

# Activation of genes involved in xenobiotic metabolism is a shared signature of mouse models with extended lifespan

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<sup>1</sup>Cellular and Molecular Biology Graduate Program, University of Michigan, Ann Arbor, Michigan; <sup>2</sup>Department of Pathology, University of Michigan, Ann Arbor, Michigan; <sup>3</sup>University of Michigan Geriatrics Center, Ann Arbor, Michigan; and <sup>4</sup>Department of Internal Medicine, Southern Illinois University, Springfield, Illinois

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**Steinbaugh MJ, Sun LY, Bartke A, Miller RA.** Activation of genes involved in xenobiotic metabolism is a shared signature of mouse models with extended lifespan. *Am J Physiol Endocrinol Metab* 303: E488–E495, 2012. First published June 12, 2012; doi:10.1152/ajpendo.00110.2012.—Xenobiotic metabolism has been proposed to play a role in modulating the rate of aging. Xenobiotic metabolizing enzymes (XME) are expressed at higher levels in calorically restricted mice (CR) and in GH/IGF-I-deficient, long-lived mutant mice. In this study, we show that many phase I XME genes are similarly upregulated in additional long-lived mouse models, including “crowded litter” (CL) mice, whose lifespan has been increased by food restriction limited to the first 3 wk of life, and in mice treated with rapamycin. Induction in the CL mice lasts at least through 22 mo of age, but induction by rapamycin is transient for many of the mRNAs. Cytochrome *P*-450s, flavin monooxygenases, hydroxyacid oxidase, and metallothioneins were found to be significantly elevated in similar proportions in each of the models of delayed aging tested, whether these were based on mutation, diet, drug treatment, or transient early intervention. The same pattern of mRNA elevation could be induced by 2 wk of treatment with *tert*-butylhydroquinone, an oxidative toxin known to activate Nrf2-dependent target genes. These results suggest that elevation of phase I XMEs is a hallmark of long-lived mice and may facilitate screens for agents worth testing in intervention-based lifespan studies.

crowded litter; caloric restriction; rapamycin; Nrf2

LIFESPAN, INCLUDING MAXIMUM LIFESPAN, can be extended in mice by genetic, dietary, and pharmacological interventions. Mutations associated with reduced action of growth hormone (GH) and/or insulin-like growth factor I (IGF-I) can increase mouse lifespan by 40% or more (8). Ames dwarf (*Prop1<sup>df/df</sup>*) (12) and Snell dwarf (*Pit1<sup>dw/dw</sup>*) (17) mice carry spontaneous loss-of-function mutations that result in improper pituitary development and a reduction in somatotrophs. Growth hormone receptor knockout (*Ghr<sup>-/-</sup>* or “GHRKO”) (14) and growth hormone-releasing hormone receptor (*Ghrhr<sup>lit/lit</sup>* or “Little”) mice (17) are also long-lived compared with their respective controls, and additional mutations affecting GH and/or IGF-I action have been shown to be associated with extended longevity (7).

Slow aging and extended lifespan can also be induced by dietary interventions. Long-term caloric restriction (CR) has been shown to extend lifespan in multiple rodent stocks (26) as well as in genetically heterogeneous mice (16). More recent evidence has shown that very short-term, transient nutrition restriction imposed prior to weaning by litter supplementation

(“crowded litter,” or CL model) also increases mean and maximal lifespans in mice (39). In this initial study, litter size was increased by 50% from 8 pups per mother to 12 pups per mother during the first 3 wk of life. Mice in the enlarged litter group were found to have lowered levels of circulating IGF-I at weaning and a small but lifelong reduction in body weight. The mechanism responsible for lifespan extension in these mice is not known but may be due to stable reprogramming of gene expression during early development, potentially including genes involved in protection from ingested and endogenous toxins. Ames mice on CR diets live longer than the same mutants fed ad libitum, suggesting that CR and the Ames mutation may extend lifespan at least partially through different mechanisms (6), although CR diets apparently do not further increase lifespan in GHRKO mice (11). Pharmacological interventions that extend mouse lifespan have only recently been described, with evidence that rapamycin can extend longevity in several stocks (2, 19, 30, 44), presumably through reduced target of rapamycin (TOR) function.

