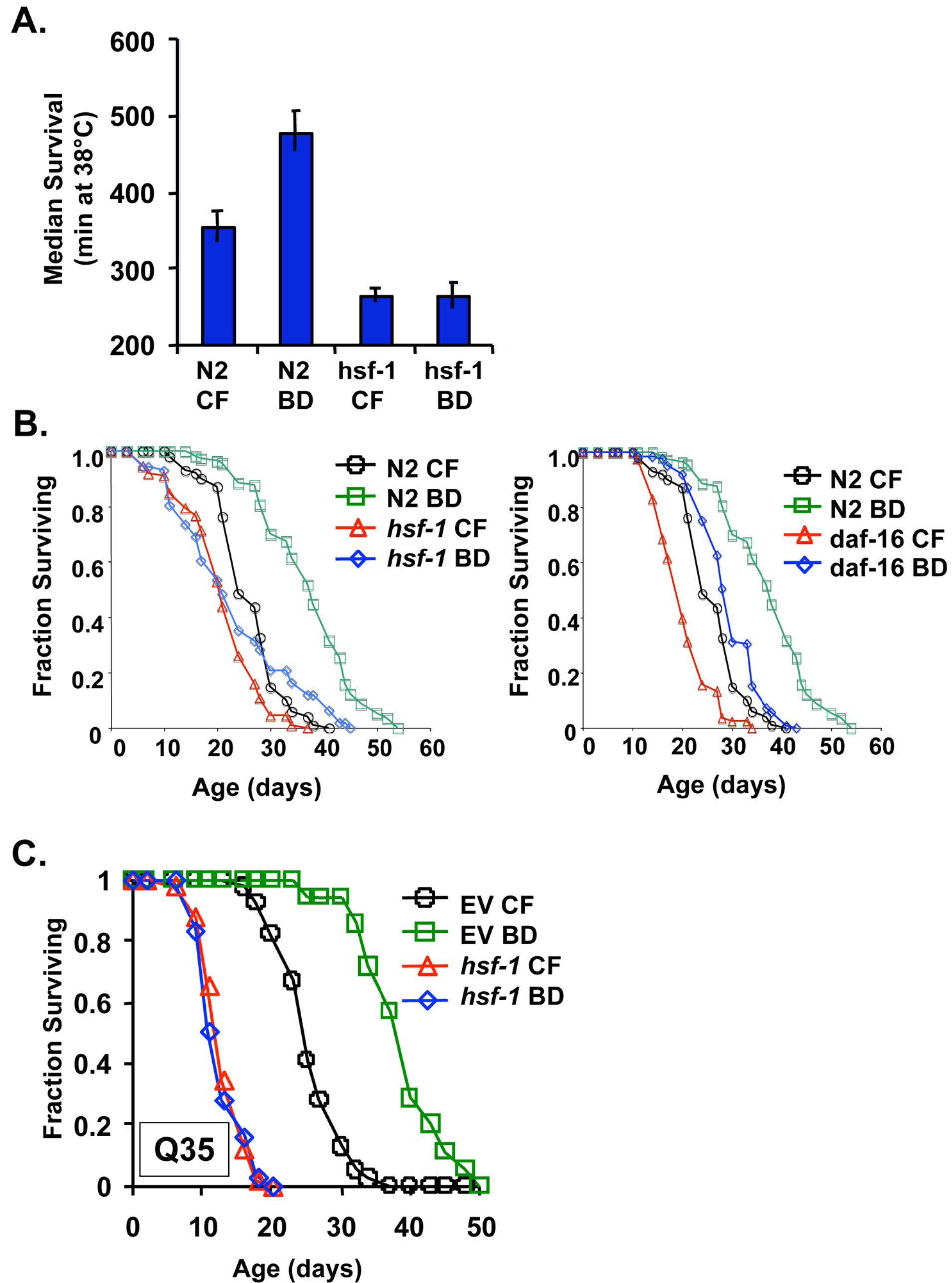

Figure 5. Heat shock factor 1 is required for enhanced thermotolerance and increased life span in response to BD

(**A**) Animals carrying a mutant allele of *hsf-1*, *hsf-1(sy441)*, fail to show increased survival at 38°C when subjected to BD. Error bars are standard error of the mean. (**B**) BD fails to increase median life span of *hsf-1*(*sy441*) animals (p=0.34) but significantly increases the life span of *daf-16*(*mu86*) animals (**C**) RNAi inhibition of *hsf-1* prevents life span extension from BD.

