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Identification of longevity-associated genes in long-lived Snell and Ames dwarf mice

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Abstract Recent landmark molecular genetic studies have identified an evolutionarily conserved insulin/IGF-1 signal transduction pathway that regulates lifespan. In *C. elegans*, *Drosophila*, and rodents, attenuated insulin/IGF-1 signaling appears to regulate lifespan and enhance resistance to environmental stress. The Ames ($Prop1^{df/df}$) and Snell ($Pit1^{dw/dw}$) hypopituitary dwarf mice with growth hormone (GH), thyroid-stimulating hormone (TSH), and prolactin deficiencies live 40–60% longer than control mice. Both mutants are resistant to multiple forms of environmental stress in vitro. Taken collectively, these genetic models indicate that diminished insulin/IGF-l signaling may play a central role in the determination of mammalian lifespan by conferring resistance to exogenous and endogenous stressors. These pleiotropic endocrine pathways control diverse programs of gene expression that appear to orchestrate the development of a biological phenotype that promotes longevity. With the ability to investigate thousands of genes simultaneously, several microarray surveys have identified potential longevity assurance genes and provided information on the mechanism(s) by which the dwarf genotypes (*dw*/*dw*) and (*df*/*df*), and caloric restriction may lead to longevity. We propose that a comparison of specific changes in gene expression shared between Snell and Ames dwarf mice may provide a deeper understanding of the transcriptional mechanisms of longevity determination. Furthermore, we propose that a comparison of the physiological consequences of the *Pit1dw* and *Prop1df* mutations may reveal transcriptional profiles similar to those reported for the *C. elegans* and *Drosophila* mutants. In this study we have identified classes of genes whose expression is similarly affected in both Snell and Ames dwarf mice. Our comparative microarray data suggest that specific detoxification enzymes of the P_{450} (CYP) family as well as oxidative and steroid metabolism may play a key role in longevity assurance of the Snell and Ames dwarf mouse mutants. We propose that the altered expression of these genes defines a biochemical phenotype which may promote longevity in Snell and Ames dwarf mice.

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Introduction

Recent discoveries using long-lived model organisms have rapidly advanced our current understanding of the biology of aging. These landmark molecular genetic studies have identified an evolutionarily conserved insulin/IGF-1 signal transduction pathway that regulates lifespan (Kimura et al. 1997; Tissenbaum and Ruvkun 1998; Kenyon 2005; Hekimi and Guarente 2003; Tatar et al. 2003). Mutations in single genes linked to this conserved endocrine pathway not only increase longevity but also appear to enhance resistance to stress (Lithgow and Walker 2002; Sampayo et al. 2003; Kenyon 2001; Lin et al. 1998).

In the worm *C. elegans*, hypomorphic mutation of an insulin/IGF-1 receptor-like gene, *daf-2*, more than doubles invertebrate lifespan and also imparts resistance to multiple forms of environmental stress (Gems and McElwee 2005; Guarente and Kenyon 2000; Murakami and Johnson 1996; Larsen 1993). In the fly *Drosophila*, mutation of the insulin-like receptor, *InR*, results in a dwarf adult phenotype and extends lifespan in female flies by greater than 80% (Tatar et al. 2001). Further, mutation of the gene chico, an ortholog of the mammalian insulin receptor substrate proteins (IRS), can extend *Drosophila* lifespan nearly 50% via a suspected increase in anti-oxidant defense (Tatar et al. 2003; Clancy et al. 2001).

In mammalian systems, attenuated insulin/IGF-1 signaling also appears to regulate lifespan and enhance resistance to environmental stress (Bartke 2005; Bartke and Brown-Borg 2004; Holzenberger 2004; Holzenberger et al. 2003; Hsieh et al. 2002a,b; Richardson et al. 2004; Brown-Borg 2003). Ames (*Prop1*$^{df/df}$) and Snell (*Pit1*$^{dw/dw}$) mice are hypopituitary dwarf mice with multiple anterior pituitary hormone deficiencies including growth hormone (GH), thyroid-stimulating hormone (TSH), and prolactin (Flurkey et al. 2001, 2002; Bartke and Brown-Borg 2004). These dwarf mice exhibit severely reduced serum insulin and IGF-1 levels, insulin hypersensitivity and live 40–60% longer than control mice (Dominici et al. 2002; Brown-Borg et al. 1996; Flurkey et al. 2001). Ames dwarf mice have enhanced antioxidant defenses in skeletal muscle, and fibroblasts isolated from both Ames and Snell dwarf mice are resistant to multiple forms of environmental stress in vitro, including UV light, heat, paraquat, H_2O_2 and heavy metal toxicity (Salmon et al. 2005; Romanick et al. 2004; Murakami et al. 2003). Similarly, GH receptor knockout (GHRKO) mice exhibit significant reductions in serum insulin and IGF-1 levels and live 30% to 40% longer than their wild-type litter mates (Liu et al. 2004; Coshigano et al. 2000). Tissue-specific deletion of the insulin receptor gene in adipocytes (FIRKO mice) lowers plasma insulin levels by more than 30% and increases lifespan by 15% to 18% in male and female mice, respectively (Bluher et al. 2003). Disruption of an intracellular substrate of the IGF-1 receptor, p66Shc, provides a 30% increase in lifespan and increased resistance to hydrogen peroxide, UV-irradiation, and paraquat toxicity (Migliaccio et al. 1999).

Further demonstrating the role of IGF-1 in regulating mammalian lifespan, female mice heterozygous for the IGF-1 receptor (Igf1r+/−) exhibit a significant 33% increase in longevity and are also resistant to oxidative stress induced by paraquat toxicity (Holzenberger et al. 2003). Interestingly, male *Igf1r+/−* mice are not resistant to paraquat, nor do they exhibit a significant increase in lifespan when compared with male wild-type mice. Finally, disrupted expression of the *klotho* gene, which encodes a circulating hormone that inhibits intracellular insulin and IGF-1 signaling, was most recently demonstrated to extend lifespan and reduce age-related pathologies (Kurosu et al. 2005).

Taken collectively, these rodent and invertebrate genetic models strongly indicate that diminished insulin/IGF-l signaling may improve resistance to exogenous and endogenous stressors and plays a central role in the determination of mammalian lifespan. These pleiotropic endocrine pathways control diverse programs of gene expression that appear collectively to orchestrate the development of a biological phenotype that promotes longevity.

The multiplicity of endocrine deficiencies of *Pit1* and *Prop1* dwarf mutants impedes the

discovery of the molecular mechanisms associated with extended lifespan in these mice. With the ability to investigate thousands of genes simultaneously, microarray technology has been instrumental in the identification of the molecular genetic characteristics of specific tissues in mammalian longevity. Several microarray surveys have been used to identify longevity assurance genes and to provide information on the mechanism(s) by which the dwarf genotypes (*dw*/*dw*) and (*df*/*df*), and caloric restriction (CR) may lead to longevity (Dozmorov et al. 2001, 2002; Gems et al. 2002; Dhahbi et al. 2004, 2005; Amador-Noguez et al. 2005). We propose that a comparison of specific differences in gene expression shared between Snell and Ames dwarf mice may provide a deeper understanding of the transcriptional mechanisms of longevity determination.

Since the insulin and IGF-1 signaling pathways appear to be an experimentally proven determinant of aging and longevity across species, we set out to identify classes of genes whose expressions are similarly affected in both Snell and Ames dwarf mice. We propose that a comparison of the physiological consequences of the *Pit1dw* and *Prop1df* mutations may reveal transcriptional profiles similar to those reported for the *C. elegans* and *Drosophila* mutants. Our comparative microarray data suggest that specific detoxification enzymes of the P_{450} (CYP) family, as well as those of oxidative and steroid metabolism, may play key roles in longevity assurance of the Snell and Ames dwarf mouse mutants.

