Published in final edited form as:

Mech Ageing Dev. 2009 June ; 130(6): 393–400. doi:10.1016/j.mad.2009.03.004.

Endocrine Regulation of Heat Shock Protein mRNA Levels in Long-lived Dwarf Mice

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Abstract

Heat shock proteins (HSPs) maintain proteostasis and may protect against age-associated pathology caused by protein misfolding. In *C. elegans*, the lifespan extension and thermotolerance in mutants with impaired insulin/IGF signals depends partly on HSP elevation. Less is known about the role of HSPs in the increased lifespan of mice with defects in GH/IGF-I pathways. We measured HSP mRNAs in liver, kidney, heart, lung, muscle and cerebral cortex from long-lived *Pit1(dw/dw)* Snell dwarf mice. We found many significant differences in HSP mRNA levels between dwarf and control mice, but these effects were complex and organ-specific. We noted 15 instances where HSP mRNAs were lower in *Pit1(dw/dw)* liver, kidney, or heart tissues, and 14/15 of these were also seen in *Ghr(-/-)* mice, which lack GH receptor. In contrast, of 12 examples where HSP mRNAs were higher in Snell liver, kidney, or heart, none were altered in *Ghr(-/-)* mice. Four liver mRNAs were depressed in both *Pit1(dw/dw)* and *Ghr(-/-)* mice, and each of these was elevated by GH injection in Ames (*Prop1(df/df)*) dwarf mice, consistent with the hypothesis that these declines depended on GH and/or IGF-I. Contributions of chaperones to longevity in mice may be more complex than those inferred from *C. elegans*.

1. Introduction

Heat shock proteins (HSPs) participate in the folding of other proteins and protect against development of age-related protein misfolding diseases (Westerheide and Morimoto, 2005; Balch et al., 2008; Morimoto, 2008). Damaged or misfolded proteins accumulate as a natural consequence of aging, which may promote a state of “chaperone overload” that challenges mechanisms maintaining proteostasis (Tatar et al., 1997; Nardai et al., 2002; Arslan et al., 2006; Balch et al., 2008). This chaperone overload can, in some circumstances, be attenuated

by increasing the abundance or activity of HSPs, which augments cellular folding capacity and buffers against declines in protein integrity with age. In *C. elegans*, for instance, HSPs protect against toxic polyglutamine aggregations (Hsu et al., 2003; Morley and Morimoto, 2004), which arise spontaneously during the course of aging in a manner that resembles human neurodegenerative disease (Brignull et al., 2007). It has been proposed that elevated HSP abundance or activity represents a longevity assurance mechanism, and indeed, over expression of heat shock genes increases lifespan in both *C. elegans* and *Drosophila* (Tatar et al., 1997; Yokoyama et al., 2002; Walker and Lithgow, 2003; Morley and Morimoto, 2004; Morrow et al., 2004; Wang et al., 2004). Moreover, mutations promoting longevity, such as the *C. elegans* *daf-2* mutations, appear to function in part by increasing HSP abundance (Hsu et al., 2003; Walker and Lithgow, 2003; McElwee et al., 2003; McElwee et al., 2004; Halaschek-Wiener et al., 2005; Lamitina and Strange, 2005; Singh and Aballay, 2006; Samuelson et al., 2007). These observations suggest that, by enhancing protein stability, increased HSP activity or abundance can have positive effects on overall lifespan. This hypothesis suggests an approach for treating age-related disease (Westerheide and Morimoto, 2005; Balch et al., 2008; Fujikake et al., 2008), but also provides an attractive explanation for associations between longevity and stress resistance in several species (Muñoz, 2003; Murakami et al., 2003; Salmon et al., 2005; Brown-Borg, 2006).

Declines in insulin/insulin-like signaling (IIS) can, in some cases, lead to increased lifespan in worms, flies and mice (reviewed in Piper et al., 2008). This conserved effect is likely mediated by multiple mechanisms, but HSPs have at least a partial role in the effect of diminished IIS on longevity (Hsu et al., 2003; Walker and Lithgow, 2003; Morley and Morimoto, 2004). In the *C. elegans* *daf-2* mutant, inhibition of IIS increases lifespan and also elevates HSP abundance, particularly the small HSPs containing the alpha-crystallin domain (Hsu et al., 2003; Walker and Lithgow, 2003; McElwee et al., 2003; McElwee et al., 2004; Halaschek-Wiener et al., 2005; Lamitina and Strange, 2005). The expression of HSP encoding genes is required for a maximal effect of *daf-2* on lifespan, since RNAi knockdown of small HSP encoding genes partially diminishes the longevity of *daf-2* mutants (Hsu et al., 2003; Morley and Morimoto, 2004). Elevated HSP expression in *daf-2* mutants appears to be mediated by activation of both DAF-16 and heat shock transcription factor (HSF). The FOXO transcription factor DAF-16 is activated by DAF-2, regulates small HSP expression (Murphy et al., 2003), and is required for HSP induction following heat shock (Hsu et al., 2003). HSF also regulates HSP expression, and is required for both increased lifespan and elevated HSP expression in *daf-2* mutants (Hsu et al., 2003). From these observations, it has been proposed that IIS normally acts to repress activity of both DAF-16 and HSF, with activation of each pathway contributing to elevated HSP expression in *daf-2* mutants (Hsu et al., 2003; Balch et al., 2008).