In this study, we sought to identify biochemical pathways that might be regulated in similar ways in mice in which lifespan has been extended by drugs, diets, or mutations. We focused on expression of genes that encode XMEs, based on previous publications that have indicated elevated expression of these genes in slow-aging mice. Meta-analysis of CR and long-lived dwarf mice microarrays revealed that phase I enzymes, particularly cytochrome *P*-450s, flavin monooxygenases, hydroxyacid oxidase, and metallothioneins were found to be elevated in liver tissue (41). These enzymes play a protective role against environmental toxins and interact with phase II conjugation enzymes, enhancing hydrophilicity and excretion rate of compounds, which are then exported into bile by downstream phase III transporters (34). Elevated systemic resistance to oxidative toxins, such as acetaminophen or paraquat, is a hallmark of long-lived dwarf (1, 5, 10) and diet-restricted mice (18) but has not been evaluated in CL or rapamycin-treated mice.

## MATERIALS AND METHODS

**Animals.** The CL, CR, rapamycin-treated, and *tert*-butylhydroquinone (tBHQ)-treated mice were of UM-HET3 stock; they were the offspring of crosses between (BALB/cByJ × C57BL/6J)F1 females and (C3H/HeJ × DBA/2J)F1 males (29).

Snell dwarf (*dw/dw*) and heterozygote (*dw/+*) control mice were bred as the progeny of (DW/J × C3H/HeJ)F1 *dw/+* females and (DW/J × C3H/HeJ)F1 *dw/dw* males; the sires had been treated with growth hormone and thyroxine to increase their fertility (47).

GHRKO mice and littermate controls were maintained in the laboratory of Dr. Andrzej Bartke at Southern Illinois University from stock produced by Dr. John Kopchick’s group at Ohio University, as

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previously described (50). The genetic background of these animals is derived from 129/Ola embryonic stem cells and includes contributions from BALB/c, C57BL/6, and C3H inbred strains.

All mice evaluated in this study were male. Animals were weaned at 3 wk of age and housed three mice (HET3, Snell) or five mice (GHRKO) per cage. All animals were given free access to food and water except for the CR cohort. Mice were maintained using standard specific pathogen-free (SPF) husbandry techniques; sentinel animals were exposed to spent bedding on a quarterly basis to check for possible pathogens, and all such tests came up negative over the course of the study.

**CL, CR, and rapamycin-treated cohorts.** CL mice were generated following the procedure described previously (39). HET3 litters were culled to eight pups (control mice) or supplemented by transfer of newborn mice to produce litters of 12 mice (CL mice). Litters were weaned at 3 wk of age and housed thereafter at three mice per cage. To control for the potentially confounding effect of differing levels of nutrition experienced in utero, litters originally including fewer than 8, eight or more than ten pups were not used. After weaning, both groups were fed control Purina 5LG6 chow ad libitum. CL mice were euthanized at 12 or 22 mo of age.

CR mice were given 80% of the amount of food consumed by AL control mice starting at 6 wk of age. At 10 wk of age, CR mice were switched to 60% AL diet and then kept at that level until they were euthanized at 12 mo of age. Previous studies have demonstrated a robust CR effect on lifespan using this protocol (6), and a similar regimen extends longevity in HET3 mice (16).

The rapamycin-treated cohort was administered 14 ppm of micro-encapsulated rapamycin in 5LG6 chow, starting at 9 mo of age, following the same protocol used in our previous reports (19, 30). Rapamycin-treated mice were euthanized at 12 or 22 mo of age.