The argument has been presented that increased longevity assurance shown by the reduction-of-function of the insulin-IGF-1 pathway activity is linked to: (a) detoxification and excretion of molecular toxins; (b) resistance to oxidative stress; and (c) conservation of existing proteins via chaperones (Gems and McElwee 2005). In mammals the four specific phases of biochemical detoxification reactions are coordinated metabolic processes that occur mainly in the liver and eliminate endogenous and exogenous toxic substances. These phases of detoxification are: (a) phase 0, organic anion trans-porting; (b) phase I, the sequential chemical modification and inactivation of xenobiotics by cytochromes P_{450}, and flavin containing monooxygenases; (c) phase II, glutathione-*S*-transferases, methyltransferases and sulfotransferases; (d) phase III, ABC transporters that involve the uptake and export, respectively, of xenobiotics (Amador-Noguez et al. 2005; Gems and McElwee 2005; Francis et al. 2003). Using high-density oligonucleotide microarrays, we have identified gene families whose activities involve detoxification reactions, oxidative metabolism and steroid metabolism. We propose that the altered expression of these genes defines a biochemical phenotype that may promote longevity in Snell and Ames dwarf mice.

Materials and methods

RNA isolation: The Pit1$^{dw/dwj}$ mice were maintained as described by Boylston et al. (2004); the Prop1 $^{df/df}$ mice were purchased from the Jackson Laboratory and maintained as a colony at the University of Texas Medical Branch, Galveston, Tex., USA. The animals were housed as described by Amador-Noguez et al. (2005). Total RNA was isolated from frozen livers of *Pit1*$^{dw/dw}$ and *Prop1*$^{df/df}$ mice and age-matched (litter mate) control animals using the RNAqueous-4PCR column-purification method according to the manufacturer's protocol (Ambion, Austin, Tex., USA). DNase-treated RNA samples (Turbo DNase, Ambion) were quantified by UV-absorbance spectrophotometry, and RNA quality assessed by capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, Calif., USA). Samples used for array hybridizations had RNA integrity numbers (RIN) greater than 8.7 (scale = 10 maximum) as computed using default assignment parameters of the Expert 2100 Bioanalyzer software package (v2.02).

Microarray analysis: For gene expression profiling, total hepatic RNA from male *Pit1*$^{dw/dwJ}$ dwarf and age-matched control mice was analyzed by hybridization to individual Affymetrix MG U74Av2 oligonucleotide arrays (>11,000 murine genes) using the following four experimental groups based on genotype and age: young *Pit1*$^{dw/dw}$ dwarf mice (4–6 months, *n*=4); young

Pit1$^{+/?}$ control mice (4–6 months, *n*=4); aged *Pit1*$^{dw/dw}$ dwarf mice (24–26 months, *n*=3); and aged *Pit1*$^{+/?}$ control mice (24–26 months, *n*=3). For *Prop1*$^{df/df}$ mice, genome-scale MG 430 2.0 oligonucleotide arrays (Affymetrix, Santa Clara, Calif., USA) representing >34,000 murine genes were used to survey hepatic transcripts; additionally, the experimental groups were enlarged, and a middle-aged group was also included. Total RNA isolated from 31 male *Prop1*$^{df/df}$ and age-matched control livers was analyzed by hybridization to separate MG 430 2.0 arrays and divided into the following six experimental groups: young *Prop1*$^{df/df}$ dwarf mice (4–6 months, *n*=5); young *Prop1*$^{+/+}$ control mice (4–6 months, *n*=5); middle-aged *Prop1*$^{df/df}$ dwarf mice (12–14 months, *n*=5); middle-aged *Prop1*$^{+/+}$ control mice (12–14 months, *n*=5); aged *Prop1*$^{df/df}$ dwarf mice (24–27 months, *n*=6); and aged *Prop1*$^{+/+}$ control mice (24–27 months, *n*=5). Preparation of target RNA, array hybridization, washing and scanning was performed by standard Affymetrix protocols at the UTMB Genomics Core Facility using 10 μg input RNA and 20 μg labeled cRNA. Arrays were scanned using an Affymetrix GeneChip Scanner 3000, and the image files were converted to probe-level data using Microarray Suite Expression Analysis (MAS 5.0) software (Affymetrix).

Preprocessing of array data, including quality assessment, background adjustment and normalization, was performed using the open-source statistical computing environment R (v2.1.1) and microarray analysis packages developed by the Bioconductor project (Irizarry et al. 2005; Gentleman and Carey 2005). Using diagnostic plots to assess array quality and visualize artifacts, we examined raw intensity data for spatial (pseudo-images) and distributional homogeneity (inter-quartile range boxplots and MA scatter plots) of probe set populations. Probe level data within each array type, i.e., U47Av2 (N=14) or MG 430 2.0 (N=31), were converted to $\log_2$-transformed expression measures using the robust multi-array average (RMA) algorithm for background correction, quantile normalization and linear model fitting of perfect match (PM) signals (Smyth 2005; Wu and Irizarry 2004). Age- and genotype-matched contrasts between dwarf and control gene expression were performed using the affylmGUI and limma (v1.8.14) statistical packages for R from Bioconductor with fitting to a general linear model and a false discovery rate (FDR) adjustment of 5% (α=0.05) (Smyth 2004). A significance threshold of FDR-adjusted $P<0.05$ was used to define differential gene expression between *Pit1*$^{dw/dw}$ and age-matched control, and between *Prop1*$^{df/df}$ and age-matched control mice. Annotation of differentially expressed probe sets was provided by Bioconductor download packages and the NetAffx bioinformatics center (Liu et al. 2003). Functional classification of genes was performed using the gene ontogeny (GO) consortium categories of biological process, cellular component, and biochemical function. To visualize distinct patterns of gene expression, the normalized data sets were subjected to hierarchical and *k*-means cluster analysis and arranged by self-organizing maps (SOMs) using Cluster and TreeView free-access software (http://rana.lbl.gov/EisenSoftware.htm). Over-represented functional classes among the differentially expressed genes were identified and displayed using the EASE statistical software package (david.niaid.nih.gov/david/ease.htm) and the GenMapp and MAPPFinder (www.genMAPP.org) open-access bioinformatics tools (Doniger et al. 2003).

Results and discussion

Genetic mutation of either *Pit1* or *Prop1* impairs pituitary development and results in similar hypopituitary phenotypes characterized by severe deficiencies in the somatotrophic, lactotrophic and thyrotrophic axes, but it also provides a significant increase in longevity. Characterization of particular transcriptional differences shared between *Pit1* and *Prop1* dwarf mice is essential for the identification of candidate genes and biochemical pathways which may be involved in the determination of mammalian lifespan. Given the potent effects of GH on this target tissue, one would expect to find similar patterns in hepatic gene expression in both dwarf mutants resulting from the combined GH- and TSH- and PRL-deficiencies of these mice. To profile transcriptional differences, two separate microarray

experiments were performed, which compared either $Pit1^{dw/dw}$ or $Prop1^{df/df}$ dwarf mice with age-matched control mice, using different Affymetrix gene chips: $Pit1^{dw}$ (Snell) by U47Av2 arrays (n=14), and $Prop1^{df}$ (Ames) by MG 430 2.0 arrays (n=31). Following background correction and normalization, separate age-matched contrasts between dwarf and control expression were performed using an empirical, Bayes-moderated, two-sample t-test assuming unequal variance and adjusted using a FDR of 5% to control for the type I error rate. A significance filter of P<0.05 was used to define differential expression, and separate gene lists were generated for each age-matched comparison of dwarf and control mice. Life-long changes in hepatic gene expression were considered those which met statistical significance for *all* age-group comparisons. Depicted in Figure 1, of greater than 45,000 probe sets on the MG 430 2.0 gene chips, 785 met statistical significance for all three age-groups of $Prop1^{df/df}$ and control mice examined, and 205 were found to be altered significantly in both young and aged $Pit1^{dw/dw}$ dwarf mice. A total of 49 unique genes was found to be differentially expressed in both *Pit1* and *Prop1* livers at all ages, relative to age-matched control animals (Table 1). These expression studies of long-lived Snell and Ames dwarf mouse mutants identify genes involved in detoxification, oxidative metabolism and steroid metabolism whose differential expression is not only characteristic of these dwarf mice but may also play an important role in the determination of mammalian longevity.