The mammalian growth hormone / insulin-like growth factor I (GH/IGF-I) pathway is orthologous to the *C. elegans* IIS pathway, but how this pathway interacts with mammalian HSPs is unclear. Like the *daf-2* mutation in *C. elegans*, inhibition of GH/IGF-I signaling in mice increases both lifespan and resistance to some types of stress (Brown-Borg et al., 1996; Flurkey et al., 2001; Coschigano et al., 2000; Coschigano et al., 2003; Murakami et al., 2003; Salmon et al., 2005; Conover and Bale, 2007). In contrast to invertebrate systems, however, IGF-I signaling in mammals is GH-dependent, and mammals have a greater diversity of HSPs, three distinct heat shock transcription factors (*Hsf1*, *Hsf2* and *Hsf4*), and two separate insulin receptors (*IR* and *Igf1r*). In addition, many of the tissues and organs of mice differ greatly from those of flies and worms in structure, function, control circuitry, and developmental biology. Therefore, it is uncertain to what extent control of HSP expression by GH and IGF-I in mice parallels that described in *C. elegans*. If such interactions are in fact conserved, inhibition of GH/IGF signaling in mice would be predicted to increase HSP expression. The evidence on this point is at present very sparse. One previous report (Maynard

and Miller, 2006) found no difference in Hsp70 levels between fibroblast cells from GH-deficient *Pit1(dw/dw)* and littermate control mice in standard growth medium, and, surprisingly, noted lower Hsp70 protein levels in the *Pit1(dw/dw)* cells after serum deprivation and H₂O₂ treatment. These *in vitro* observations contrast with those of Brown-Borg (2006), who reported that Hsp70 protein was elevated in kidney tissue from GH-deficient *Prop1(df/df)* Ames dwarf mice.

In our study, we investigated the influence of GH/IGF-I signaling mutations on HSP expression in six tissues from long-lived dwarf mice (liver, kidney, heart, lung, muscle and brain). Our analysis involves three long-lived mutant mouse genotypes (*Pit1(dw/dw)*, *Prop1(df/df)*, *Ghr(-/-)*), each of which exhibits increased lifespan and delayed aging with inhibited GH/IGF-I signaling (Brown-Borg et al., 1996; Flurkey et al., 2001; Coschigano et al., 2003). *Pit1(dw/dw)* and *Prop1(df/df)* mice have impaired embryonic development of the anterior pituitary, leading to primary loss of GH, TSH, and prolactin production, with lower serum IGF-I levels secondary to the GH deficiency (Brown-Borg et al., 1996; Flurkey et al., 2001). *Ghr(-/-)* mice have a narrower endocrine deficiency involving GH-insensitivity, without alteration of other endocrine pathways (Coschigano et al., 2003). Our main interests were to test the working hypothesis that the diminished GH/IGF-I signaling in these mice would lead to elevated expression of multiple HSP genes in multiple tissues, and to make comparisons among the long-lived mutants in order to analyze the relative importance of TSH, PRL, GH and IGF-I pathways in the control of HSP gene expression.

2. Methods

2.1. Mice

All mice were maintained in specific pathogen-free facilities and provided *ad libitum* access to tap water and Purina Mouse Chow. Pathogen-free status was verified by exposing sentinel mice to spent bedding from colonies and checking sentinels for antibodies and parasites. No positive test results were obtained during the study period. The *Pit1(dw/dw)* mice, along with wild-type littermates, were bred and housed in facilities at the University of Michigan. The *Prop1(df/df)* and *Ghr(-/-)* experimental and wild-type control mice were bred and housed in facilities at Southern Illinois University. The *Pit1(-/-)* and *Ghr(-/-)* mutations were generated on DW/J *Pit1^{dw}* × C3H/HeJ *Pit1^{dw-J}* and C57BL/6J genetic backgrounds, respectively. *Prop1(df/df)* mice are maintained on a heterogeneous genetic background (Schaible and Gowen, 1961). All mice used for experiments were between 3 and 12 months of age. Tissue samples were collected only from male mice and fibroblast cell lines were derived from both male and female mice. Treatment protocols were approved by animal care and use review boards at University of Michigan and Southern Illinois University.

2.2. GH-Treatment Protocol

Porcine growth hormone (pGH) (Sigma, St. Louis, MO) treatment of approximately 7 month old *Prop1(df/df)* mice and wild-type littermates was carried out over a six week period at a dose of 4 µg per gram of body weight per day. Separate groups of *Prop1(df/df)* mice and wild-type littermates were given injections of saline. All treated mice were males. Animals were sacrificed for tissue collection 24 hours after the final pGH (or saline) injection (*n* = 9). The efficacy of pGH-treatments was verified by weekly measurements of body weight, which indicated 30% increase of body weight in GH-treated *Prop1(df/df)* mice in comparison to only 5% of body weight change in saline-treated littermates during 6 weeks treatment. Additionally, significant responses in blood chemistry measures indicated responses to GH treatment (e.g., insulin, glucose, triglycerides, glucose, FFA; data not shown).

2.3. Fibroblast Cell Lines

Primary fibroblast cell lines from *Pit1(dw/dw)* mice and wild-type littermates were derived from tail skin biopsies taken prior to 5 months of age (Salmon et al., 2005; Leiser et al., 2006; Maynard and Miller, 2006). Donor mice were anesthetized with isoflurane and a tail skin biopsy was taken (3 - 5 mm in length). Biopsies were immediately placed in DMEM (high glucose with L-Glutamine, GIBCO Cat. No. 11965) and stored briefly at 4°C until further processing. Biopsies were rinsed in 70% ethanol, immersed in PBS, diced with a sterile scalpel, and digested overnight at 37°C (10% CO₂) in complete media and collagenase (400 U/ml). Complete media was 90% DMEM and 10% Fetal Bovine Serum (HyClone Cat. No. SH30396.03), supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and fungizone (0.25 µg/ml). After overnight incubation, cells were dislodged from collagenase-digested tissue by passage through a sterile filter, isolated by centrifugation, and suspended in complete media. Approximately 2.5×10^5 passage 0 (P0) cells were seeded in 4.5 mL of complete media for a period of 6-7 days at 37°C (10% CO₂) (half the media was replaced after the first 3 days). After 6-7 days, cells were rinsed in PBS, detached by incubating cells in 0.05% Trypsin-EDTA (GIBCO 25300) for five minutes at 37°C. Trypsin digestions were halted by adding complete media to detached cells and approximately 0.75×10^6 P1 cells were seeded in new 75 cm² flasks and 9 ml of complete media. Following an additional 6-7 days, the passaging procedure was repeated and approximately 1×10^6 P2 cells were seeded in 75 cm² or 175 cm² sterile flasks.

