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Molecular mechanisms underlying genotype-dependent responses to dietary restriction

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Summary

Dietary restriction (DR) increases lifespan and attenuates age-related phenotypes in many organisms; however, the effect of DR on longevity of individuals in genetically heterogeneous populations is not well characterized. Here we describe a large-scale effort to define molecular mechanisms that underlie genotype-specific responses to DR. The effect of DR on lifespan was determined for 166 single-gene deletion strains in *Saccharomyces cerevisiae*. Resulting changes in mean lifespan ranged from a reduction of 79% to an increase of 103%. Vacuolar pH homeostasis, superoxide dismutase activity, and mitochondrial proteostasis were found to be strong determinants of the response to DR. Proteomic analysis of cells deficient in prohibitins revealed induction of a mitochondrial unfolded protein response (mtUPR) which has not previously been described in yeast. Mitochondrial proteotoxic stress in prohibitin mutants was suppressed by DR via reduced cytoplasmic mRNA translation. A similar relationship between prohibitins, the mtUPR, and longevity was also observed in *Caenorhabditis elegans.* These observations define conserved molecular processes that underlie genotype-dependent effects of DR that may be important modulators of DR in higher organisms.

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Keywords

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Introduction

Dietary restriction (DR, also referred to as caloric restriction or CR) is the most widely studied intervention for slowing aging and enhancing healthspan in diverse species (Kennedy *et al.* 2007). Defined as a reduction in nutrient availability without malnutrition, DR can increase lifespan in yeast, nematodes, fruit flies, and other invertebrates. In both mice and rats, DR has been found to increase lifespan and delay the onset of a variety of age-related phenotypes. Positive health effects of DR have also been reported in a primate, the rhesus macaque, where DR reduced age-related mortality and lowered the incidence of age-related disease (Colman *et al.* 2009). If, and to what degree, DR will slow aging in humans remains to be determined, though studies to date indicate some health benefits of DR consistent with delayed aging in humans (Lefevre *et al.* 2009).

Several genetic pathways have been suggested to play a role in mediating the health and longevity effects of DR. These include growth hormone and insulin/IGF-1-like signaling, sirtuin activity, and signaling through the mechanistic target of rapamycin (mTOR) (Kenyon 2010). mTOR signaling in particular has become an intensely studied longevity pathway, as inhibition of mTOR by the drug rapamycin is sufficient to increase lifespan in mice, even with treatment begun at 600 days of age (Harrison *et al.* 2009). Genetic studies indicate that inhibition of mTOR is likely involved in lifespan extension by DR in yeast, nematodes, and flies, though the relationship between mTOR and DR in mammals is yet to be directly examined (Johnson *et al.* 2013).

Despite abundant data indicating that DR can slow aging across evolutionarily divergent species, examples exist where DR has had no effect or has caused a reduction in lifespan. For example, one study that examined DR in mice recently derived from the wild found that mean lifespan was not extended in that strain (Harper *et al.* 2006). In another study the effect of DR on 42 recombinant inbred mouse lines resulted in a distribution of responses ranging from 98% extension to 68% reduction in lifespan (Liao *et al.* 2010). Recently, two independent studies of DR in rhesus macaque, spanning more than three decades, resulted in strikingly different outcomes: one found significant reductions in mortality due to age-related causes in the DR group (Colman *et al.* 2009) while the other found no change in survival, though DR improved measures of healthspan (Mattison *et al.* 2012). While multiple factors likely influenced the outcome in these studies, together they support the idea that genotype plays a critical role in determining the effect DR has on longevity. The molecular processes underlying genotype-dependent responses to DR remain largely unexplored.

In this study we used the budding yeast *Saccharomyces cerevisiae* as a model to explore the interaction between genotype and the effect of DR on lifespan. Yeast replicative lifespan (RLS) is defined as the number of daughter cells a mother cell is capable of producing before irreversibly exiting the cell cycle (Mortimer & Johnston 1959). DR in *S. cerevisiae* is achieved by reducing the glucose concentration in the medium from 2% to 0.5% or lower and has been shown to extend RLS in multiple strain backgrounds (Longo *et al.* 2012). Here we examined the effect of DR at 0.05% glucose on the RLS of 166 strains, each lacking a single non-essential gene. Similar to the prior study using inbred mouse lines (Liao *et al.* 2010), a distribution was observed ranging from dramatic decreases to substantial increases

in lifespan relative to that observed for wild-type cells. Gene ontology (GO) analysis of genes associated with significantly positive or negative changes in RLS by DR revealed multiple conserved molecular processes associated with response to DR. Disruption of vacuolar/lysosomal pH homeostasis and mitochondrial superoxide dismutase (*SOD2*) lead to a dramatic shortening of RLS by DR. In contrast, cells lacking mitochondrial prohibitins (*PHB1*, *PHB2*) are short-lived under control conditions and show a large lifespan extension in response to DR, a relationship that is conserved in *Caenorhabditis elegans*.

Results

To investigate the interaction between genotype and DR, we analyzed the effect of DR at 0.05% glucose on approximately 100 randomly selected single gene deletion strains. Additional strains were added to the screen either randomly or by selecting within previously tested functional categories (e.g. vacuolar ATPases) to validate earlier results. Strains with growth properties inconsistent with annotations in the Saccharomyces Genome Database (www.yeastgenome.org) were excluded from further analysis. In total, RLS data for 166 strains are reported here. The resulting distribution showed a median DR-induced increase in mean RLS of 3.9% with changes ranging from a 79% reduction to a 103% increase (Figure 1A, Tables S1-S2). DR resulted in statistically significant changes to RLS (multiple testing corrected $q < 0.05$) for 82 (49%) strains and non-significant effects from DR for the remaining 84 strains (Tables S1-S2). The absolute effect of DR (mean RLS on DR medium minus mean RLS on control medium) shows a similar distribution (Figure S1A).

We considered the possibility that the effect of DR could be influenced by the overall fitness of the strains. To explore this, we compared the effect of DR on RLS in each strain to two measures of fitness: the RLS of the strain under control conditions and maximal growth rate (an indicator of general fecundity). In both cases there is no significant correlation between fitness and the effect of DR measured by either percent or absolute change (Figure 1B-C, Figure S1B-C).