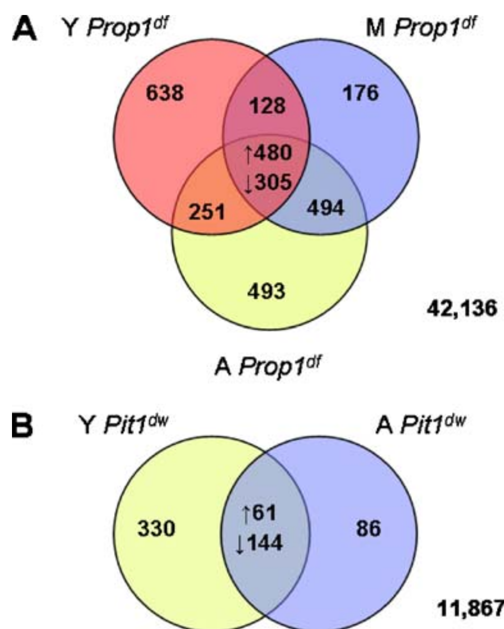

Figure 1 Venn diagrams of significant differences in gene expression in $Pit1^{dw}$ and $Prop^{df}$ microarray studies. Separate age-matched contrasts between dwarf and control expression measures were performed. Using a significance filter of FDR-adjusted P<0.05 to define differential expression, we generated gene lists for each age-matched comparison. Represented by the intersections, 785 met statistical significance for all three age groups of $Prop1^{df/df}$ and control mice, and 205 were found to be altered significantly in both young and aged $Pit1^{dw/dw}$ dwarf mice

Detoxification, oxidative metabolism and steroid metabolism: the cytochrome P_{450} signature

Our working hypothesis is based on the concept that the physiological processes of metabolic detoxification, oxidative metabolism and mitochondrial dysfunction play a major role in mammalian aging and longevity determination (Gems and McElwee 2005; Amador-Noguez et al. 2005; Boylston et al. 2004). The cytochrome P_{450} enzymes that fall into phase I of xenobiotic metabolism (detoxification) are composed of a super-family of proteins and are classified into different families in accordance with the degree of amino acid sequence similarity in their protein structures (Rendic 2002). These enzymes are responsible for metabolic detoxification reactions involving oxidative, peroxidative and reductive metabolic transformation of drugs, environmental chemicals and natural products.

The data in Table 1 show that there are six cytochrome P_{450} xenobiotic metabolizing genes that show significant changes in mRNA levels in both Snell and Ames dwarf mouse livers. Five of these genes were upregulated in young mice of both Snell and Ames dwarf mutants, and were maintained at this elevated level of expression in aged mice. Those genes upregulated in Snell and Ames dwarf mutants are *Cyp2b10*, *Cyp2b9*, *Cyp2b13*, *Cyb4a10* and *Cyp4a14*. One of the six genes, *Cyp7b1*, was strongly downregulated in both mutants. Interestingly, these are genes that

Table 1 Differentially expressed genes in homozygous *Pit1*^{dw} and *Prop1*^{df} livers.

Gene title	M (log_2 difference)					Adjusted *P*				
	Pit1^{dw}		*Prop1*^{dw}			*Pit1*^{dw}		*Prop1*^{dw}		
	6 months	24 months	6 months	12 months	24 months	6 months	24 months	6 months	12 months	24 months
Flavin containing monooxygenase 3	3.55	3.67	3.67	4.57	4.77	*7.8E-11*	*1.1E-10*	*1.6E-11*	*9.6E-14*	*1.2E-14*
Cytochrome P450, family 2, subfamily b, polypeptide 13	2.56	5.00	5.00	4.96	4.99	*1.9E-08*	*2.7E-08*	*3.5E-17*	*4.3E-17*	*1.0E-17*
Hydroxysteroid dehydrogenase-5, delta<5>-3-beta	−5.33	−5.37	−5.37	−5.24	−5.52	*1.2E-08*	*8.8E-08*	*1.8E-08*	*3.7E-08*	*7.4E-09*
Major urinary protein 3	−2.96	−3.23	−3.23	−3.60	−2.45	*2.0E-09*	*5.1E-08*	*7.7E-09*	*1.1E-09*	*9.0E-07*
Cytochrome P450, family 4, subfamily a, polypeptide 14	2.45	5.76	5.76	6.14	4.84	*3.5E-06*	*1.7E-07*	*3.7E-12*	*7.4E-13*	*9.4E-11*
Hydroxyacid oxidase (glycolate oxidase) 3	1.84	4.61	4.61	4.84	4.61	*8.2E-06*	*4.9E-07*	*3.7E-12*	*9.7E-13*	*1.4E-12*
Epidermal growth factor receptor	−1.63	−1.37	−1.37	−1.10	−1.63	*2.3E-06*	*4.3E-06*	*1.2E-07*	*9.3E-06*	*3.4E-09*
Kidney expressed gene 1	−2.80	−3.78	−3.78	−4.03	−2.38	*1.9E-08*	*3.9E-06*	*1.4E-08*	*4.7E-09*	*2.9E-05*
Isocitrate dehydrogenase 2 (NADP+), mitochondrial	1.20	1.59	1.59	1.12	1.10	*1.6E-04*	*5.4E-04*	*1.6E-11*	*2.5E-08*	*2.3E-08*
Sulfotransferase family 2A, dehydroepiandrosterone (DHEA)-preferring, member 2	1.57	7.29	7.29	7.49	6.81	*7.9E-04*	*4.3E-06*	*2.1E-20*	*9.2E-21*	*4.1E-20*
Solute carrier family 16 (monocarboxylic acid transporters), member 7	1.18	1.50	1.50	1.56	1.51	*1.6E-04*	*8.5E-04*	*2.0E-07*	*1.4E-07*	*1.3E-07*
Complement component 9	−2.60	−1.90	−1.90	−1.49	−1.17	*1.3E-07*	*6.7E-04*	*5.6E-07*	*4.5E-05*	*4.9E-04*
Elastase 1, pancreatic	−1.28	−1.46	−1.46	−1.54	−1.88	*4.0E-05*	*8.0E-04*	*2.0E-04*	*1.7E-04*	*5.1E-06*
Major urinary protein 5	−2.74	−3.13	−3.13	−2.85	−1.47	*1.5E-08*	*4.9E-07*	*4.9E-08*	*4.3E-07*	*1.8E-03*
ERO1-like beta (S. cerevisiae)	−1.29	−0.90	−0.90	−0.80	−0.57	*5.9E-07*	*4.3E-05*	*9.4E-06*	*9.3E-05*	*2.1E-03*
Serine (or cysteine) proteinase inhibitor, clade A (anti-trypsin), member 12	−2.32	−1.47	−1.47	−1.76	−2.74	*2.2E-05*	*1.3E-05*	*2.3E-03*	*5.4E-04*	*3.6E-07*
Serine (or cysteine) proteinase inhibitor, clade A, member 3K	−1.54	−4.90	−4.90	−4.29	−3.17	*1.6E-03*	*4.5E-03*	*9.6E-10*	*1.7E-08*	*2.4E-06*
Cytochrome P450, family 7, subfamily b, polypeptide 1	−1.81	−4.40	−4.40	−4.62	−4.49	*5.7E-04*	*6.4E-03*	*1.3E-09*	*8.1E-10*	*5.0E-10*
Purkinje cell protein 4-like 1	1.39	1.79	1.79	1.76	1.44	*9.9E-04*	*4.7E-03*	*1.4E-04*	*2.9E-04*	*1.4E-03*
Cytochrome P450, family 2, subfamily b, polypeptide 9	2.15	4.59	4.59	3.88	3.53	*3.0E-03*	*6.0E-03*	*4.5E-06*	*9.9E-05*	*1.7E-04*