Cells were harvested by incubation with Trypsin-EDTA and split into replicate 25 cm² flasks for use in serum deprivation experiments. In serum deprivation experiments, approximately 1×10^6 harvested P3 cells were seeded in 25 cm² flasks and 4.5 mL of complete media. After 24 hours, medium was drained from one replicate 25 cm² flask, cells were rinsed with DMEM, and 4.5 mL of serum free medium was added (DMEM, 0.02 g/mL bovine serum albumin, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone). For a second replicate 25 cm² flask, the medium was drained and replaced with complete medium. Cells from both treatments were then incubated for 20-24 hours at 37 °C prior to RNA extraction.

2.4. Total RNA Extraction and RT-PCR

RNA extractions were performed using reagents provided in RNeasy Mini Kits (Qiagen Cat. No. 74104). For tissue samples, freshly dissected tissues were immediately submerged in RNAlater solution (Ambion Cat. No. 7020) or frozen in liquid nitrogen and transferred to -80°C for long term storage. Frozen tissue samples were transitioned to -20°C by storage in RNAlater-ICE solution (Ambion Cat. No. 7030). Tissue samples were placed in a small vessel containing Lysis RLT Buffer and disrupted using a rotor-stator homogenizer. Following centrifugation, supernatants were transferred to RNeasy columns for wash steps, along with an on-column DNase digestion. For cell cultures, this same procedure was followed, except Lysis RLT Buffer was added directly to 25 cm² flasks and the lysate was homogenized using QIAshredder spin columns (Qiagen Cat. No. 79654). Quantification and quality analysis of extracted RNA was performed using a NanoDrop spectrophotometer. Sample RNA concentrations were estimated by averaging two NanoDrop measurements, and working RNA dilutions of 4 ng/µl were prepared with RNase-free water.

Real time one-step RT-PCR reactions of 20-25 µl were set up on ice using Master Mix and RT Mix reagents provided in SYBR green detection kits (Qiagen Cat No. 204243). Approximately 8 ng of total RNA per sample was used for each reaction, with bioinformatically validated Qiagen primer assays for genes of interest (Supplemental Data File 1). RT-PCR amplifications were carried out using a Corbett Rotor-Gene 3000 thermocycler unit, with samples arranged on a rotating disc designed to limit block effects and enhance uniformity among sample wells. Following the completion of each run, melting curve analysis was performed, and we examined

derivative plots to ensure that only target and reference genes were amplified (without non-specific amplification, primer dimers or contaminating DNA). We used β -Actin (*Actb*) as a reference gene for all analyses and thus estimate expression relative to abundance of the *Actb* transcript. All results are based upon 6 or more biological replicates per treatment (i.e., tissues were not pooled among mice), with 2-3 RT-PCR reactions per biological replicate serving as technical replication.

Target genes included at least one representative from several classes of mammalian heat shock genes, including heat shock transcription factors (*Hsf1*, *Hsf2*, *Hsf4*), high molecular weight HSPs (*Hsp1*, *Hsp90aa*), 70 kDa HSPs (*Hspa1b*, *Hspa5*, *Hspa9*), 40 - 60 kDa HSPs (*Hspd1*, *Hsp47*), DnaJ co-chaperones (*Dnajb11*, *Dnaja3*) and low molecular weight HSPs (*Hspb1*, *Hspb2*, *Hspb7*, *Hspb8*, *Hspb9*, *Cryab*, *Hspe1*). In choosing targets, we emphasized genes encoding small HSPs, since this group of chaperones has most frequently been found to be up regulated by the *daf-2* mutation in *C. elegans* (Hsu et al., 2003; Walker and Lithgow, 2003; McElwee et al., 2004; Halaschek-Wiener et al., 2005; Lamitina and Strange, 2005; Singh and Aballay, 2006; Samuelson et al., 2007). In particular, the alphaB-crystallin small heat shock protein *Cryab* is orthologous to the *C. elegans* *hsp-16* protein family, and members of this family are most strongly induced in *daf-2* mutants (i.e., *hsp-16.1*, *hsp-16.2*, *hsp-16.11*, *hsp-16.41*, *hsp-16.48*, *hsp-16.49*) (Hsu et al., 2003; Halaschek-Wiener et al., 2005). Our analysis, however, includes HSPs of varying molecular weights, because the *C. elegans* *daf-2* mutation has also been found to increase expression of 40 - 100 kDa HSP family members (i.e., *hsp-1*, *hsp-3*, *R151.7*) (Halaschek-Wiener et al., 2005; Singh and Aballay, 2006). In addition, we consulted microarray studies that have previously examined the effects of the *Pit1* (*dw/dw*) and *Prop1* (*df/df*) mutations in liver tissue (Amador-Noguez et al., 2004; Boylston et al., 2006), and these data suggested that non-small HSPs, including *Hspa5*, *Dnaja3* and *Dnajb11*, were down regulated by both *Pit1* (*dw/dw*) and *Prop1* (*df/df*) in liver.