In contrast to the aforementioned studies in mice and primates, the genetic variation in each yeast strain is known and limited to a single gene, allowing for the study of mechanisms underlying these differential responses. GO analysis of genes associated with significant positive or negative response to DR revealed enrichment of specific cellular processes. Genes involved in pathways regulating cellular and organellar pH, ion homeostasis, and oxidative stress response were significantly enriched in strains whose RLS was reduced by DR (Figure 1D, S2-3, Tables S3-S5). In contrast, genes involved in mitochondrial function were highly enriched in strains where RLS was increased by DR (Figure 1E, S4, Table S6).

To further characterize the nutrient response profile of the strains strongly affected by DR, we examined RLS for select single gene mutants across a range of glucose concentrations from 2% to 0.005%. These include gene deletions corresponding of the mitochondrial porin, *POR1* (Figure S5A), the mitochondrial inner membrane (MIM) chaperone complex component, *PHB1* (Figure 2A), the vacuolar ATPase subunits encoded by *VMA21* (Figure S5B) and *VMA6* (Figure S5C), and the mitochondrial superoxide dismutase, *SOD2* (Figure 2B), among others (Figure S5D). Each of these strains shows a dose-dependency in their response to DR.

To determine the molecular mechanisms underlying these genotype-specific responses to DR, we first examined *sod2Δ* cells. One effect of DR in yeast is an increase in mitochondrial respiration. Given the role of Sod2 in antioxidant defense, we considered the possibility that the effects of DR on *sod2Δ* cells may result from the shift toward respiratory metabolism

and an inability to detoxify superoxide radicals associated with respiration. We observed a similar reduction in the RLS of *sod2Δ* cells on non-fermentable carbon sources (Figure 2C, Table S7), consistent with this model.

The induction of respiration in yeast is largely mediated by the transcription factor Hap4 which regulates expression of many nuclear encoded mitochondrial proteins (Lin *et al.* 2002). In otherwise wild-type cells, deletion of *HAP4* had little effect on the response to DR (Figure S6A-B). In contrast, deletion of *HAP4* completely suppressed the lifespan shortening effect of DR in *sod2Δ* cells (Figure 2D, Table S8). Addition of the antioxidant ascorbic acid (AA) to the media also suppressed the short RLS of both *sod2Δ* and *sod1Δ* cells on 2% glucose and DR media (Figures 2E-F, S5E-F Table S9). Further supporting this model, we observed that deletion of *SOD2* causes sensitivity to the superoxide generating compound paraquat under control conditions and that *sod2Δ* cells show an increased sensitivity to paraquat compared to wild-type cells when subjected to DR (Figure 2G). Together these data demonstrate that mitochondrial antioxidant capacity is important for lifespan extension when respiration is induced and that defects in these processes cause DR to become harmful (Figure 2H).

Among the strains showing the most positive response to DR were many genes coding for proteins involved in mitochondrial function. These include Por1 and both subunits of the mitochondrial prohibitin complex, Phb1 and Phb2 (Figure 1A inset, S5B, Table S2), which behaved identically in each assay performed in these studies (referred to as *phbΔ* hereafter). DR thus appears to be particularly beneficial in the context of at least certain forms of mitochondrial dysfunction. We focused on understanding the mechanistic basis for robust lifespan extension in *phbΔ* cells.

The prohibitin complex is an inner mitochondrial membrane complex highly conserved from yeast to mammals. This complex participates in mitochondrial protein folding, electron transport chain assembly, and regulation of mitochondrial proteases (Merkwirth & Langer 2009). Prohibitin mutants have been previously reported to have short RLS and reduced mitochondrial membrane potential (Coates *et al.* 1997), phenotypes confirmed in this study (Figures 2A, S5D, S7, and Table S7). Because the prohibitin complex functions in the mitochondria, we tested if increased mitochondrial respiration causes the RLS extension from DR in *phbΔ* cells. We generated double mutant cells lacking the prohibitin complex and the transcription factor Hap4, discussed above. Although these cells are deficient for growth on a non-fermentable carbon source, they show a robust lifespan extension from DR at 0.05% glucose that is indistinguishable from prohibitin single mutant cells (Figures 3A, S8A, Table S8). A similar effect is seen when prohibitin deficiency is combined with deletion of the gene encoding the Rieske iron-sulfur protein, Rip1, which is required for mitochondrial electron transport chain function (Figures 3A, S6C, S8B, Table S8). Together these data demonstrate that the mechanism of RLS extension by DR in the *phbΔ* mutants is distinct from that underlying decreased RLS in *sod2Δ* cells.

In addition to a shift toward mitochondrial respiration, DR in yeast is associated with induction of autophagy. We considered the possibility that induction of autophagy by DR might increase lifespan in prohibitin mutants; however, deletion of genes necessary for autophagy had no effect on the increase in RLS of *phbΔ* cells on DR (Figures 3B, S6D-E). Thus, we conclude that the robust RLS increase by DR does not result from increased autophagy in *phbΔ* cells.

To further characterize the mechanism of RLS extension by DR in prohibitin deficient yeast, we examined the effect of prohibitin deficiency on the mitochondrial proteome. Here DR was accomplished by growing cells in 3% glycerol (DR-Gly), a non-fermentable carbon

source that induces many of the same physiological effects as 0.05% glucose including robust RLS extension of *phbΔ* cells (Figure 3C). These conditions were necessary to prevent exhaustion of the carbon source during liquid culture and allow for growth of sufficient cell mass to harvest mitochondria for mass spectrometry (Figure 3D). As anticipated, peptides corresponding to proteins up-regulated by the metabolic shift to respiration were higher in both wild-type cells and prohibitin mutants in DR-Gly samples (Figure S9-10). Several peptides were differentially abundant in mitochondria from prohibitin mutants relative to wild-type cells under control conditions but not DR-Gly (Figures 3E-F, Figure S9-10, Table S10), demonstrating a rescue of phenotype at the level of the proteome. Prohibitin mutants in control conditions had elevated levels of mitochondrial heat shock proteins and chaperones associated with the mitochondrial unfolded protein response (mtUPR), a response described in nematodes and mammals (Haynes & Ron 2010). To our knowledge this is the first description of a coordinated mtUPR in yeast. The observed induction of chaperones appeared to be restricted to the mtUPR, as chaperones associated with cytoplasmic or endoplasmic reticulum (ER) proteotoxic stress were not induced (Figure S9C). In organisms with a recognized mtUPR, the response involves induction of nuclear-encoded mitochondrial stress proteins including Hsp60 (Cpn60), Hsp10 (Cpn10), and mtHsp70 (Ssc1 in yeast, Hsp-6 in worms). Each of these was significantly increased in mitochondrial fractions from *phbΔ* samples grown in control medium but not in cells grown in DR-Gly (Figures 3F, S9C, S10). Elevation of Hsp60 in prohibitin mutants under control but not DR conditions was verified by western blot using whole-cell lysates (Figure 3G-H). These data demonstrate that a mitochondrial specific response, similar to the mtUPR observed in other species, occurs in prohibitin deficient yeast cells and that DR suppresses this phenotype.