Cytochrome P450, family 4, subfamily a, polypeptide 10	1.78	3.85	3.85	4.97	5.25	*9.7E-03*	*1.4E-04*	*2.1E-06*	*3.3E-08*	*7.1E-09*
Esterase 31	−3.82	−3.69	−3.69	−3.94	−2.26	*2.7E-07*	*9.9E-03*	*1.4E-07*	*6.1E-08*	*2.2E-04*
Hydroxysteroid (17-beta) dehydrogenase 2	−1.19	−1.25	−1.25	−1.25	−0.61	*1.3E-05*	*4.8E-04*	*2.9E-06*	*6.1E-06*	*1.1E-02*
Insulin-like growth factor binding protein, acid labile subunit	−2.04	−2.62	−2.62	−2.41	−0.96	*1.5E-08*	*4.9E-07*	*3.4E-08*	*2.5E-07*	*1.2E-02*
Major urinary protein 1/2	−5.40	−2.99	−2.99	−3.11	−1.28	*7.8E-07*	*6.5E-05*	*7.4E-07*	*6.4E-07*	*1.3E-02*
Phosphatidic acid phosphatase 2a	0.47	0.59	0.59	0.68	0.51	*5.1E-04*	*4.4E-05*	*3.9E-03*	*1.6E-03*	*9.4E-03*
NAD(P)H dehydrogenase, quinone 1	0.45	0.62	0.62	0.60	0.79	*5.0E-03*	*8.5E-03*	*5.7E-04*	*1.4E-03*	*2.2E-05*
Dopachrome tautomerase	−0.89	−2.87	−2.87	−2.39	−2.03	*4.3E-03*	*1.0E-02*	*8.5E-06*	*2.1E-04*	*7.2E-04*
Insulin-like growth factor 1	−2.77	−3.16	−3.16	−3.11	−1.12	*5.0E-08*	*2.1E-07*	*5.3E-08*	*1.0E-07*	*1.7E-02*
Guanine nucleotide binding protein, alpha 14	−0.68	−1.34	−1.34	−1.30	−1.01	*3.5E-03*	*1.4E-02*	*4.0E-06*	*1.1E-05*	*2.0E-04*
Major urinary protein 4	−3.05	−0.62	−0.62	−0.50	−0.37	*2.1E-09*	*8.1E-08*	*1.5E-04*	*2.8E-03*	*1.5E-02*
Complement component factor h	−0.74	−0.95	−0.95	−0.98	−0.71	*1.5E-03*	*1.3E-03*	*2.2E-03*	*2.7E-03*	*1.7E-02*
Cytochrome P450, family 2, subfamily c, polypeptide 38	0.94	0.99	0.99	1.51	1.22	*3.6E-04*	*6.2E-03*	*2.1E-02*	*7.6E-04*	*3.0E-03*
Leukemia inhibitory factor receptor	−1.41	−1.88	−1.88	−1.65	−1.22	*1.6E-03*	*8.5E-03*	*5.7E-04*	*3.9E-03*	*1.8E-02*
UDP glucuronosyltransferase 2 family, polypeptide B5	−1.04	−0.89	−0.89	−0.92	−0.48	*7.8E-05*	*3.3E-02*	*4.0E-07*	*3.6E-07*	*1.6E-03*
Serine (or cysteine) proteinase inhibitor, clade E, member 2	−0.33	−2.00	−2.00	−1.65	−1.53	*1.6E-02*	*2.0E-02*	*4.5E-06*	*1.3E-04*	*1.8E-04*
Complement component 4 (within H-2S) /// sex-limited protein	−1.58	−1.20	−1.20	−1.54	−1.33	*2.8E-04*	*4.6E-02*	*8.6E-05*	*4.7E-06*	*2.0E-05*
Cysteine sulfinic acid decarboxylase	−0.54	−3.76	−3.76	−3.68	−1.73	*2.6E-02*	*2.4E-02*	*6.8E-10*	*1.1E-09*	*2.1E-04*
Angiotensinogen	1.04	0.93	0.93	0.91	0.65	*1.7E-04*	*4.9E-02*	*1.5E-05*	*4.3E-05*	*1.2E-03*
Dipeptidylpeptidase 7	−0.33	−0.66	−0.66	−0.88	−0.44	*2.0E-02*	*8.9E-03*	*9.4E-04*	*4.9E-05*	*2.2E-02*
Very low density lipoprotein receptor	0.63	0.69	0.69	0.62	0.25	*1.6E-04*	*7.1E-03*	*1.8E-06*	*1.8E-05*	*4.5E-02*
Tumor protein p53 inducible nuclear protein 2	−1.63	−1.87	−1.87	−1.17	−0.69	*4.5E-05*	*2.9E-02*	*3.7E-07*	*6.5E-04*	*2.6E-02*
UDP-glucose pyrophosphorylase 2	1.27	1.07	1.07	0.75	0.81	*3.8E-03*	*4.9E-02*	*5.5E-05*	*5.8E-03*	*1.3E-03*
Cyclin-dependent kinase inhibitor 1C (P57)	0.84	0.59	0.59	0.36	0.37	*1.4E-03*	*2.5E-02*	*1.2E-04*	*2.8E-02*	*8.9E-03*
Mannose binding lectin (A)	−1.09	−0.74	−0.74	−0.58	−0.35	*3.3E-04*	*1.8E-02*	*7.9E-05*	*2.4E-03*	*4.3E-02*
Glutathione *S*-transferase, mu 3	0.64	1.82	1.82	1.02	0.96	*3.4E-03*	*4.3E-06*	*7.7E-05*	*3.9E-02*	*2.6E-02*
B-cell translocation gene 1, anti-proliferative	0.68	0.83	0.83	0.83	0.79	*2.3E-02*	*4.3E-02*	*3.9E-03*	*5.9E-03*	*3.9E-03*
Transcobalamin 2	0.43	0.40	0.40	0.35	0.53	*1.9E-02*	*4.5E-02*	*3.6E-03*	*2.0E-02*	*1.4E-04*
Acetyl-coenzyme A dehydrogenase, medium chain	0.36	0.32	0.32	0.37	0.53	*3.3E-02*	*9.2E-04*	*3.4E-02*	*2.0E-02*	*3.3E-04*

A significance threshold of *P* (FDR-adjusted) <0.05 was used to define differential gene expression between $Pit1^{dw/dw}$ and age-matched control, or $Prop1^{df/df}$ and age-matched control mice. A total of 49 unique genes was found to be differentially expressed at all ages in comparisons of both *Pit1* and *Prop1* dwarf mice with age-matched control animals. *M* is the difference in $\log_2$-transformed expression measures between dwarf and control, i.e., dwarf minus control, and the *P* values shown were adjusted using a FDR of 5%.

encode proteins involved in detoxification (*Cyp2b*) and fatty acid metabolism (*Cyp4a* family). In all cases the levels of expression of these genes were established in the livers of young mutants and maintained in the middle aged (Ames) and aged mutants. These data suggest that the metabolic patterns of certain detoxification processes, and fatty acid and steroid metabolism in the long-lived mutants are established by young adulthood and are stabilized throughout their life cycle.

The phenobarbital-inducible P_{450}s-*Cyp 2b9*, *Cyp 2b10* and *Cyp 2b13* genes are upregulated in both Snell and Ames dwarf mutants

Phenobarbital (PB) has long been known to induce drug-metabolizing enzymes in the liver (Damon et al. 1996). The microarray analyses indicate that *Cyp2b9*, *Cyp2b10* and *Cy2b13*, three of four known phenobarbital-inducible P_{450} genes, are upregulated specifically in young and aged dwarfs. Others have shown that *Cyp2b13* is also upregulated by PB, although this gene is not upregulated in the dwarfs (Nemoto and Sakurai 1995; Stupans et al. 1984). Thus, the increased, stabilized level of expression of P_{450} detoxification processes provide increased protective mechanisms against xenobiotic toxins. These phase I xenobiotic detoxifiers are excellent candidates for longevity assurance genes.

The life-long increase in hepatic expression of phase I xenobiotic detoxifiers in these mutants indicates that these protective metabolic processes are established and stabilized in the young dwarfs and further supports the hypothesis that enhanced capacity for detoxification may serve as a contributing factor in the longevity of the dwarf mutants. We propose that the early and sustained metabolic detoxification processes of phase I genes play an important and basic role in longevity determination in both Snell and Ames dwarf mice.