2.5. Statistical Analyses

For each target heat shock gene, statistical analyses were performed on the average Δ Ct values calculated for individual mice, where Δ Ct is the difference in cycle threshold value between target and reference genes. Δ Ct values are proportional to gene expression level on a log-scale, and are therefore appropriate for statistical tests sensitive to outliers. To evaluate whether the *Pit1* (*dw/dw*) or *Ghr* (*-/-*) mutations significantly influenced expression of heat shock genes, t-tests were used to evaluate whether Δ Ct values differed significantly between experimental and control groups. In some cases, an outlier was present among Δ Ct values, and for these genes we have instead reported results from the nonparametric Wilcoxon sign test (Table 1). For tests involving the *Pit1* (*dw/dw*) mutation, we report results from two-tailed tests, and adjusted for multiple comparisons among the 19 heat shock genes using the Benjamini Hochberg procedure (Benjamini and Hochberg, 1995). For tests involving the *Ghr* (*-/-*) mutation, we report results from one-tailed t-tests, since analyses of the *Pit1* (*dw/dw*) mutation provided an expectation in terms of whether genes would be up or down regulated.

3. Results

3.1 Heat shock gene expression is altered in long-lived *Pit1* (*dw/dw*) Snell dwarf mice

It was predicted that loss of GH/IGF-I signaling would promote increases in mRNA levels for multiple chaperone proteins in multiple organs. To evaluate this possibility, we used RT-PCR methods to compare levels for 19 such mRNAs in liver, kidney, heart, lung, muscle, and brain for *Pit1* (*dw/dw*) mutant mice and their littermate controls ($n = 6$ of each genotype). Figure 1 shows the results as bars encompassing each 95% confidence interval; bars that overlap the reference line (showing a dwarf/control ratio of 1) indicate the absence of statistical significance at a nominal p-value of 0.05. Based on this criterion, our initial screen identified

36 significant heat shock gene expression differences (shaded bars in Figure 1). At a type I error rate of 5%, only 5 or 6 of the 114 comparisons would be expected to reach $p < 0.05$ by chance alone. As a conservative measure, however, we also carried out Benjamini-Hochberg multiple test corrections, and found that 20 of the 40 significant differences remained significant after this adjustment (solid bars in Figure 1).

Contrary to our working hypothesis, the *Pit1(dw/dw)* mutation did not promote a uniform elevation of heat shock gene expression in multiple organ types (Figure 1). Rather, results show that *Pit1(dw/dw)* mice have significant increases, or significant decreases, in expression of many HSP and HSP-related mRNAs, with the pattern of change varying greatly from organ to organ. Most significant effects of *Pit1(dw/dw)* were associated with liver, kidney and heart, while fewer genes were altered by *Pit1(dw/dw)* in lung, muscle and brain (Figure 1). In kidney, *Pit1(dw/dw)* tended to decrease heat shock gene expression, with nine genes down regulated by *Pit1(dw/dw)* and only one gene up regulated by *Pit1(dw/dw)*. In cardiac tissue, the opposite pattern emerged, and seven heat shock genes were up regulated by *Pit1(dw/dw)* (Figure 1). In liver, *Pit1(dw/dw)* increased the expression of four genes (e.g., *Hsp47*, *Hspa9*), but also decreased the expression of five others (e.g., *Hspb1*, *Hsph1*). Among the 19 genes examined, there were seven genes for which *Pit1(dw/dw)* increased expression in one tissue, while decreasing expression significantly in another tissue (Figure 1). These results document complex tissue-specific regulatory mechanisms controlling heat shock gene expression in young adult mice.

3.2 Partial overlap of *Pit1(dw/dw)* and *Ghr(-/-)* effects in kidney and liver

Table 1 shows the result of an analysis, in *Ghr(-/-)* tissues, of the 36 mRNAs for which we detected a significant difference between *Pit1(dw/dw)* mice and wild type littermates. In the kidney, each of the 9 HSP mRNAs that showed a significant decline in *Pit1(dw/dw)* mice also declined in kidney tissue from *Ghr(-/-)* mice (e.g., *Dnajc3*, *Hsph1*), which is consistent with GH-regulation of these genes in this organ. Likewise, in liver, four of the five HSP mRNAs that were significantly lower in *Pit1(dw/dw)* also showed a significant decline in *Ghr(-/-)* mice (*Dnajc3*, *Hspa5*, *Hsph1* and *Dnabp11*). In contrast, none of the four liver genes that were significantly elevated in *Pit1(dw/dw)* mice (*Hspb7*, *Hspa9*, *Hsp47* and *Hsf4*) was altered by the *Ghr(-/-)* mutation. Similarly, none of the seven genes elevated in the heart of *Pit1(dw/dw)* mice was altered significantly in the *Ghr(-/-)* animals. The data from liver, heart, and kidney are consistent with the idea that diminution of chaperone genes in *Pit1(dw/dw)* mice is mediated, directly or indirectly, by GH, but that elevation of mRNA levels in these mice is not dependent on GH effects.

In lung, mRNA for the two small HSPs, *Hspb7* and *Hspb8*, showed large and significant increases in both *Pit1(dw/dw)* and *Ghr(-/-)* mice, suggesting that small HSPs, at least in lung, may be elevated by loss of GH. This was an exception to the general pattern described above, where decreases in HSP mRNAs in the *Pit1(dw/dw)* system were replicated in *Ghr(-/-)* mutants more often than increases in HSP mRNA.