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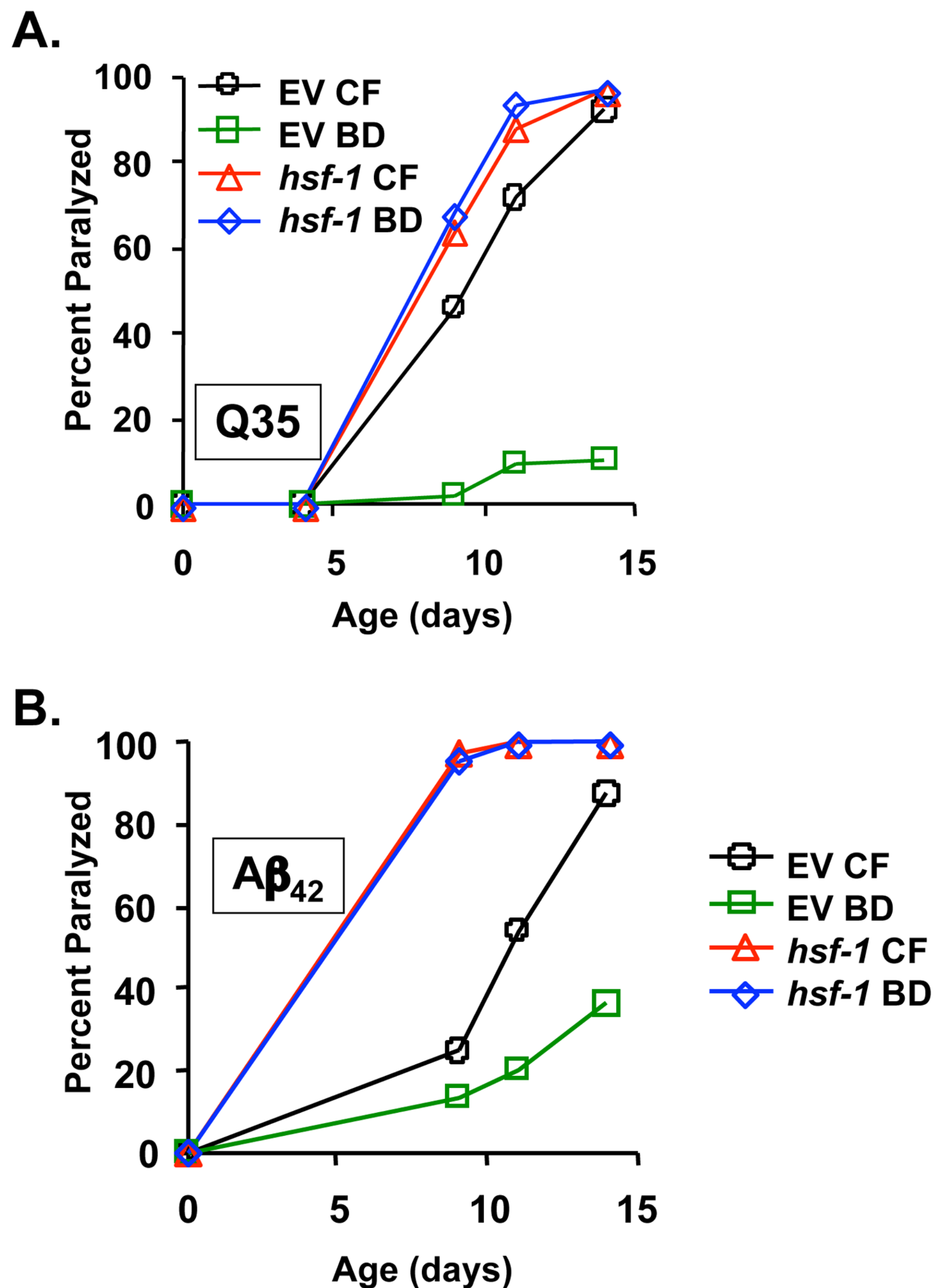

Figure 6. Heat shock factor 1 is required for suppression of proteotoxicity in response to BD
(**A**) BD significantly reduces paralysis of Q35YFP animals grown on empty vector bacteria ($p=1.1 \times 10^{-11}$), but does not reduce paralysis of Q35YFP animals grown on *hsf-1* RNAi ($p=0.51$). (**B**) BD significantly reduces paralysis of $A\beta_{42}$ animals grown on empty vector bacteria ($p=8.9 \times 10^{-14}$), but does not reduce paralysis of $A\beta_{42}$ animals grown on *hsf-1* RNAi ($p=0.1$).

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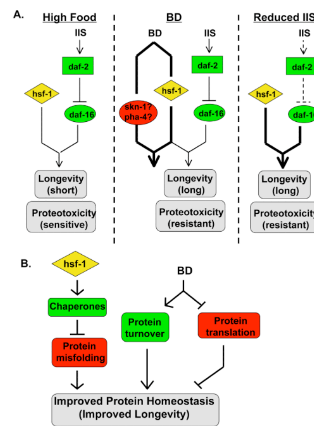

Figure 7. Models for the role of *hsf-1* in determining longevity and sensitivity to proteotoxicity
(A). The effect of BD is mediated by *hsf-1*. Under conditions of abundant food, the dietary restriction pathway is inactive and *hsf-1* activity and insulin/IGF-1-like signaling (IIS) are at normal levels. This leads to short life span and sensitivity to proteotoxicity. Under DR conditions, *hsf-1* is activated along with other DR-responsive proteins (perhaps *skn-1* and *pha-4*) to increase life span and suppress proteotoxicity. In mutants with reduced IIS, the DR pathway is inactive, but life span and resistance to proteotoxicity are increased through activation of *daf-16*. (B) BD and hsf-1 function in parallel to alter protein homeostasis. *hsf-1* activity upregulates chaperone activity, which decreases the amount of misfolded proteins *in vivo*. At the same time, BD might both increase the rate of protein turnover and decrease the synthesis of new protein. These three factors would combine to improve protein homeostasis, decreasing the rate of aging.