Mechanisms of regulation of P_{450}*Cyp2b* Phase I detoxification genes

The *Cyp2b-9* and *-10* responses to PB are regulated by multiple factors. Ca^{2+} via the Ca^{2+}-calmodulin-dependent kinase and PKC are positive regulators, while the cAMP/PKA pathway, cytokines (IL-1β) and GH are negative regulators (Marc et al. 2000; Galisteo et al. 2000; Shapiro et al. 1994; Abdel-Razzak et al. 1995). Although little is known about the activity of these factors in the dwarfs, the GH deficiency may play a role in the upregulation of these genes. In fact, both male and female sex hormones and glucocorticoid activate *Cyp2b-9* and *-10* in C57BL/6 mouse livers, suggesting that endogenous hormone levels regulate the constitutive level of expression of these genes (Nemoto and Sakurai 1995). Thus, since both *Cyp2b-9* and *-10* are isozymes of testosterone 16α-hydroxylase, enzymes that play a key role in testosterone biosythesis, their increased activity may delay the age-associated decrease in testosterone levels. Furthermore, the absence of negative regulation by GH deficiency in the dwarfs may be responsible for the upregulation of the basal level of expression of *Cyp2b9* and *Cyp2b10*. Changes in 3β-hydroxysteroid metabolism have been shown to occur in the dwarfs that support a role of altered testosterone metabolism as a potential factor in longevity determination in the dwarf (see below).

The CYP 4a Family

P_{450}CYP4a10 and Cyp4a14—fatty acid metabolism The Cyp4a enzymes are fatty acid hydroxylases that play an important role in the metabolism of various endogenous lipid substrates, such as fatty acids and arachidonic acid (Leclerq et al. 2000). The genes of this family are inducible by peroxisome proliferators, suggesting that the enzymes could catalyze the production of reactive oxygen and lipid peroxides during peroxisome proliferation. Moreover, *Cyp4a* genes are co-regulated with other genes that encode proteins involved in β- and ω-oxidation of fatty acids (e.g., acetyl CoA-oxidase and ketothiolase), transport (liver fatty acid-binding proteins and acetyl-CoA-binding protein) and stress response (chaperonin, T-complex proteins 1a). The functions(s) of these genes, therefore, may include activities associated with longevity determination.

This suggests that CYP4A proteins are key intermediaries in an adaptive response to perturbation of hepatic lipid metabolism (Kroetz et al. 1998; Leone et al. 1999) and that the upregulation of CYP4A enzymes may be a physiological response that prevents aberrant lipid accumulation. With respect to their stress response activities, oxidative stress and lipid peroxidation levels in liver result in lipid peroxidation end-products that are strong chemoattractants for inflammatory cells. The data in Table 1 show significant and specific increases in the levels of *Cyp4a10* and *Cyp4a14* in both mutants. Notably, the physiological functions of these enzymes fall into the category of oxidative metabolism that prevents aberrant lipid accumulation and production of inflammatory cell chemoattractants. The increased activity of these genes in the Snell and Ames mutants establishes a physiological status that favors longevity.

The global regulatory activity of PGC-1α on PPAR targets occurs in response to food deprivation. This suggests that PGC-1α and its downstream targets may play a key role in establishing longevity characteristics. In addition to P_{450}s, PGC-1α regulates both ligand-dependent and -independent activation of many nuclear receptors that play a role in energy and drug metabolism. PPARα, a target of PGC-1α, regulates responses to fasting, including fatty acid β- and ω-oxidation, gluconeogenesis and ketogenesis. Thus, the targeted regulation by PGC-1α establishes some of the physiological responses to CR and suggests that both PGC-1α and *PPARα* genes may be longevity assurance gene candidates.

The possible significance of these genes in longevity determination is indicated by the fact that the *Cyp4a10* and *Cyp4a14* genes are also targeted in the livers of CR mice and have been shown to serve as beneficial factors in CR (in mouse liver) (Corton et al. 2004), because of their physiological regulation of lipid metabolism, inflammation and cell growth. The activities of both *Cyp4a10* and *Cyp4a14* genes are dependent on PPARα. Their beneficial functions involve the activation of fatty acid ω-hydroxylases by xenobiotics, fasting and diabetes in wild-type mice (Simpson 1997). CR increases the expression of *Cyp4a10* and *Cyp4a14*, which are ω-oxidation genes. These data suggest an increased dependence on fatty acids as an energy source and are consistent with metabolic effects of fasting on fatty acid metabolism regulated by PGC-1α (Puigserver and Spiegleman 2003). The data in Table 1 show that both *Cyp4a10* and *Cyp4a14* mRNA levels are increased in the Ames and Snell dwarf mutants and that the increase is established in the young long-lived mutants.

The direct relationship of PPARα regulatory functions to longevity determination is exemplified by its role in prevention of liver damage by the hepatotoxicant, thioacetamide. The mechanism of CR protection is believed to be due to enhanced liver repair due to increased cell proliferation. In general PPARα is required for energy metabolism production that is needed for tissue repair (Anderson et al. 2002; Shankar et al. 2003). Thus, it is proposed that the increased expression of PGC-1α in CR-induced hepatoprotection contributes to longevity via its activation of PPARα (Corton et al. 2000). We also propose that an important metabolic pathway characteristic of longevity may involve global PGC-1α regulatory functions that mediate coordinated responses by the targeted protective genes such as the P_{450}s and other genes that mediate protective processes.

The upregulation of these phase 1 Cyp2 and Cyp4 family genes suggests that the mechanisms that regulate the transcription levels may be altered in the dwarf mutants. Studies on the transcription factors that regulate the induction of *Cyp4a* genes have shown that the nuclear orphan constitutive active receptor (CAR) serves as a transcription blocker that prevents *Cyp4a10* and *Cyp4a14* induction by PB (Ueda et al. 2002). These studies showed that *Cyp4a10* and *Cyp4a14* are induced only in CAR-null mice, indicating that CAR is a negative regulator of these genes. Thus, the constitutive increase of these genes in the long-lived dwarf livers suggests that the blocking ability of CAR may be attenuated in these mutants. The inducibility of these genes by PB, and the fate of CAR in these mutants have not been studied. Analysis of the status of CAR activity and PB inducibility should provide basic information on understanding the role and mechanism of *Cyp4* genes in the longevity determination in these mice.

Although studies indicate that PPARα regulates *Cyp4a10* and *Cyp4a14*, PPARγ also appears to be an important regulator of P_{450} Cyps. The *Cyp4a14* gene is highly inducible after treatment with peroxisome proliferator MCP while the *Cyp4a12* gene, whose activity is not altered in the dwarfs, is strongly repressed by MCP (Heng et al. 1997). In fact, the upregulation of *Cyp4a14* and downregulation of *Cyp4a12* is typical of the wild-type response to MCP. The peroxisome-proliferation type characteristics exhibited by the unstimulated dwarf are consistent with our observation that PPARγ is upregulated and that a major characteristic of the dwarf involves regulation of fatty acid and steroid synthesis (Boylston et al. 2004).

The P_{450}Cyp7b1 enzyme The data in Table 1 show that the mRNA level of *Cyp7b1* is downregulated in the dwarfs. CYP7B is solely responsible for the conversion of DHEA to its metabolite 7α-OH-DHEA (Dulos et al. 2005a,b), both in vivo and in vitro. In vitro studies suggest that 7α-OH-DHEA has immunostimulatory activity (Morfin 2002), e.g., it prevents the immunosuppressive effects of glucocorticoids. This suggests that an imbalance between immunostimulating activity of 7α-OH-DHEA and immunosuppressive endogenous glucocorticoids may contribute to a sustained inflammation. On the other hand, strong downregulation of *Cyp7b1* in the dwarfs suggests a decrease in immunostimulatory activity and may play a role in the delay of the aging of the immune response processes (Flurkey et al. 2001, 2002).

Proinflammatory cytokines TNFα, IL-1α, IL-1β and IL-17 increase CYP7B activity. Since the enhanced CYP7B activity and formation of 7α-OH-DHEA contribute to a chronic inflammatory response, the decreased mRNA levels of *Cyp7b1* in the dwarfs may result in a lower level of chronic inflammation and reduced state of chronic stress. These are characteristics that correlate well with our molecular–metabolic model of longevity.