Our dataset for muscle and brain is limited, because only 3 mRNAs were altered significantly, in each of these tissues, in the *Pit1(dw/dw)* mice. Of the six HSP mRNAs whose expression changed in skeletal muscle or brain (2 increases, 4 decreases), none showed significant alteration in *Ghr(-/-)* mice. The limited effects of *Pit1(dw/dw)* on HSP mRNAs in muscle and brain therefore seem unrelated to GH levels. We cannot, however, rule out the possibility that such *Pit1(dw/dw)* effects are somehow related to maternal GH signals during fetal development or early lactation, to which *Ghr(-/-)* mice are insensitive.

3.3 GH injections mitigate effect of *Prop1(df/df)* mutation on HSP gene expression in liver

Our observations on *Pit1(dw/dw)* and *Ghr(-/-)* mice suggested that diminished expression of *Dnajc3*, *Hspa5*, *Hsph1* and *Dnajb11* in liver might reflect lower GH or IGF-I levels. To test this idea, we evaluated expression of each of these four mRNAs in liver of GH-deficient *Prop1(df/df)* Ames dwarf mutants that had been exposed to GH injections for a period of six weeks. As shown in Figure 2, expression of each gene was diminished in liver of *Prop1(df/df)* mutants compared to their littermate controls ($P < 0.01$); thus the pattern seen in *Pit1(dw/dw)* and *Ghr(-/-)* mutants was replicated in the Ames *Prop1(df/df)* dwarf. GH-treatment of these mutant mice significantly increased expression of *Hspa5*, *Hsph1*, *Dnajb11* and *Dnajc3* (two-tailed t-test; $p < 0.01$) (Figure 2), but had no significant effect on *Hspb1* expression (two-tailed t-test; $p = 0.25$) (data not shown). These findings are therefore consistent with the idea that the lower level of these four HSP mRNAs in liver of *Pit1(dw/dw)*, *Prop1(df/df)*, and *Ghr(-/-)* mutants reflects action of GH and/or IGF-I on hepatic cells. Surprisingly, we also noted that GH-treatment decreased expression of each of these four HSP mRNAs in wild-type mice (ANOVA Genotype by Treatment Interaction, $P < 0.048$; posthoc t-test significant at $P < 0.05$ for *Hsph1*, *Hspa5* and *Dnajb11*, but not significant for *Hspb1* and *Dnajc3*). This may reflect differences in GH-sensitivity between wild type and *Prop1(df/df)* mice, or an interaction between GH and other endocrine factors that are diminished in the *Prop1(df/df)* mutant (e.g., thyroid stimulating hormone, prolactin).

3.4 *Pit1(dw/dw)* does not increase HSP expression in stress-resistant fibroblast cells

Skin-derived fibroblast cells from *Pit1(dw/dw)* mice are resistant to multiple forms of stress (Murakami et al., 2003; Salmon et al., 2005). The *C. elegans daf-2* mutant is also stress resistant, and this resistance depends partly upon elevated HSP expression (Walker and Lithgow, 2003; Morley and Morimoto, 2004). We therefore evaluated whether stress-resistance of *Pit1(dw/dw)* fibroblasts could be attributable to elevated HSP expression (Figure 3). Our initial analysis indicated that, in complete media culture, two small HSPs (*Hspb1* and *Hspb7*) are significantly down regulated in fibroblasts from *Pit1(dw/dw)* mice, while *Hsp90aa* and *Hspa1b* were significantly up regulated (Figure 3A). Since these effects were minor, we evaluated whether effects of *Pit1(dw/dw)* were enhanced by a 24 hr. period of serum deprivation, which is known to augment the stress resistance of *Pit1(dw/dw)* fibroblasts (Murakami et al., 2003). Following serum withdrawal, however, the four genes for which expression was modulated by *Pit1(dw/dw)* in complete media were no longer differentially expressed between cells from *Pit1(dw/dw)* and wild-type mice (Figures 3B – 3E). Additionally, of the 15 other heat shock genes we examined, none were significantly altered by the *Pit1(dw/dw)* mutation following serum deprivation (data not shown).

4. Discussion

Augmented levels of heat shock proteins (HSPs) can increase the stress resistance and longevity of invertebrate organisms (Tatar et al., 1997; Yokoyama et al., 2002; Walker and Lithgow, 2003; Morley and Morimoto, 2004; Morrow et al., 2004; Wang et al., 2004). In the *C. elegans daf-2* mutants, inhibition of insulin/insulin-like signals (IIS) elevates HSP expression, which contributes to both stress resistance and extended lifespan (Hsu et al., 2003). In mouse, inhibition of the orthologous GH/IGF-I pathway also increases organismic lifespan and cellular stress resistance, but the role of HSPs in these effects is uncertain. We have evaluated HSP expression patterns in several tissues from long-lived mice with diminished GH/IGF-I signaling. We started with two working hypotheses. First, based on the nematode results, we expected elevation of most HSP genes in multiple tissues. Second, because *Pit1(dw/dw)* and *Ghr(-/-)* mice are both long-lived, and both have low GH and IGF-I signals, we expected tissues from these mice to show similar patterns of alteration in HSP gene expression.