DR has previously been associated with reduced cytoplasmic translation (Kaeberlein & Kennedy 2008), a response we also observed in wild-type cells grown in glycerol (Figure 4A). We considered the possibility that DR might reduce mitochondrial proteotoxic stress and extend the RLS of prohibitin mutants through reduced translation of cytoplasmic proteins destined for import into the mitochondria. Deletion of the large ribosomal subunit *RPL20B* provides a robust genetic model for reduced cytoplasmic translation (Figure S11A). We found that deletion of *RPL20B* in *phbΔ* cells increased lifespan to the same degree as DR (Figure 4B).

The RLS of wild-type cells can be extended by deletion of genes encoding components of the large ribosomal subunit but generally not by deletion of small ribosomal subunit genes (Steffen *et al.* 2008) (Figures S11A-B). This is thought to occur through activation of the transcription factor Gcn4, which appears to be activated by large but not small subunit disruption. RLS extension by deletion of *RPL20B* in otherwise wild-type cells requires Gcn4 (Steffen *et al.* 2008) (Figure S11C). In contrast, loss of *GCN4* did not prevent RLS extension by *RPL20B* deletion in *phbΔ* cells (Figure 4C). Additionally, deletion of the small ribosomal subunit *RPS7A*, which does not induce *GCN4*, extended RLS in *phbΔ* but not wild-type cells (Figure S11B,D). Together these results suggest that reduced global mRNA translation associated with *RPL20B* deletion, rather than activation of *GCN4*, is responsible for RLS extension in *phbΔ* cells.

To further test this model we examined the effect of deletion of the gene encoding the ribosomal S6 kinase homolog, Sch9, on RLS in *phbΔ* cells. Sch9 promotes mRNA translation in response to nutrient availability (Jorgensen *et al.* 2004). Deletion of *SCH9* is sufficient to increase RLS and dramatically reduce translation under control conditions (Figure 4A, S6F), and lifespan extension by DR is partially mediated by reduced Sch9 activity (Kaeberlein *et al.* 2005b). Similar to DR, deletion of *SCH9* robustly reduced levels of translation and increased RLS in *phbΔ* cells (Figure 4A,D).

Finally, we examined the effect of the translation inhibitor cycloheximide on RLS. Cycloheximide binds to the E site of eukaryotic ribosomes, inhibiting mRNA translation through a mechanism distinct from loss of ribosomal subunits and having no affect on mitochondrial translation (Ennis & Lubin 1964). 50 ng/mL cycloheximide had little effect on the RLS of wild-type cells, as previously reported (Steffen *et al.* 2008), but increased the RLS of prohibitin-deficient cells significantly and to a degree similar to loss of *RPS7A* (Figure 4E). Together this data demonstrate that a reduction in cytoplasmic mRNA translation is sufficient to increase the RLS of cells deficient in mitochondrial prohibitins and that, although regulation of mRNA translation is important for RLS extension in both wild-type and prohibitin deficient cells, the mechanisms are at least partially distinct (Figure 4F).

RNAi knockdown of the worm ortholog of PHB2 shows a synergistic lifespan extension when combined with a genetic model of DR in *Caenorhabditis elegans* (Artal-Sanz & Tavernarakis 2009), suggesting that the effect of prohibitin deficiency on the response to DR is conserved. We therefore asked whether the mechanistic basis for this interaction is conserved. We performed knock-down of *phb-2* by RNAi in the nematode *C. elegans* and observed a robust induction of both commonly used mtUPR reporter strains which express GFP under the control of the *hsp-6* and *hsp-60* promoters (Yoneda *et al.* 2004) (Figure 5A-D, S12). No induction was observed using *hsp-4* or *hsp-16* promoter driven GFP, reporters of the cytoplasmic and ER UPR's respectively (Figure S13A-B). The basal level and induction of *Hsp60* was attenuated by deletion of *rsks-1* (Figure 5F), which encodes the nematode ortholog of Sch9 and, as in yeast (Steffen *et al.* 2008), reduces mRNA translation in *C. elegans* (Pan *et al.* 2007). *Rsks-1* RNAi did not attenuate induction of the cytoplasmic or ER UPR's by heat shock, indicating that the effect on *hsp-60* induction was unlikely to be a direct result of the general reduction in translation caused by knockdown of *rsks-1* loss (Figure S13C-D). Finally, prohibitin knockdown shortens the lifespan of wild-type (N2) animals but significantly extends lifespan in animals lacking *rsks-1*, which are already long-lived (Figure 5E, Table S12). Thus the effect of prohibitin deficiency on lifespan and mitochondrial stress, the response to DR, and the underlying mechanisms for that response appear to be shared between yeast and *C. elegans*.

Discussion

While DR attenuates aging in a number of organisms there is growing recognition that the effects of DR are strongly influenced by genetic background. Here we present the effect of DR on the lifespans of 166 yeast strains, each lacking a single non-essential gene. The data presented here are consistent with a study in mice examining effects of 40% DR on survival in 41 recombinant inbred mouse strains (Liao *et al.* 2010). Though that study examined a small number of animals for each genotype (5 per condition), the distribution of effects from DR is strikingly similar to our data from the 166 yeast deletion strains.

While our study examined only single gene deletions, which likely underrepresents genetic variation in natural populations, our approach has the distinct advantage that the primary genetic factor is known for each strain examined. The data clearly indicate that genetic context plays a large role in determining the effect of DR on lifespan, and in this study we have begun to elucidate the molecular processes that influence this response. We define specific molecular processes that underlie both positive and negative responses to DR. Each of the molecular processes identified here is highly conserved from yeast to humans, suggesting that similar mechanisms could underlie genotype-specific responses to DR in higher eukaryotes. In the case of the prohibitins, their shared effects on mitochondrial protein homeostasis and longevity in *C. elegans* further support this notion. Our study examined a limited number of single deletion strains, representing a subset of molecular

processes. Further study of additional strains will likely reveal additional cellular processes that modify the response to DR.