**tBHQ administration.** tBHQ-treated food was prepared fresh weekly in 500-g batches in our laboratory using powdered Purina 5001 chow. tBHQ (Sigma-Aldrich) was added into powdered chow at 1% wt/wt. A Knox gelatin solution (4% wt/wt, relative to chow weight) was prepared in ddH<sub>2</sub>O, heated to 60°C, and allowed to cool to 30°C. The gelatin solution was then added to the powdered chow-tBHQ mix and refrigerated overnight. Young adult male HET3 mice, 6 mo of age, were administered 1% tBHQ-treated food, or control food prepared in the same manner without drug, for 2 wk prior to euthanasia.

**RNA isolation from liver.** Liver tissue was washed with ice-cold PBS, snap-frozen in liquid nitrogen, and stored at -80°C. Sectioned liver samples were suspended in TRIzol reagent (Life Technologies), homogenized for 30 s, and then sonicated for 30 s using 10-s pulse with 5-s rest intervals on ice. RNA was isolated by phenol-chloroform extraction to yield total RNA following the manufacturer's instructions. Samples were dissolved in 500  $\mu$ l of RNase-free ddH<sub>2</sub>O, and RNA concentration was quantified by spectrophotometry using a NanoDrop 1000. Samples were then stored at -80°C until later use.

**Real-time RT-PCR.** Quantitative real-time RT-PCR analysis was performed by two-step reaction by first generating cDNA with the iScript cDNA synthesis kit (Bio-Rad Laboratories). Amplification was measured using SYBR Green I (Bio-Rad Laboratories) and a RotorGene 6000 cyclor (Corbett Research). After an initial denaturation step (95°C for 180 s), amplification was performed over 45 cycles of denaturation (95°C for 10 s), annealing (58°C for 6 s), and elongation (72°C for 13 s). The  $\Delta\Delta C_T$  comparative method ( $\Delta C_{T\text{target}} - \Delta C_{T\text{actin}}$ ) was used to determine relative gene expression levels. Primer sequences are available upon request.

**Statistical analyses.** All bar graphs show mean values  $\pm$  SE normalized to the levels seen in the corresponding control animals. Differences between treatment groups were evaluated using a two-tailed, unpaired *t*-test, with  $P < 0.05$  as the criterion for statistical significance. Scatterplot correlation analysis was performed using Spearman's rank correlation coefficient, with  $P < 0.05$  as the criterion for significance.

## RESULTS

**Litter crowding during weaning results in stable upregulation of xenobiotic metabolism genes.** Previous work had shown that CL mice given limited access to food for the 3 wk period between birth and weaning lived 18% longer than control mice (39). To evaluate the extent to which patterns of gene expression in CL mice might resemble those in CR mice, we measured mRNA levels in liver for genes involved in phase I detoxification of xenobiotic chemicals. Of 19 such mRNAs evaluated, 17 were found to be elevated in liver of 12-mo-old CL mice, and six of these were significantly elevated in a comparison of six CL mice to six controls (see Fig. 7). In parallel, we measured mRNA levels in 12-mo-old CR mice of the same genetically heterogeneous stock (see Fig. 7). Comparison of the mRNA levels in CL and CR mice showed a close correspondence (Fig. 1A), with a correlation  $R = 0.79$  ( $P < 0.001$ ). These results show that a brief period of food restriction limited to the first 3 wk of life is sufficient to establish an altered pattern of liver gene expression that is retained for at least 11 mo after the end of the intervention and that the pattern resembles that produced by long-term CR.

To see whether similar overexpression of genes involved in xenobiotic metabolism was also characteristic of mice in which slowed aging was due to genetic or pharmacological interventions, we evaluated the same set of mRNAs in 12-mo-old Snell mice and in HET3 mice given a diet containing rapamycin (19, 30) from 9 mo of age. Results shown in Fig. 1, B and C, demonstrate that the pattern of genes overexpressed in CL and CR mice also characterizes age-matched Snell and rapamycin-treated mice. The gene expression pattern in the CL mice is