Both of these expectations were refuted by the data. Some HSP mRNAs are indeed elevated in *Pit1(dw/dw)* tissues, but many others are significantly lower, and furthermore the pattern seen in *Ghr(-/-)* differs from that of *Pit1(dw/dw)* mice in many respects. Compared to controls, HSP mRNA levels in *Pit1(dw/dw)* mice were typically increased in heart tissue, were decreased in kidney tissue, and showed a mixture of increases and decreases in liver. For these organs, all but one of the decreases in HSP mRNA (14 of 15) characteristic of *Pit1(dw/dw)* mice were also significantly altered in the *Ghr(-/-)* animals, suggesting that the lower HSP mRNA levels were indeed a consequence, direct or indirect, of lower GH and/or IGF-I signals. Of the 12 elevations of HSP mRNAs seen in these tissues of *Pit1(dw/dw)* mice, none were seen in *Ghr(-/-)* mice, suggesting that these elevations probably do not reflect alterations in GH or IGF-I alone. Changes in HSP mRNA levels in lung, muscle, and brain were fewer, and inconsistent in direction, although it is noteworthy that the elevations of *Hspb7* and *Hspb8* in *Pit1(dw/dw)* lung are also seen in pulmonary tissue of *Ghr(-/-)* mice. We found no HSP, of the 19 evaluated, that showed a consistent change in each of the six tissues examined, although mean levels of *Hsph1* were lower in five of the six tissues (significant in three of these), and *Hspb7* showed a trend towards higher expression in five tissues (significant in three). Ames dwarf mice (*Prop1(df/df)*) injected with GH showed a significant increase in mRNA for the four liver HSP genes whose levels are depressed in *Pit1(dw/dw)* and *Ghr(-/-)* mice, strongly suggesting that depression of these four mRNAs in liver is dependent on GH.

We do not exclude the possibility that other endocrine factors, besides GH, also contribute to the transcriptional regulation of HSPs *in vivo*. Clearly, some effects of *Pit1(dw/dw)* are not shared by the *Ghr(-/-)* mutation (Table 1). These effects could be attributable to circulating levels of prolactin (PRL) and/or thyroid stimulating hormone (TSH), which are greatly diminished in *Pit1(dw/dw)* mice, but normal in *Ghr(-/-)* mice. For tissues we evaluated, the transcriptional effects of PRL and TSH have not been well-studied, but there is evidence that these hormones can alter HSP expression within other organ systems. In particular, PRL has been found to increase HSP expression in prostate (Robertson et al., 2003), ovary (Stocco et al., 2001), mammary gland (Briskin et al., 2002), rat Nb2-11C lymphoma cells (Bole-Feysot et al., 2000) and human SKBR3 breast cancer cells (Perotti et al., 2008). Likewise, the expression of *Hspa5* (also *BiP*) is induced by TSH supplementation within rat thyroid epithelial cells (Endo et al., 1991). Increased HSP expression in cardiac tissue is the strongest effect of *Pit1(dw/dw)* that is not shared by *Ghr(-/-)*. This effect may be attributable to loss of PRL and/or TSH signals in *Pit1(dw/dw)* mice, and may also be consequential for longevity, since HSP expression in heart is thought to protect against oxidative stress damage (Lau et al., 1997; Martin et al., 1997; Yan et al., 2002). Surprisingly, *Pit1(dw/dw)* did not also increase HSP expression in skeletal muscle (Fig. 1), which shares a similar cell type composition with the heart. Effects of *Pit1(dw/dw)* on cardiac HSP expression may therefore relate to a histological feature of cardiac tissue that is distinct from skeletal muscle (e.g., abundance of mitochondria, distribution of transverse tubules and sarcoplasmic reticulum).

Taken together, our findings suggest a difference between the effects of GH/IGF-I signaling on HSP expression in mice, and the effects of IIS signaling on HSP expression in worms. The inhibition of IIS in the *C. elegans daf-2* mutant increases expression of small heat shock proteins (*hsp-12.3*, *hsp-12.6*, *hsp-16.1*, *hsp-16.2*, *hsp-16.11*, *hsp-16.41*, *hsp-16.48*, *hsp-16.49* and *sip-1*) (Hsu et al., 2003; Walker and Lithgow, 2003; McElwee et al., 2004; Lamitina and Strange, 2005; Samuelson et al., 2007), as well as heat shock genes that encode HSP70 and HSP90 family members (*hsp-1*, *hsp-3*, *R151.7*) (Halaschek-Wiener et al., 2005; Singh and Aballay, 2006). These results represent effects of IIS mutations on systemic heat shock gene expression in *C. elegans*, using RNA pools derived from cohorts of multiple isogenic individuals, but the available data do not provide much insight into possible differences among cells and tissues in expression of HSPs. Our system allows dissection of tissue-specific responses, and suggests that the effects of diminished GH and/or IGF-I signals on HSP gene

expression in mice do not resemble, and are in certain respects more complicated, than those described in *daf-2* mutants of *C. elegans*. This may also mean that dissimilar stress resistance mechanisms exist in *daf-2* worms and *Pit1(dw/dw)* mice. For instance, increased HSP expression contributes to stress resistance in *daf-2* mutants (Walker and Lithgow, 2003; Morley and Morimoto, 2004), but we have found little evidence that HSP expression is elevated in stress-resistant *Pit1(dw/dw)* fibroblast cells (see Fig. 3).

The expression of many HSPs is regulated by heat shock factor 1 (HSF1) (Trinklein et al., 2004), and it is possible that GH or IGF-I might exert its effects on HSP mRNA by regulation of the amount, localization or availability of this transcription factor. In *C. elegans*, the FOXO transcription factor DAF-16 appears to regulate HSP expression, and it has been proposed that DAF-16 interacts with HSF to regulate expression of heat shock genes in *C. elegans* (Hsu et al., 2003). Our data set does not indicate that alterations in HSP mRNAs in tissues of long-lived mice are coordinated with (or by) HSF1 mRNA levels, but the data do not address models in which alterations in HSF1 protein translation, stability, localization, aggregation, or post-translational modification might participate in GH/IGF-I modulation of HSP gene expression. Several other transcription factors are plausible candidates for coordinate expression of HSP levels in mice. For instance, in kidney, 7 of the 9 genes down-regulated by *Pit1(dw/dw)* and *Ghr(-/-)* have a promoter binding site for the ATF6 transcription factor, which regulates expression of heat shock genes involved in the unfolded protein response (e.g., *Hspa5*; Kudo et al., 2008).