Conserved features of the mitochondrial unfolded protein response in yeast

Evidence for a conserved mtUPR had not previously been extended to yeast (Martinus *et al.* 1996) despite conservation of the primary mitochondrial stress response proteins including Hsp60, Ssc1 (mtHSP70), Hsp10, and Mdj1 (mtDnaJ) (Deloche *et al.* 1997). In *C. elegans*, studies of the mtUPR have relied almost exclusively on the $hsp\text{-}6_{pr}$::GFP and $hsp\text{-}60_{pr}$::GFP reporter strains used in this study. Induction of these reporters in response to mitochondrial proteotoxic stress requires several factors, including ubiquitin-like protein Ubl-5, the matrix peptide exporter Haf-1, and the Dve-1 transcription factor (Haynes & Ron 2010). Interestingly, we were unable to detect a change in mRNA levels of *HSP60* in prohibitin-deficient cells, suggesting that the regulation of the mtUPR may involve post-transcriptional regulation of protein levels (data not shown). Although the factors that mediate this response appear to be different in yeast, the degree to which the signaling and regulatory components of the mtUPR are conserved will be important to assess.

The observation that prohibitin deficiency induces a mitochondrial proteotoxic stress response and shortened lifespan in both yeast and *C. elegans* is of particular interest. The importance of nuclear-mitochondrial signaling in aging was first demonstrated by the retrograde response in yeast (Kirchman *et al.* 1999). More recently, the mtUPR has been shown to be required for lifespan extension following electron transport chain (ETC) RNAi in *C. elegans* (Durieux *et al.* 2011). This raises the question of why induction of the mtUPR is associated with enhanced longevity in some cases, such as knockdown of cytochrome c oxidase 1 (*cco-1*) by RNAi (Durieux *et al.* 2011), and reduced longevity in other cases, such knockdown of *phb-2* ((Artal-Sanz & Tavernarakis 2009) and (Figure 5D)). One possibility is that the nature of the mitochondrial proteotoxic stress may differ between prohibitin-deficient cells and cells deficient in components of the ETC. An alternative explanation is that situations where induction of the mtUPR is associated with extended lifespan are examples of hormesis. In these scenarios, induction of the mtUPR in the absence of a lifespan-shortening defect may result in an increase in chaperones that promote or facilitate longevity. Prohibitin loss may represent a case where the window of hormesis has been exceeded and the underlying defect reduces longevity. This explanation is consistent with the fact that prohibitin deletion is developmentally lethal in *C. elegans* and mice (Artal-Sanz *et al.* 2003) and that conditional post-developmental brain-specific knockout of prohibitin in mice results in neurodegeneration (Merkwirth *et al.* 2012). This type of dosage-dependent hormetic relationship has been described for the ER-UPR and heat shock, where mild stress is capable of extending lifespan in a setting where more intense stress is lifespan limiting.

During preparation of this manuscript (Houtkooper *et al.* 2013) reported a reduction in life span in mice and nematodes with defective mitochondrial translation. These observations were attributed to an imbalance in mitochondrial protein homeostasis and activation of the mitochondrial unfolded protein response. Our data suggest that induction of the mtUPR in yeast and nematodes deficient for prohibitins indicates a failure to maintain protein homeostasis in the MIM coupled with a robust induction of the mtUPR. Prohibitins are localized to the MIM where they have been implicated in a variety of processes. Notably, prohibitins negatively regulate the mitochondrial AAA (m-AAA) protease comprised of Afg3 and Yta12 in the MIM (Steglich *et al.* 1999). The m-AAA proteases are producers of mitochondrial peptides in yeast and worms that act to mediate mitochondria to nucleus signaling. The peptides produced by these proteases are exported from the mitochondria by Mdl1 and Mdl2 in yeast (Young *et al.* 2001), while peptides produced in mitochondria in worms are exported by Haf-1, an exporter reported to be necessary for induction of the mtUPR (Haynes *et al.* 2010). Intriguingly, it has recently been demonstrated that induction

of the mtUPR in *C. elegans* can be accompanied by a reduction in cytoplasmic translation through a parallel pathway involving GCN-2 (Baker *et al.* 2012). This implies that reduced cytoplasmic translation is a homeostatic process that evolved to promote proteostasis in the face of a mitochondrial proteotoxic stress. Interestingly, deletion of *AFG3* extends RLS and decreases cytoplasmic mRNA translation in wild-type yeast (Delaney et al. 2012), while prohibitin deletion does not reduce translation (this study) and is synthetically lethal with both *AFG3* and *YTA12* ((Osman et al. 2009) and Figure S14).Together, this suggests that a functional complex of the m-AAA proteases may be necessary for induction of the mtUPR in yeast, that disruption of this complex prevents the response to mitochondrial proteotoxic stress and leads to inviability in *phbΔ* cells, and that the translation reduction mediated by loss of *AFG3* may be mechanistically related to the reduced translation resulting from mitochondrial proteotoxic stress in worms. The availability of a yeast model in which to study the mtUPR should facilitate addressing these important questions.

Genotype determines the mechanisms by which DR modulates longevity

Another important observation from this study is that, in addition to the magnitude and direction of effect, the mechanisms by which DR influences longevity can change depending on genetic context. For example, in *sod2Δ* cells, induction of mitochondrial respiration dramatically shortens lifespan while in wild-type (Kaeberlein *et al.* 2005a) or prohibitin mutant cells (this study) induction of respiration has little or no effect on RLS extension by DR. The effect of respiratory deficiency on RLS under both control and DR conditions varies among different laboratory yeast strains (Kirchman *et al.* 1999; Lin *et al.* 2002); natural diversity in antioxidant systems may contribute to these varied responses.

Our observations in *sod2Δ* cells are similar to previous reports examining the effects of DR in a transgenic mouse model of amyotrophic lateral sclerosis (ALS). G93A animals express a mutant form of human *SOD1* and display progressive limb weakness and paralysis due to loss of spinal motor neurons. When subjected to a 40% reduction in caloric intake, G93A animals experienced increased oxidative stress and accelerated disease onset and progression (Hamadeh *et al.* 2005; Patel *et al.* 2010). In our screen, *sod1Δ* cells subjected to DR also had a reduced RLS (Figure S15), though the extremely short RLS of these cells on both normal and DR media prohibited epistasis experiments. We did, however, observe a robust increase in RLS of *sod1Δ* and *sod2Δ* cells when ascorbic acid or NAC were added to either standard or DR media (Figure 2E-F, S5E-F). Together, the findings in yeast and mice support a model where, under conditions of reduced antioxidant capacity, DR is detrimental to both life span and health span by increasing oxidative damage (Figure 2H).