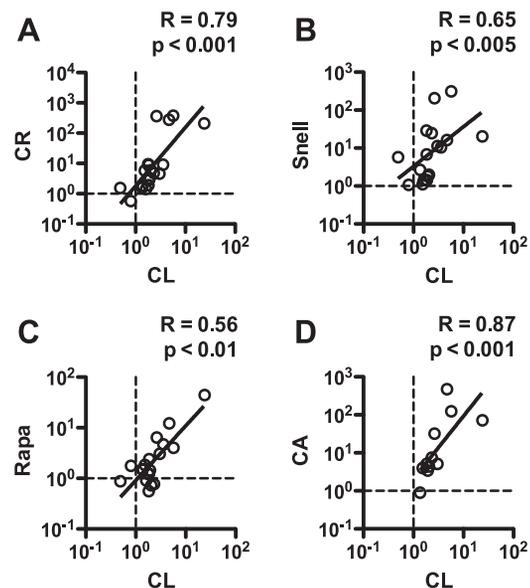


Fig. 1. Scatterplots of mRNA levels for enzymes involved in phase I detoxification of xenobiotics in liver, expressed as ratios to their respective control mice, for crowded litter (CL) mice compared with calorie-restricted (CR) mice (A), Snell mice (B), rapamycin-treated mice (C), and published effects of exposure to CA (D) (1). Each symbol represents a different mRNA. All mice were 12 mo old, except 2–4 mo for the CA group. Each panel also shows the Spearman correlation coefficient  $R$  and its associated  $P$  value. Calculated values are means of  $n = 6$  experimental mice compared with  $n = 6$  of the relevant age-matched control. Diagonal lines show best least squares linear fit. Note that vertical axis scales differ among panels to provide legible detail for each of the data sets compared.

also similar to that seen in mice exposed to cholic acid (CA), a known inducer of xenobiotic detoxification genes; Fig. 1D compares our results from CL mice with published levels (1) for 2- to 4-mo-old mice of the C57BL/6J background tested after 7 days of exposure to 2% CA. Thus, elevation of genes involved in phase I xenobiotic detoxification is characteristic of CL, CR, Snell, and rapamycin-treated mice and is similar to the pattern induced by short-term exposure to a bile acid.

*Elevated levels of many xenobiotic metabolism genes are seen at both 12 and 22 mo of age in CL mice.* To see if the gene expression pattern observed in 12-mo-old CL mice remained stable through adult life, we measured gene expression in 22-mo-old animals and compared the mRNA levels with those of control mice at 12 or 22 mo (Fig. 2). All six genes that were significantly elevated at 12 mo of age in CL liver remained elevated in 22-mo-old CL mice; five of those six contrasts were statistically significant relative to 12-mo-old control mice. Four of those six mRNAs, i.e., Cyp2b2, Cyp2b13, Fmo3, and Hao3, were also found to be significantly higher in 22-mo controls than in 12-mo controls. Because aging leads to elevated expression of these genes in control mice, the levels in control mice at 22 mo are similar to those of the CL mice at this age. Those four genes are thus increased in the first year of life by the CL intervention, and then in later life by the aging process itself in mice not subjected to CL.

*The duration of rapamycin treatment has differential effects on xenobiotic metabolism gene expression.* Mice treated with rapamycin for 3 mo, starting at 9 mo of age, had elevated expression of 14 of the 19 phase I xenobiotic metabolism genes evaluated in liver of 12-mo-old mice (see Fig. 7), and nine of those were significantly elevated compared with age-matched controls. To see whether rapamycin, like the CL intervention, leads to long-lasting activation of these mRNAs, we evaluated liver of 22-mo-old mice that had been treated with rapamycin from 9 mo onward. Figure 3 shows the results for each of the nine mRNAs found to be significantly elevated in the 12-mo-

old rapamycin mice. Seven of those nine mRNAs were at lower levels in 22-mo-old rapamycin mice than they had been at 12 mo of age, and four were significantly lower. Indeed, three of these, Cyp2c38, Cyp4a10, and Por, were at significantly lower levels in 22-mo-old rapamycin-treated mice than in age-matched controls, and a fourth mRNA (Hao3) was also significantly lower in the rapamycin-treated mice than in 22-mo-old controls. Other mRNAs elevated by rapamycin in the 12-mo-old mice, however, such as Cyp1a1 and Fmo3, remained elevated in the 22-mo-old mice. Among the nine mRNAs significantly activated by rapamycin at 12 mo of age, six showed a significant age-associated increase among control mice, reminiscent of age-related increases in genes sensitive to CL (see Fig. 2).