Our data set helps address a more general question: which changes in gene expression are common, and thus possibly causal, in multiple models of extended longevity in mice? Each of the long-lived genotypes we have studied here involves diminution of GH and/or IGF-I signals, and it would therefore not be surprising to see many commonalities in expression pattern. More impressive would be a list of gene expression changes seen in multiple models of extended longevity that do not share an obvious mechanism. *Hsph1* (also called *Hsp105* or *Hsp110*) may deserve special attention in this context. *Hsph1* is a high molecular weight HSP that functions in cellular stress response and apoptosis pathways (Yamagishi et al., 2008). In our study, *Hsph1* was down regulated in liver by three longevity mutations (*Pit1(dw/dw)*, *Prop1(df/df)*, *Ghr(-/-)*), and in both kidney and heart in the two mutants evaluated (*Pit1(dw/dw)* and *Ghr(-/-)*). *Hsph1* expression is likewise reduced in many tissues of mice exposed to the anti-aging effects of caloric restriction, including liver, heart, white adipose tissue, hypothalamus and colon (Swindell, 2008). The data on *Dnajc3* (also called *p58IPK*) also deserve follow-up studies. Liver expression of *Dnajc3* was diminished by all three of the longevity mutants studied (*Pit1(dw/dw)*, *Prop1(df/df)* and *Ghr(-/-)*), and was also lower in kidney, muscle, and heart of the *Pit1(dw/dw)* mice. *Dnajc3* is a stress-inducible co-chaperone critical for survival in response to ER stress (Rutkowski et al., 2007), and there is evidence that this role of *Dnajc3* in cellular stress response can have widespread physiological effects. For instance, mice that lack *Dnajc3* have severely reduced body fat levels, low body weight and are diabetic, most likely due to apoptosis of pancreatic β -cells (Ladiges et al., 2005).

The contribution of HSPs to mammalian health and longevity may vary across tissue types or cellular contexts. In humans, genetic association studies have identified polymorphisms of genes encoding HSPs that correlate with longevity and age-related disease (Altomare et al., 2003; Debler et al., 2003; Singh et al., 2004; Wu et al., 2004), but mechanisms that underlie these associations are uncertain. HSP expression appears to enhance overall proteome stability (Balch et al., 2008), and may counteract age-related conditions resulting from accumulation of certain disease proteins (e.g., Fujikake et al., 2008; Steele et al., 2008). On the other hand, much evidence suggests that HSP expression can be deleterious, particularly by facilitating malignant transformation in some tissues (Dai et al., 2007; Min et al., 2007; Dong et al., 2008). Indeed, caloric restriction is the best established intervention for increasing lifespan and

combating age-related disease, but in mice, caloric restriction promotes widespread diminution of HSP expression (Swindell, 2008). HSP expression may therefore have both positive and negative effects, depending upon the cellular context. We have found that loss of GH/IGF-I signals has substantial, but complex effects on HSP expression in long-lived dwarf mice. These mice exhibit an increase in lifespan along with lower incidence of kidney disease, cataracts and joint disease, as well as fatal neoplastic disease, such as lymphoma and adenocarcinoma (Ikeno et al., 2003; Vergara et al., 2004). Further investigation of heat shock gene regulation in GH/IGF-I signaling mutants may thus provide insight into the physiological effects of HSPs and their influence on age-related disease among organ systems.

Acknowledgments

We thank Maggie Lauderdale and Jessica Sewald for technical and husbandry assistance. Two anonymous reviewers provided helpful comments on this manuscript. This work was supported by grants AG023122 (RAM), AG024824 (RAM), and AG198899 (AB). WRS is supported by NIA training grant T32-AG00114. J.J.K. is supported in part by the State of Ohio's Eminent Scholars Program, which includes a gift from Milton and Lawrence Goll and by WADA, and NIH (AG19899, DK075436 and CA099904).

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Figure 1.

Pit1(dw/dw) effects on heat shock gene expression in liver, kidney, heart, lung, muscle and brain. For 19 heat-shock genes, expression differences were evaluated between *Pit1(dw/dw)* mice and wild-type littermates ($n = 6$ of each genotype). Fold-change estimates were calculated, with estimates greater than 1 indicating higher expression in *Pit1(dw/dw)* mice, and estimates less than 1 indicating diminished expression in *Pit1(dw/dw)* mice. Boxes shown in the chart outline 95% confidence intervals associated with each fold-change estimate. Filled red boxes represent fold-change estimates significantly greater than 1 following adjustment for multiple testing (adjusted P-value < 0.05). Likewise, filled green boxes represent fold-change estimates significantly less than 1 following multiple testing adjustments. Boxes partially shaded red or

green represent estimates significantly greater or less than 1 based upon nominal P-values of 0.05 (without multiple testing adjustment).

Figure 2.

Growth hormone effects on hepatic heat shock gene expression in GH-deficient *Prop1(df/df)* mice and wild-type littermates ($n = 9$ of each genotype). *Prop1(df/df)* and wild-type mice were injected with GH or saline for six weeks and livers were collected 24 hours following the final injection (see Methods). We evaluated expression of four heat shock genes (*Hsph1*, *Hspa5*, *Dnajb11* and *Dnajc3*), each of which was significantly down regulated by both the *Pit1(dw/dw)* and *Ghr(-/-)* mutations in liver (see Table 1). We also evaluated expression of one heat shock gene (*Hspb1*) significantly down regulated by only the *Pit1(dw/dw)* mutation (data not shown). Significance stars shown in each figure indicate significant effects of GH-treatment in either wild-type or *Prop1(df/df)* mice ($P < 0.05$; one or two-tailed t-test).