Recently, Hughes *et al* (Hughes & Gottschling 2012) reported a reduction in RLS in *vmaΔ* cells subjected to DR. This report provides an independent confirmation of the data reported herein for some strains in our screen. In contrast to our findings in *sod2Δ* cells, DR-mediated decrease in RLS of *vmaΔ* cells resulted from altered amino acid storage within the vacuole rather than increased respiration. Therefore, although both *sod2Δ* and *vmaΔ* cells both experience reductions in RLS in response to DR, it appears that two distinct mechanisms are responsible.

Another example of mechanistic differences in the longevity effect from DR is evident from the fact that a general decrease in mRNA translation is sufficient to extend the RLS of prohibitin mutants but not wild-type cells. Gcn4 is required for RLS extension from mutants with reduced mRNA translation in wild-type yeast (Steffen *et al.* 2008), but not in prohibitin mutants. We propose that mitochondrial proteotoxic stress limits the RLS of prohibitin mutants and that a general inhibition of cytoplasmic mRNA translation alleviates this defect. This model is consistent with a prior study reporting that inhibition of cytoplasmic mRNA translation can suppress the short RLS and mitochondrial degeneration of cells expressing a

dominant-negative allele of the adenine nucleotide translocase Aac2 (Wang *et al.* 2008). Thus, although DR is able to extend lifespan in both wild-type and prohibitin mutants, the mechanistic underpinnings for lifespan extension appear distinct.

In addition to providing insight into the role of genetic variation in the response to DR, the data and approach presented here may also suggest novel therapeutic strategies for a subset of genetic diseases. For example, the robust positive effect of DR in several strains lacking a nuclear encoded mitochondrial protein (15 of the top 20 responders) may indicate that DR, or DR mimetics such as rapamycin, may be beneficial in mitochondrial disorders. Although speculative, this idea merits further consideration and testing. In the case of the prohibitin mutants in yeast and worms, the mechanistic basis for this effect appears to involve attenuation of proteotoxic stress in the mitochondria through a global reduction in mRNA translation. It will therefore be important to determine whether a similar mechanism is at play in the other mitochondrial mutants showing a robust response to DR, as well as whether the mtUPR is induced in diseases of mitochondrial dysfunction.

Conclusions

Genetic variation modulates the effect of DR on longevity, as well as the molecular mechanisms underlying these effects. Here we have shown that variation in mitochondrial antioxidant capacity, vacuolar pH homeostasis, and mitochondrial protein homeostasis can profoundly influence the response to DR in yeast resulting in effects on median lifespan ranging from −79% to +103%. With the continued development of DR mimetics for therapeutic use against diseases of aging, these observations demonstrate the importance of obtaining a better understanding of the molecular processes that are likely to underlie individual, genotype-dependent differential responses to DR in mammals.

Methods

Strains and Media

Yeast strains were derived from the BY4742 (*MATα MET15, his3D1Δ, leu2DΔ, lys2DΔ, ura3DΔ*) and BY4741 (*MAT***a** *LYS2, his3D1Δ, leu2DΔ, lys2DΔ, ura3DΔ)* backgrounds. The *MAT***a** and *MATα* ORF deletion collections and parental strains were obtained from Open Biosystems. Additional strains were generated by PCR-based gene disruption as previously described (Steffen *et al.* 2008). A complete list of all strains is provided in Table S8.

Cells were grown on standard YEP agar containing 1% yeast extract and 2% peptone with 2% glucose (YPD) unless otherwise noted. Unless stated otherwise, DR was accomplished by reducing the glucose content of the YEP medium to 0.05%, as described (Kaeberlein *et al.* 2004). Alternative forms of DR used in this study included 0.5% glucose, 0.1% glucose, 0.005% glucose, and 3% glycerol in YEP. Cycloheximide (Sigma-Aldrich, St. Louis MO) was dissolved in 100% ethanol at 100mg/ml and cycloheximide, ascorbate, and NAC were added to YEP-agar as previously described (Steffen *et al.* 2008).

All *C. elegans* strains used in this study were obtained from the Caenorhabditis Genetic Center (CGC). Strains used were SJ4100 (zcIs13[*hsp-6p*r: :GFP]), SJ4058 (zcIs9[*hsp-60p*r: :GFP]), CL2070 (dvIs[*hsp-16.2p*r: :GFP]), RB1206 (*rsks-1(ok1255)* III) and N2 wild-type.

Replicative Lifespan (RLS) Assay

RLS experiments were performed as described (Steffen *et al.* 2009). Frozen stocks were streaked onto YPD plates two days prior to the start of the experiments. All strains were grown on experimental media prior to start of RLS experiments. Virgin daughter cells

obtained from an overnight patch. Cells were incubated at 30°C and examined for the formation of new daughter cells every 2-4 hours. Statistical significance for individual experiments was determined using a Wilcoxon Rank-Sum test and defined as $p < 0.05$. q-value multiple testing correction was utilized to determine significance across the 166 single gene mutant set using the open source software R (R Development Core Team, 2008) with the plug-in QVALUE (Kall *et al.* 2009). All experiments were blinded so that individuals performing microdissection had no knowledge of the genotype or media composition.

Polysome Profiles

Polysome profiles were performed as previously described (Steffen *et al.* 2008). Briefly, log phase cultures were arrested by quick chilling and addition of 100μg/ml cycloheximide and pelleted. Pellets were washed once in 10ml lysis buffer (25mM Tris-HCl pH 7.5, 40mM KCl, 7.5mM $MgCl_2$, 1mM DTT, 0.5mg/ml heparin, 100μg/ml cycloheximide), resuspended in 1ml lysis buffer, and lysed by shearing using glass beads. Triton X-100 and sodium deoxycholate were each added to a final concentration of 1%. Samples were incubated 5 min on ice and centrifuged to separate cell debris. Protein content was determined by absorbance at 260nm on a Nanodrop spectrometer (Thermo Scientific). 20 OD units were resuspended in 1ml Lysis Buffer containing 1% Triton X-100 and 1% sodium deoxycholate. Samples were loaded onto 11ml linear 7%-47% sucrose gradients (50mM Tris-HCl pH 7.5, 0.8M KCl, 15mM $MgCl_2$, 0.5mg/ml heparin, and 100μg/ml cycloheximide) and centrifuged 2 hours at 39,000rpm at 4°C. Gradients were collected from the top and analyzed at 254nm.