The DHEA-sulfotransferase 2A enzyme The data in Figure 2 and Table 1 show that sulfotransferase 2A, a DHEA-preferring enzyme, exhibits the highest level of upregulation, i.e., ~7-fold in both Snell and Ames dwarfs. DHEA-sulfotransferase 2A1 is a phase II metabolizing and detoxifying enzyme, with substrate preference for physiological hydroxysteroids, diverse drugs and other xenobiotics. Recent studies have shown that transcription of DHEA-sulfotransferase 2A1 is markedly enhanced in senescent male rat liver, and that caloric restriction retards this increase (Echchgadda et al. 2004). This upregulation has been attributed to the age-associated loss of expression of the liver androgen receptor, a negative regulator of this gene. Interestingly, the Sult2A1 gene is induced by the pregnane X receptor (PXR), which is a xeno-sensing nuclear receptor that is activated by endobiotic and xenobiotic chemicals. Thus, it is argued that repression of androgen receptor and induction of PXR act in coordination to mediate the senescence-associated and xenobiotic-mediated stimulation of Sult2A1. Increased expression of this gene is both age and longevity associated and may be a factor in an adaptive response that ensures optimal metabolism of xenobiotic substrates in aged tissue. A major difference, however, between the normal aging process and dwarf longevity is the fact that sulfotransferase activity is strongly upregulated in the young Ames dwarf and remains at its elevated level throughout the life cycle. This suggests that the protective function of this phase II detoxifying enzyme(s) occurs in the young dwarfs and is consistent with the role of phase II proteins in establishing the physiological factors that favor longevity.

The 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase gene family

The 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD) isoenzymes are responsible for the oxidation and isomerization of Δ^5-3β-hydroxysteroid dehydrogenase precursors into Δ^4-ketosteroids, thus catalyzing an essential step for the formation of all classes of active steroid hormones: the adrenal steroid hormones, cortisol, corticosterone and aldosterone; and the gonadal

steroid hormones, progesterone, testosterone and estradiol (Couet et al. 1992). The 3β-HSD enzymes exist in multiple isoforms in rodents and humans, each a product of a distinct gene. The 3β-HSD gene family exhibits differential patterns of tissue- and cell-specific expression and regulation involving multiple signal transduction pathways that are activated by several growth factors, steroids and cytokines (Herrmann et al. 2002). There are six isoforms in this family that fall into two functionally distinct groups. Group 1 is comprised of 3β-HSD I, III and VI (and most likely II), which function as NAD^+-dependent dehydrogenase/isomerases and are therefore essential for the biosynthesis of active steroid hormones. Group II, composed of 3β-HSD IV and V function as NADPH-dependent 3-ketosteroid reductases and are involved in the inactivation of steroid hormones such as dihydrotestosterone (DHT).

Affymetrix microarray analyses of liver mRNAs from Snell and Ames dwarf mice have clearly shown that the ketosteroid reductase, 3β-HSD-V, is dramatically downregulated in both dwarf mutants at all ages (Table 1 and Figure 2). In fact, both Snell and Ames mice show that 3β-HSD-V is the only member of this family that is dramatically downregulated (Boylston et al. 2004; Amador-Noguez et al. 2005). These data suggest that the inactivation of testosterone, which increases with age in the wild-type mouse livers, may be significantly slowed in both of these long-lived dwarf mice and serves as an example of delayed aging.

It is interesting that 3β-HSD-V is exclusively expressed in the male liver, with expression first detected at 30–40 days post-natally, i.e., during pubertal development (Abbaszade et al. 1995; Payne et al. 1997). The downregulation of this gene in males, to the very low level of expression

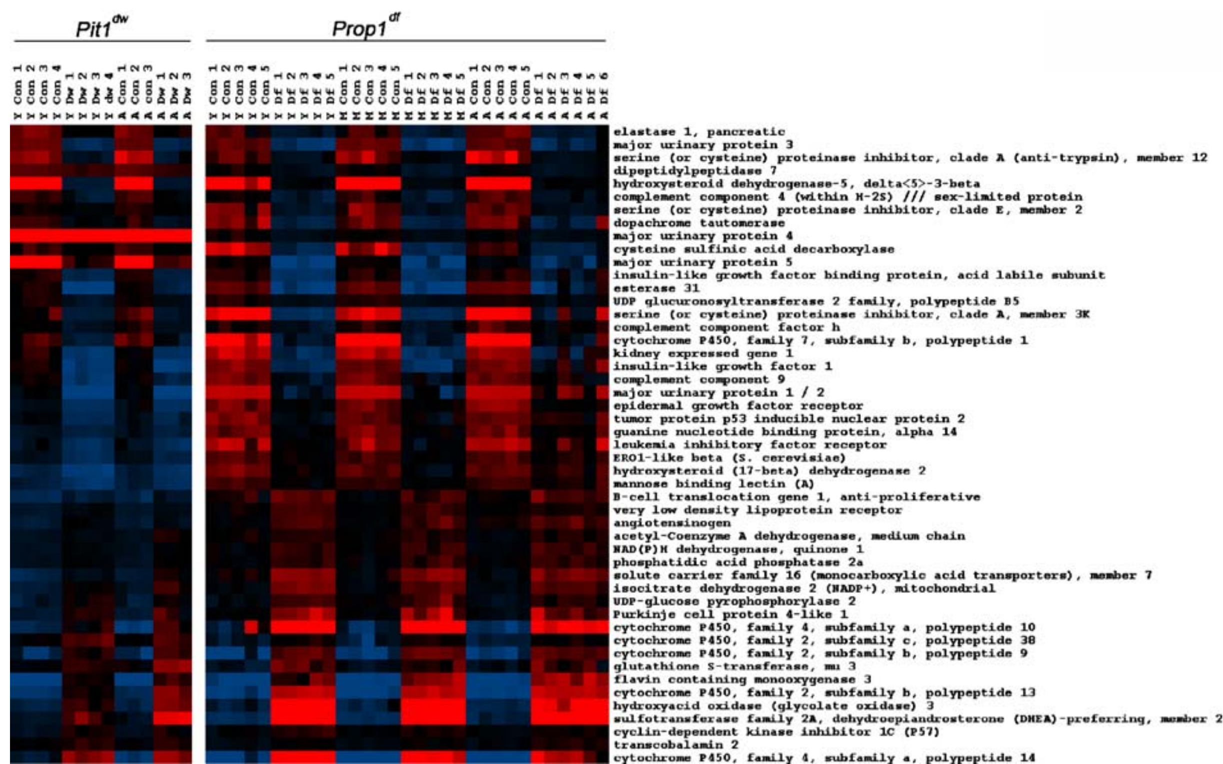

Figure 2 Shared hepatic transcriptional profile for homozygous *Pit1*^{dw} and *Prop1*^{df} dwarf mice. To visually summarize patterns of gene expression across all arrays, we subjected log_2-transformed expression measures for the 49 genes to cluster analysis. Using 100,000 iterations and 50 nodes, we constructed a self-organizing map (SOM) of these differentially expressed genes. Relative level of gene expression: *blue* low, *black* intermediate, *red* high expression level

detected in female livers, is an example of the sexual dimorphism and tendency for the adult male dwarf to exhibit female genetic characteristics (Amador-Noguez et al. 2005).

The recent report of the clustered downregulation of 3β-HSD-II, III and VI suggests that this regulatory event may be mediated by a specific transcription factor(s), shared by all of these genes. Both STAT5 and STAT6 play a regulatory role in activation of 3β-HSD-II, and both IL-4 and IL-13 induce 3β-HSD-I gene expression, through STAT6 activation; 3β-HSD-II gene regulation also involves the interaction with the orphan nuclear receptors steroidogenic factor-1 and DAC-1 (X-chromosome gene). The complexity of these regulatory processes is indicated by the speculation that the mechanisms involving the functional cooperation between STATs and nuclear receptors may involve their potential interaction with other pathways such as GATA proteins (Herrmann et al. 2002).