Figure 3.

Pit1(dw/dw) effects on heat shock gene expression in skin-derived fibroblast cells. (A) For 19 heat-shock genes, expression differences were evaluated between cells from *Pit1(dw/dw)* and wild-type mice cultured in complete media ($n = 8$ of each genotype). Fold-change estimates were calculated, with estimates greater than 1 indicating elevated expression in *Pit1(dw/dw)* mice, and estimates less than 1 indicating diminished expression in *Pit1(dw/dw)* mice. Boxes outline 95% confidence intervals associated with each fold-change estimate. Boxes partially shaded red or green represent estimates significantly greater or less than 1 based upon nominal P-values of 0.05 (without multiple testing adjustment). (B – E). The expression of the four genes regulated by *Pit1(dw/dw)* in complete media was examined following 20–24 hours of

serum withdrawal. In each plot, open circles / dotted lines represent log-expression measurements from *Pit1(dw/dw)* cells, and filled circles / non-dotted lines represent log-expression measurements from wild type cells. Stars indicate significant differences between *Pit1(dw/dw)* and wild-type fibroblasts (nominal P-value < 0.05).

Table 1

Effects of *Pit1(dw/dw)* and *Ghr(-/-)* mutations on heat shock gene expression in liver, kidney, heart, lung, muscle and brain. An initial evaluation of Snell dwarf mice identified 36 cases in which the *Pit1(dw/dw)* mutation had significant effects on heat shock gene expression (Figure 1). For each of these significant results, effects of the *Ghr(-/-)* mutation were evaluated in the corresponding gene and tissue type. The table lists the fold-change expression ratio estimated for each gene-tissue combination (mutant / wild type), with p-values listed in parentheses (two-sided t-test or sign test). Bold-faced entries indicate corresponding effects between the *Pit1(dw/dw)* and *Ghr(-/-)* mutations on heat shock gene expression. Note that for cases in which expression is altered in the same direction between mutants, a two-sided p-value of 0.10 is significant according to a one-sided significance test. All comparisons are based upon $n = 6$ mutant and $n = 6$ wild type animals.

Tissue	Gene	<i>Pit1(dw/dw)</i>	<i>Ghr(-/-)</i>
Liver	<i>Hspb7</i>	2.92 (0.007)	0.45 (0.240)
	<i>Hspa9</i>	1.95 (0.045)	0.71 (0.093)
	<i>Hsp47</i>	1.90 (0.008)	1.02 (0.937)
	<i>Hsf4</i>	1.73 (0.007)	0.93 (0.261)
	<i>Dnajc3</i>	0.66 (0.047)[†]	0.59 (0.082)
	<i>Hspa5</i>	0.62 (0.030)	0.53 (0.041)
	<i>Hsph1</i>	0.57 (0.048)	0.57 (0.093)
	<i>Hspb1</i>	0.57 (0.029)	0.87 (0.485)
	<i>Dnajb11</i>	0.55 (0.026)	0.60 (0.058)
Kidney	<i>Hspb7</i>	1.61 (0.031)	0.78 (0.180)
	<i>Hspe1</i>	0.64 (0.007)	0.46 (0.001)
	<i>Hspb8</i>	0.64 (0.037)	0.60 (0.026)
	<i>Hsf2</i>	0.60 (0.005)	0.65 (0.011)
	<i>Dnajb11</i>	0.56 (0.002)	0.73 (0.071)
	<i>Hspa5</i>	0.55 (0.006)	0.55 (0.001)
	<i>Dnajc3</i>	0.53 (0.001)	0.53 (0.013)
	<i>Hsp90aa</i>	0.52 (0.002)[†]	0.71 (0.065)
	<i>Hsph1</i>	0.50 (0.016)	0.58 (0.019)
	<i>Hspd1</i>	0.46 (0.010)	0.43 (0.004)
Heart	<i>Hspa1b</i>	1.97 (0.003)	0.94 (0.861)
	<i>Hspb1</i>	1.91 (0.003)	0.73 (0.065)
	<i>Hsp90aa</i>	1.84 (0.004)	0.84 (0.317)
	<i>Hsf4</i>	1.76 (0.015)	0.88 (0.568)
	<i>Hsf2</i>	1.69 (0.031)	1.11 (0.485)
	<i>Dnajc3</i>	1.37 (0.015)	0.75 (0.026)
	<i>Cryab</i>	1.33 (0.048)	0.72 (0.132)
	<i>Hsph1</i>	0.54 (0.005)	0.63 (0.065)
Lung	<i>Hspb7</i>	3.19 (0.019)	2.24 (0.065)
	<i>Hspb8</i>	1.94 (0.008)	2.61 (0.003)
	<i>Cryab</i>	0.74 (0.012)	2.15 (0.005)
Muscle	<i>Hsp47</i>	1.62 (0.026)	0.79 (0.093)
	<i>Hspb1</i>	0.77 (0.017)	0.83 (0.364)

Tissue	Gene	<i>Pit1(dw/dw)</i>	<i>Ghr(-/-)</i>
	<i>Dnajc3</i>	0.62 (0.003)	0.97 (0.485)
Brain	<i>Hspb8</i>	1.85 (0.004)	0.78 (0.331)
	<i>Hspb2</i>	0.53 (0.002)	0.88 (0.653)
	<i>Cryab</i>	0.47 (0.006)	0.82 (0.394)