Isolation of Mitochondrial Proteins and Mass Spectrometry

Mitochondrial proteins were isolated from log phase cultures as described previously (Meisinger *et al.* 2000). Briefly, cell pellets were washed once in DI water and resuspended in DTT buffer (100mM Tris-H_2SO_4 pH9.4, 10mM dithiothreitol). Samples were incubated 15 minutes at 30°C, resuspended in Lyticase Buffer (1.2M sorbitol, 20mM potassium phosphate pH 7.4, 1U/ml lyticase), and incubated for 10 minutes at 30°C. Following incubation, samples were resuspended in homogenization buffer (0.6M sorbitol, Tris-HCl pH 7.4, 1mM EDTA), homogenized using a dounce homogenizer, and centrifuged 10 min at 600g. The supernatant was removed and spun for 10min at 6500g.

Samples were normalized to 20μg/100μL and mixed at a 1:1 with 0.1% RapiGest/50mM Ammonium Bicarbonate pH 7.8/5mM DTT and incubated at 50°C for 30 min. IAA was added to 15mM and samples were incubated, covered from light, at RT for 30 min. Samples were digested by adding trypsin to a final concentration of 1:50 enzyme:protein and incubated for one hour at 37°C with shaking. HCl was added to a final concentration of 200mM, samples were incubated at 37°C for 45 min with shaking, then spun at 14,000rpm for 10min. The supernatant containing peptides was moved to a new tube and stored at −20°C until analysis. LC-MS method and proteomic data analysis was performed as described in Supplemental Methods.

C. elegans UPR Reporter Induction

Synchronized eggs were obtained by treating adult animals with hypochlorite. Eggs were grown on EV, *phb-2*, or *cco-1* RNAi at 20° C on Nematode Growth Media (NGM) containing AMP and IPTG (Sutphin & Kaeberlein 2009). At day 3, animals were assayed for GFP expression by fluorescent microscopy using a Zeiss SteREO Lumar.V12 (Thornwood, NY, USA) microscope. Image analysis was performed using ImageJ.

Western Blotting

Yeast protein was extracted by freeze-fracture and homogenization in RIPA buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% deoxycholate, 0.1% SDS, and 1× protease inhibitor cocktail (Cat. #05892791001; Roche)). Yeast Hsp60 was detected by immunoblot using anti-Hsp60 (SPA-807, Stressgen) and GAPDH using anti-GAPDH (ab9485) both at a 1:1,000 dilution in 1% milk TBST. GFP reporter worms were homogenized in RIPA buffer using dounce homogenizers. Proteins were detected by immunoblot using anti-GFP (ab69312, Abcam, or sc-9996, Santa Cruz Biotechnology) at a 1:2,500 dilution in 1% milk TBST and anti-GAPDH antibodies (ab9485; Abcam).

C. elegans Lifespan Experiments

C. elegans lifespans were performed as previously described (Sutphin & Kaeberlein 2009). Nematodes were maintained at 20°C on NGM. Synchronized eggs obtained by treating adult animals with hypochlorite were grown on NGM plates containing 1 mM IPTG, 25 μg/ml carbenicillin, seeded with HT115(DE3) bacteria transformed with either the pL4440 vector or the *phb-2* RNAi construct. At larval stage 4 worms were transferred to plates with 50 μM FUDR to prevent hatching of progeny. Lifespans were performed at 20°C. Cohorts were examined every 1-3 days using tactile stimulation to verify viability of animals. Ruptured animals were not censored from experiments. Animals lost due to foraging were not included in the analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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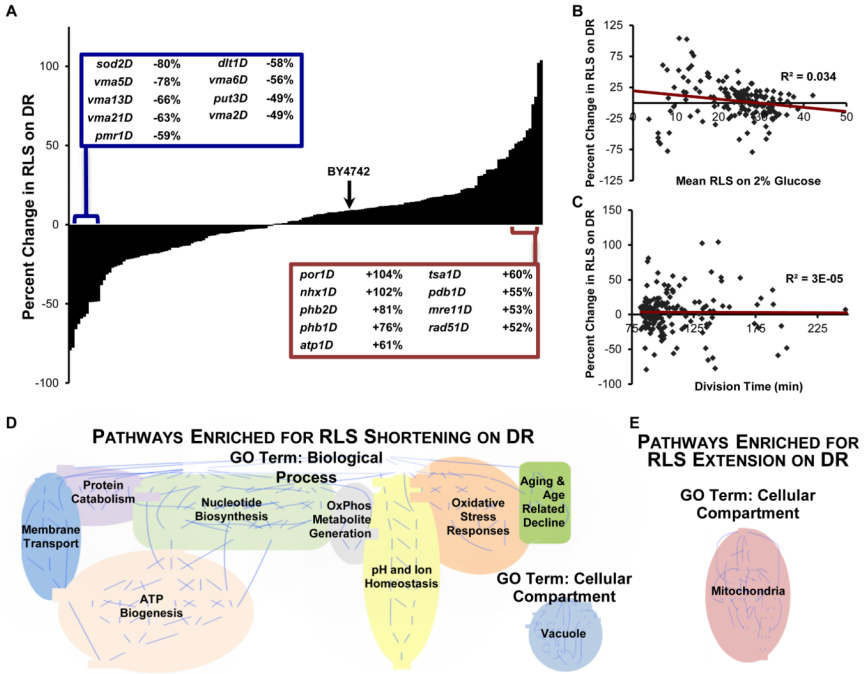

Figure 1. Genotype-dependent variation in yeast RLS response to dietary restriction

(A) RLS of 166 single deletion strains subjected to DR at 0.05% glucose. RLS varied from 80% reduction to 104% extension. Percent change in RLS on DR did not correlate to general fitness as measured by mean lifespan on 2% glucose (B) or division time (C). Gene ontology analysis of mutants with RLS significantly shortened (D) or extended (E) by DR revealed specific molecular processes involved in determining the direction of the response. Life span data and statistics provided in Table S1. GO analysis data provided in Tables S3-5.