The 3β-HSD isoenzymes are membrane-bound proteins located in ER and mitochondria, depending on the tissue (Sauer et al. 1994; Simard et al. 2005; Berchtold 1977). The fact that these enzymes are present in the inner mitochondrial membrane (Chapman and Sauer 1979) raises the question of whether their coordinated downregulation is a part of an overall downregulation of mitochondrial activity in the long-lived mutants. Co-precipitation studies have shown that 3β-HSD is in a functional steroidogenic complex with P_{450} side-chain cleavage enzyme (P_{450} ssc) in the inner mitochondrial membrane. This complex provides the enzyme with immediate substrate metabolized from cholesterol transported across the mitochondrial membrane (Cherradi et al. 1995). Our previous studies have indicated that the cholesterol pathway is downregulated in the Snell dwarf (Boylston et al. 2004). Thus, the coordinated downregulation of 3β-HSD and cholesterol biosynthesis strongly suggests a downregulation of some clustered mitochondrial functions involving steroid metabolism. Interestingly, mitochondrial vs ER localization is tissue-specific, e.g., the 3β-HSD is restricted to mitochondria in the testis and, possibly, in the mouse liver mitochondria.

Hepatic 3β-HSD expression is important for the biosynthesis and inactivation of steroids. The adult mouse liver expresses 3β-HSD types II, III and V, with type III predominating. Mouse type I 3β-HSD is the predominant form in fetal liver until post-natal day 1, when type III is induced. On day 40 the male-specific type V is detected. Thus, the mouse liver plays a key role in the fetal and adult development of the isomerase biosynthetic activities (I and II) and ketosteroid activities (V). The fact that 3β-HSD-V is detected on post-natal day 40 and is male-specific suggests that the liver plays a key role in the inactivation of 3β-HSD-V. Furthermore, the delay of testosterone inactivation by 3β-HSD-V in the dwarfs suggests that the maintenance of hormonal level plays a role in longevity.

Studies have shown that both GH and prolactin (Prl) downregulate the 3-ketoreductase activity of 3β-HSD-V in mouse liver (Naville et al. 1991; Keeney et al. 1993). Thus, since Snell and Ames dwarfs have GH and Prl deficiencies, the dramatic downregulation of 3β-HSDs in these mutants does not correlate with the regulatory role of these hormones on the expression of these genes. These data suggest that there are other factors that mediate the downregulation of these genes in the absence of GH and Prl.

The flavoenzymes

The flavin-containing monooxygenases The flavoenzymes are flavin adenine dinucleotide (FAD)-dependent and flavin mononucleotide (FMN)-dependent proteins. These enzymes have the unique ability to catalyze a wide range of biochemical reactions that involve the dehydrogenation of metabolites in one- and two-electron transfers from and to redox centers in the activation for oxidation and hydroxylation reactions (Fraaije and Mattevi 2000). Flavin-containing monooxygenases (FMOs) are a family of NADPH- and FAD-dependent enzymes that catalyze the oxygenation of a wide variety of compounds containing nucleophilic nitrogen, sulfur and phosphorus heteroatoms (Zhang and Cashman 2005; Cashman 1995, 2005; Ziegler 1988; Henerson et al. 2004). The functional diversity of this phase I family is determined by the

expression of five genes, FMO1 to FMO5, and their variants. The data in Figure 2 and Table 1 show that FMO3 is the only member of this family that is strongly upregulated in male Snell and Ames dwarf livers. Furthermore, the increased abundance of FMO3 mRNA is established in the young dwarfs and persists throughout their life cycle. It has been proposed that the upregulation of *Fmo1*, *3* and *5* in the wild-type male mouse liver during puberty, and their downregulation in adulthood, are presumably due to hormonal influences (Janmohamed et al. 2004; Latter et al. 2002). The male dwarfs, however, show that *Fmo3* is strongly upregulated in young adult livers and remains upregulated throughout their life-cycle. Thus, their longevity is associated with the maintenance of *Fmo3* expression and the failure of adult factors or processes to downregulate the gene. This is indicative of the maintenance of pre-adult or puberty level characteristics, thus suggesting that aging characteristics are delayed in the dwarfs. Interestingly, the increase in FMO3 in male dwarf livers is another example of the trend toward female patterns of expression in the dwarfs and an example of sexual dimorphism (Amador-Noguez et al. 2005).

The majority of drug and exogenous chemical metabolism by the FMOs occurs in the liver and kidney. A comparison of FMO1 to FMO5 mRNA levels in humans has shown that FMO3 mRNA is mainly detected in the adult liver in significantly larger amounts than in other tissues such as fetal and adult brain and small intestine (Zhang and Cashman 2005).

Mammalian FMOs are hepatic microsomal enzymes that utilize oxygen and NADPH to oxygenate a wide range of sulfur- and nitrogen-containing xenobiotics, and, in general, any chemical containing a soft nucleophile that gains access to the peroxyflavin intermediate (FADOOH) is a potential substrate (Krueger and Williams 2005; Massey 1994). In the first step of the catalytic cycle, FAD undergoes 2-electron reduction by NADPH. The reduced flavin reacts rapidly with molecular oxygen to form a stable peroxyflavin intermediate that is poised to react with a suitable nucleophile. This nucleophilic attack on FADOOH results in one atom of molecular oxygen being transferred to the substrate and one atom to form water. The rate-limiting steps in the catalytic cycle are thought to be the breakdown of the FADOH pseudo-base or the release of $NADP^+$.

The structural features of the FAD pocket minimizes uncoupling/leakage of ROS from the breakdown of FADOOH. As FMO is present at high concentrations in the hepatic endoplasmic reticulum, a significant production of super-oxide anion radical or hydrogen peroxide from the decomposition of the FADOOH would be detrimental. Thus, it is speculated that the FMO must have evolved a mechanism to protect nucleophilic sites (e.g., methionine, cysteine) from oxidative attack by the peroxyflavin. This decreased tendency to generate ROS by the FMOs supports the hypothesis that the FMO-mediated detoxifications, because of their minimal ROS production, are excellent longevity assurance gene candidates. Furthermore, the increased activity of FMO3 in Snell and Ames dwarfs suggests that the physiological functions of FMO3 are an important factor in longevity—possibly through its ability to minimize uncoupled leakage of ROS from the breakdown of FADOOH.

The developmental regulation of expression of longevity assurance genes may be an important factor in lifespan because of the potential to establish protective characteristics in early life. For example, in wild-type mice, FMO3 expression is switched on in the liver after birth (Cherrington et al. 1998). This important developmental process has the potential to protect the liver from environmental hazards during early, crucial, post-natal development. This concept is consistent with the hypothesis that activation of detoxification processes during early post-natal development plays a major role in longevity development because of their protective functions.

With respect to its potential protective properties, the hepatic distribution of mRNAs encoding FMOs 1–5 is similar to that of other phase I enzymes such as the cytochrome P450 (CYPs). Most of these enzymes are more highly expressed in the perivenous region of the liver (Lindros 1997). This location of FMO3 may serve to protect the liver acinus from xenotoxic damage (Janmohamed et al. 2004).

Table 2 Functional classes of genes comprising the shared hepatic expression profile between homozygous Pit1^{dw} and Prop1^{df} dwarf mice.