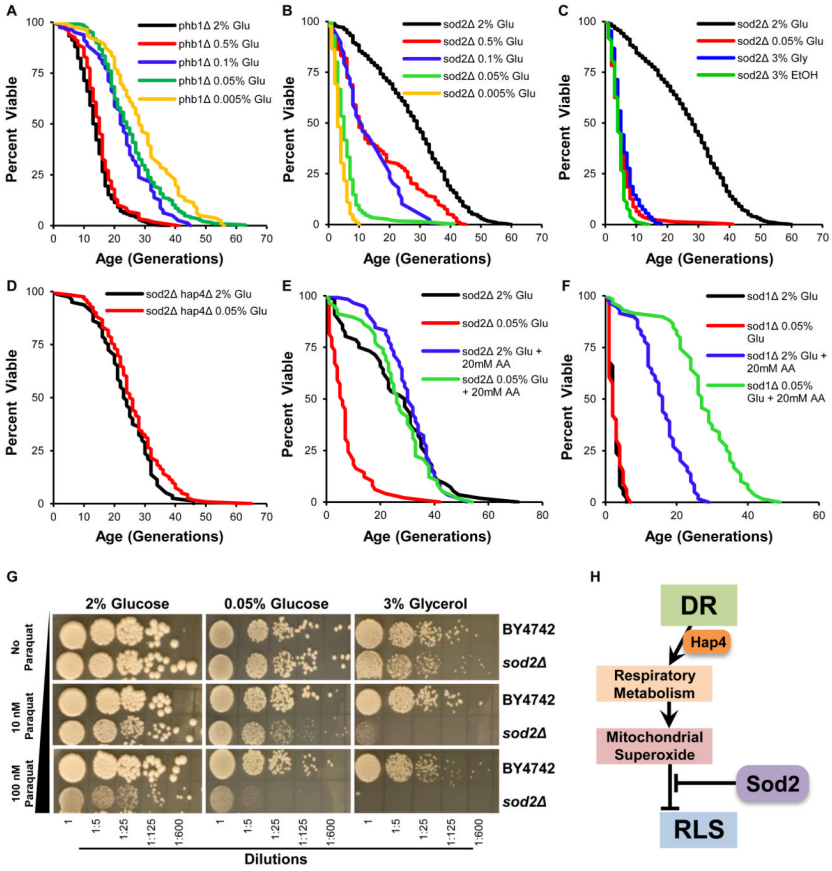

Figure 2. DR shortens the RLS of*sod2Δ*cells through the induction of respiratory metabolism

(A-B) Select mutants grown on DR demonstrate dose-dependency in both positive and negative effects of DR. (C) Growth of *sod2Δ* cells on respiratory media mimics the effect of DR on RLS. (D) Deletion of the transcription factor Hap4, which mediates the induction of respiration by DR, prevents the lifespan shortening effects of DR on *sod2Δ* cells. (E-F) RLS of *sod2Δ* and *sod1Δ* cells is increased on 2% and 0.05% glucose by addition of 20mM ascorbic acid. (G) *sod2Δ* cells are sensitive to the mitochondrial oxidative stress inducer paraquat and this sensitivity is increased under respiratory growth conditions. (H) This data supports a model by which respiratory growth induced by DR results in increased oxidative stress in *sod2Δ* cells, resulting in decreased RLS. Summary life span data are presented in Tables S7-8.

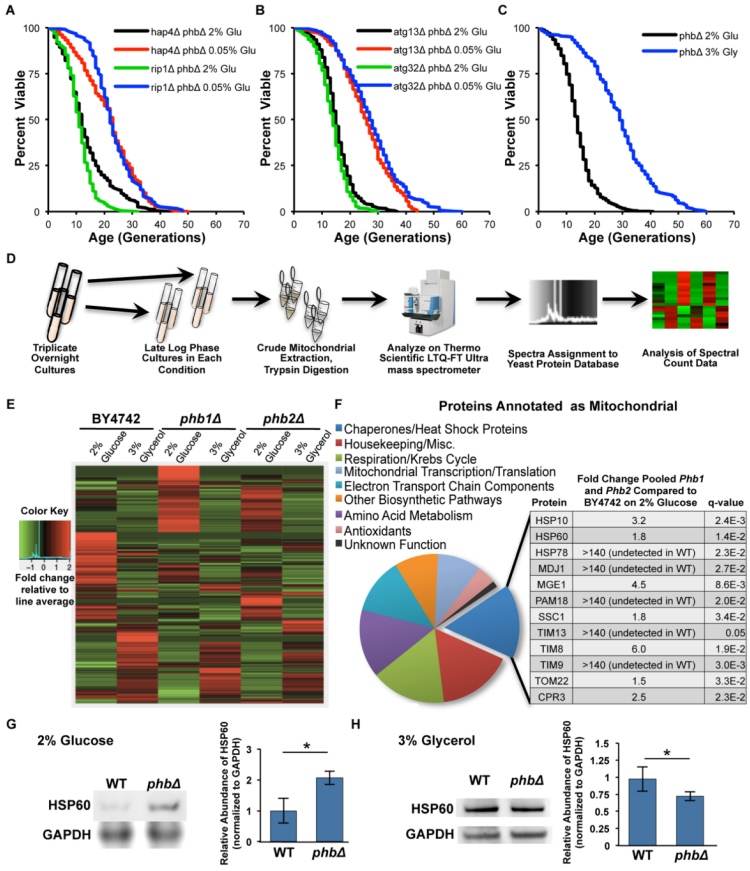

Figure 3. The DR mediated increase in RLS in*phbΔ*cells is not a result of increased respiration or autophagy and*phbΔ*cells show markers of mitochondrial proteotoxic stress which is attenuated by DR

(A) Inhibition of respiration, by deletion of HAP4 or RIP1 does not prevent the increased RLS resulting from DR in *phbΔ* cells. (B) Deletion of genes associated with autophagy does not prevent the RLS increase resulting from DR. (C) Growth on the non-fermentable carbon source, 3% glycerol, extends the RLS of *phbΔ* cell RLS to the same extent as 0.05% glucose. (D) Crude-mitochondrial isolation method schematic. (E) *phbΔ* cells show a significantly altered mitochondrial proteome when grown on 2% glucose but appear very similar to wild-type cells when grown in 3% glycerol. (F) Peptides annotated as mitochondrial were upregulated in *phbΔ* cells in 2% glucose. (G-H) Western blotting of whole cell protein isolates verifies induction of the mitochondrial chaperone Hsp-60 in *phbΔ* cells in 2% glucose and an attenuation of this in 3% glycerol. Summary life span data and proteomics are presented in Tables S1, S7-9.

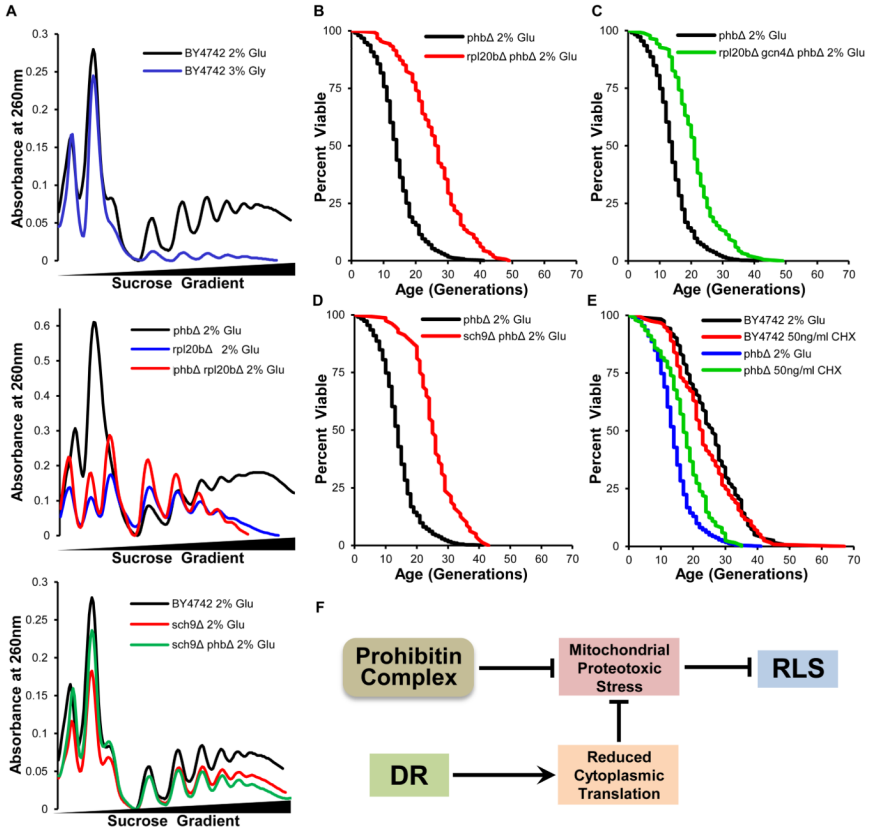

Figure 4. Increased RLS of cells deficient in prohibitin by DR is mediated by decreased translation

(A) Polysome profiles of BY4742 cells on 2% glucose and 3% glycerol (top graph), *phbΔ,* *rpl20bΔ,* and *phbΔ rpl20bΔ* cells (middle graph), and BY4742, *phbΔ*, and *sch9Δ phbΔ* (bottom graph). (B) Deletion of the large ribosome subunit component Rpl20b increases RLS of *phbΔ* cells. (C) Deletion of GCN4 does not prevent the extensions of RLS in *rpl20bΔ phbΔ* cells. (D) Loss of Sch9 extends RLS of *phbΔ* cells to a an extent similar to DR. (E) Inhibition of translation by cycloheximide slightly, but significantly extends RLS of *phbΔ* cells. (F) Proposed model by which DR extends the RLS of *phbΔ* cells by reducing mitochondrial proteotoxic stress via reduced cytoplasmic translation. Summary life span data are presented in Tables S1 and S8.

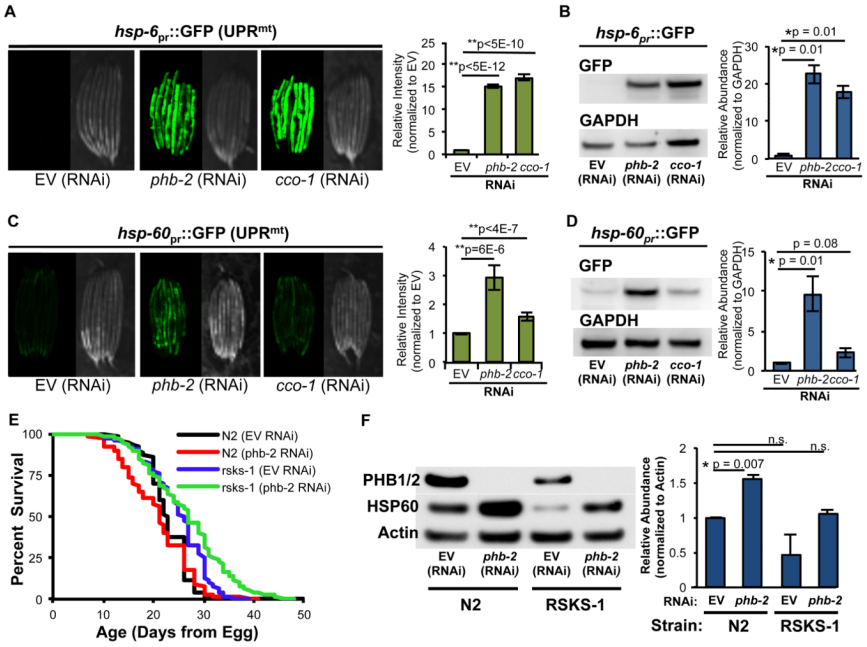

Figure 5. The effects of prohibitin deficiency on the mtUPR and response to DR are conserved in*C. elegans*

Fluorescent microscopy of *hsp-6*$_{pr}$*::GFP* (A) and *hsp-60*$_{pr}$*::GFP* (C) animals treated with empty vector (EV) (left panel), *phb-2* (middle panel), or *cco-1* (right panel) RNAi. Treatment with *phb-2* and *cco-1* RNAi induced expression of *hsp-6* and *hsp-60* (A). These findings were confirmed by Western blotting of whole protein extracts isolated *hsp-6*$_{pr}$*::gfp* and *hsp-60*$_{pr}$*::gfp* animals treated with EV, *phb-2*, or *cco-1* RNAi (B, D). (E) *phb-2* RNAi shortens the lifespan of wild-type animals, but extends the lifespan of animals lacking RSKS-1. Summary life span data and statistics are presented in Table S12. (F) Deletion of *rsks-1* decreases basal levels and attenuates the induction of *hsp-60* in animals fed *phb-2* RNAi.