Gene ontology category	Gene classification	List hits	Array hits	EASE score
Biological process	Steroid metabolism	8	104	1.8E-06
	Lipid metabolism	12	375	5.9E-06
	Electron transport	10	240	6.9E-06
	Carboxylic acid metabolism	11	331	1.3E-05
	Organic acid metabolism	11	332	1.4E-05
	Complement activation	5	35	4.5E-05
	Steroid biosynthesis	6	75	6.5E-05
	Lipid biosynthesis	7	178	5.0E-04
	Humoral defense mechanism	5	65	5.1E-04
	Fatty acid metabolism	6	138	1.1E-03
	Humoral immune response	5	94	2.0E-03
	Xenobiotic metabolism	4	65	6.2E-03
	Response to xenobiotic stimulus	4	66	6.4E-03
	Complement activation, classical pathway	3	23	7.7E-03
	Physiological process	39	5,720	2.4E-02
	Hormone metabolism	3	42	2.4E-02
	Response to external stimulus	11	898	2.9E-02
	Response to pest/pathogen/parasite	6	302	2.9E-02
	Cholesterol metabolism	3	47	3.0E-02
	Sterol metabolism	3	51	3.5E-02
	Coenzyme and prosthetic group metabolism	4	126	3.6E-02
	Embryonic morphogenesis	3	58	4.4E-02
	Steroid catabolism	2	8	4.6E-02
	Estrogen metabolism	2	8	4.6E-02
	Olfaction	3	60	4.7E-02
	Complement activation , alternative pathway	2	9	5.1E-02
	Chemosensory perception	3	68	5.9E-02
	Perception of chemical substance	3	68	5.9E-02
Molecular function	Monooxygenase activity	7	66	2.5E-06
	Oxidoreductase activity	14	471	2.6E-06
	Oxidoreductase activity, acting on paired donors , with incorporation or reduction of molecular oxygen	6	67	4.9E-05
	Pheromone binding	3	7	7.5E-04
	Odorant binding	3	11	1.9E-03
	Protease inhibitor activity	5	92	2.3E-03
	Endopeptidase inhibitor activity	5	92	2.3E-03
	Enzyme inhibitor activity	6	156	2.4E-03
	Catalytic activity	27	2,718	2.9E-03
	Serine-type endopeptidase inhibitor activity	4	62	6.2E-03
	Complement activity	3	22	7.8E-03
	Oxidoreductase activity, acting on NADH or NADPH , NAD or NADP as acceptor	3	27	1.2E-02
	Alkane 1-monooxygenase activity	2	2	1.2E-02
	Oxidoreductase activity, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen	3	28	1.2E-02
	Oxidoreductase activity, acting on CH-OH group of donors	4	83	1.4E-02
	Oxidoreductase activity , NAD or NADH as one donor, and incorporation of one atom of oxygen	3	32	1.6E-02

Table 2 continued.

Gene ontology category	Gene classification	List hits	Array hits	EASE score
	Oxidoreductase activity , reduced iron-sulfur protein as one donor , and incorporation of one atom of oxygen	2	3	1.8E-02
	Defense/immunity protein activity	4	108	2.8E-02
Cellular component	Extracellular	25	1,888	2.1E-06
	Microsome	8	116	2.2E-06
	Vesicular fraction	8	117	2.3E-06
	Extracellular space	23	1,689	6.3E-06
	Membrane fraction	9	315	2.1E-04
	Cell fraction	10	407	2.3E-04
	Endoplasmic reticulum	8	341	2.0E-03
	Peroxisome	3	66	4.9E-02
	Microbody	3	66	4.9E-02

The 49 genes found to be differentially expressed in both *Pit1* and *Prop1* dwarf livers (5% FDR-adjusted $P<0.05$) were subjected to statistical analysis for over-represented functional classes provided by the GO and KEGG databases using the open-access package EASE (david.niaid.nih.gov/david/ease.htm). *List hits* refers to the number of genes from the list of 49 genes, and *array hits* denotes the total number of genes on the array mapping to that category. The statistical analysis was performed using the genes represented on the MG U74Av2 array.

Expression of *Fmo3* in adult mouse liver is also gender-specific. Its expression increases in wild-type male liver during the development of sexual maturity, while in females its expression is high and is neither induced nor affected by age. For example since *Fmo3* is not inducible in the females, its high level of protective function is constitutive and may be an important factor in longevity determination of wild-type mice as well as in the increased longevity in female dwarf mutants. Interestingly, it is specifically down-regulated in the liver of normal adult male mice only (Cherrington et al. 1998; Janmohamed et al. 2004). The fact that the female liver contains about 80-times as much FMO3 mRNA as male liver may be a characteristic that contributes to the longer lifespan in females.

Since both Snell and Ames dwarf mice have GH, Prl and TSH deficiencies, hormonal regulation may be a key factor in the regulation of FMO3 expression. For example, FMO3 is downregulated in adult male liver by testosterone (Falls et al. 1997). Thus, if the downregulation of 3β-HSD-V does result in slowing of the inactivation of testosterone, the upregulation of *Fmo3* must overcome the negative regulatory effect by testosterone. On the other hand, studies have shown that 17-β-estradiol (Coecke et al. 1998b) and thyroid hormones (Coecke et al. 1998a) down-regulate the expression of FMOs. This negative regulation favors the increase in Fmo3 activity. Because of the potential importance of Fmo3 in longevity determination, the roles of these hormones in the dwarfs must be clarified.

In addition, the generation of hydrogen peroxide by FMO could also play an important physiological role in control of the overall redox state of the cell and in expression of genes controlled by hydrogen peroxide or the cellular redox potential. Importantly, although active toward many of the same substrates, CYP and FMO often produce distinct metabolites. In general FMO oxygenation results in metabolites with decreased pharmacological and toxicological properties. This is a unique characteristic of the FMO3 that strongly supports its potential role in decreased oxidative stress associated with longevity determination, and as a longevity assurance gene candidate.

FMN dependent glyoxylate oxidase The data in Table 1 show that glycolate oxidase 3 is upregulated by ~4.5-fold in the Ames and Snell dwarf livers. Glycolate oxidases, also known as hydroxyl acid oxidases, are tissue-specific peroxisomal FMN-dependent enzymes that oxidize glycolate to glyoxylate (fatty acid α-oxidation) with concomitant production of H_2O_2 (Recalcati et al.

2003). There are three human 2-hydroxy fatty acid oxidases that are involved in the oxidation of 2-hydroxy fatty acids and may also contribute to the general pathway of fatty acid α-oxidation (Jones et al. 2000). It has been reported that oxidative stress, induced by either glutathione depletion or post-ischemic reperfusion in rat liver, causes a decrease in Hao1 rate of transcription and mRNA pool levels. This oxidative stress-linked downregulation suggests a mechanism that may prevent H_2O_2 formation in liver peroxisomes and furthermore represents a physiological mechanism and function of this family that regulates the activity of ROS-producing and/or ROS-targeted proteins. Thus, the regulation of glycolate oxidase activity by ROS may play a key role in the regulation of constitutive levels of ROS production, which suggests an important function involving the physiological role for the balanced activity of ROS signaling. The upregulation of glycolate oxidase in the dwarf mutants may, therefore, be due to the decreased level of oxidative stress in the mutants. This may play a key role in the regulation of balanced signaling activity of ROS-targeted genes and contribute to the regulation of the overall redox state of the cell.

Conclusions

The microarray analyses of various models of aging and longevity have provided compelling evidence that the physiological processes of oxidative metabolism, oxidative and inflammatory stress, and metabolic detoxification play a basic role in aging and longevity determination. A comprehensive listing of the biological processes associated specifically with both Snell and Ames dwarf mice is shown in Table 2. These data strongly indicate the need for understanding the molecular and physiological mechanisms affected by oxidative stress (ROS) that determine the development of such physiological characteristics as resistance to oxidative stress and inflammation, and increased levels of detoxification. It is important to have a comprehensive understanding of the mechanisms that regulate and stabilize the activities of clusters of genes that are implicated by the microarray analyses, as these should provide some understanding of the role of transcription, translation, protein turnover and post-translational modifications that contribute to longevity. In particular, oxidative stress caused by ROS production by organellar dysfunction (mitochondrial/peroxisomal/endoplasmic reticulum) is a powerful factor that affects each of these biochemical processes. The identification of physiological functions (i.e., families of genes whose activities are targeted and regulated by ROS and whose activities affect aging and longevity) also raises the question of the mechanisms of these physiological functions.

Although genetic mutants have served as important models for studies on aging and longevity, the significance of tissue-specific aging in complex organisms and the role of epigenetic mechanisms and micro-environmental changes, especially on stem cell programming, is an important area of future research. The microarray and proteomics high-throughput technologies provide the opportunities to understand the global physiological environment of aging and longevity. They provide the information and resources for future investigations in understanding specific biological processes that define the molecular mechanisms of aging and longevity.

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