*The Nrf2 activator tBHQ induces xenobiotic metabolism genes.* To see if some of mRNAs that were elevated in these slow-aging mice could be activated acutely, we evaluated liver mRNA in young adult HET3 mice after 2 wk of exposure to tBHQ, which induces the transcription factor Nrf2 (32). All 19 of the mRNAs tested showed an elevation in tBHQ-treated mice, and 13 of those showed statistically significant increases (see Fig. 7). The scatterplots shown in Fig. 4 compare the patterns of phase I xenobiotic mRNAs in tBHQ-treated mice with those of 12-mo-old CR, CL, Snell, and rapamycin-treated mice, together with data from a published study of young adult CA-treated mice. There are strong and significant correlations with tBHQ exposure and the pattern of mRNAs seen in the CR, CL, Snell, and CA-treated models, but changes in xenobiotic metabolism in rapamycin-treated mice do not resemble those seen in tBHQ-treated mice (Fig. 4D). In addition, we observed that ER-enriched microsomes isolated from liver tissue of tBHQ-treated mice had higher cytochrome P-450 enzymatic activity relative to control fed mice, as measured by the formation of resorufin (Steinbaugh and Miller, unpublished data).

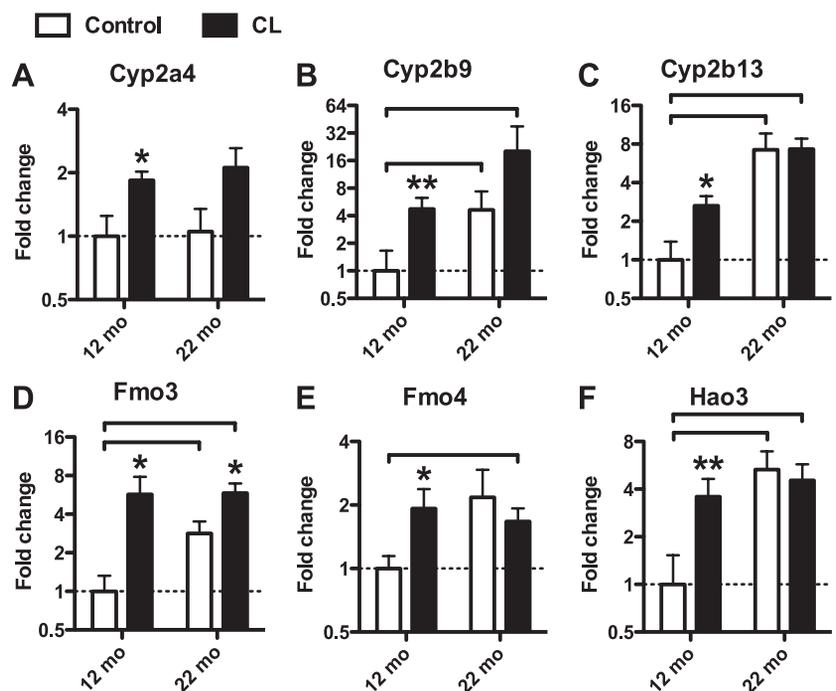


Fig. 2. CL mice maintain elevated levels of liver phase I mRNAs through 22 mo of age. Each panel shows 1 of the 6 mRNA species that was significantly elevated in 12-mo-old CL mice. Bars indicate means  $\pm$  SE for CL and age-matched control mice, in each case normalized to the average level of mRNA in 12-mo-old control animals;  $n = 6$  mice per treatment, except  $n = 5$  for 22-mo-old CL mice. Asterisks over CL bars indicate significant differences from age-matched controls (2-tailed  $t$ -test, \* $P < 0.05$ , \*\* $P < 0.01$ ). Horizontal lines indicate significant differences vs. levels in 12-mo-old control mice.

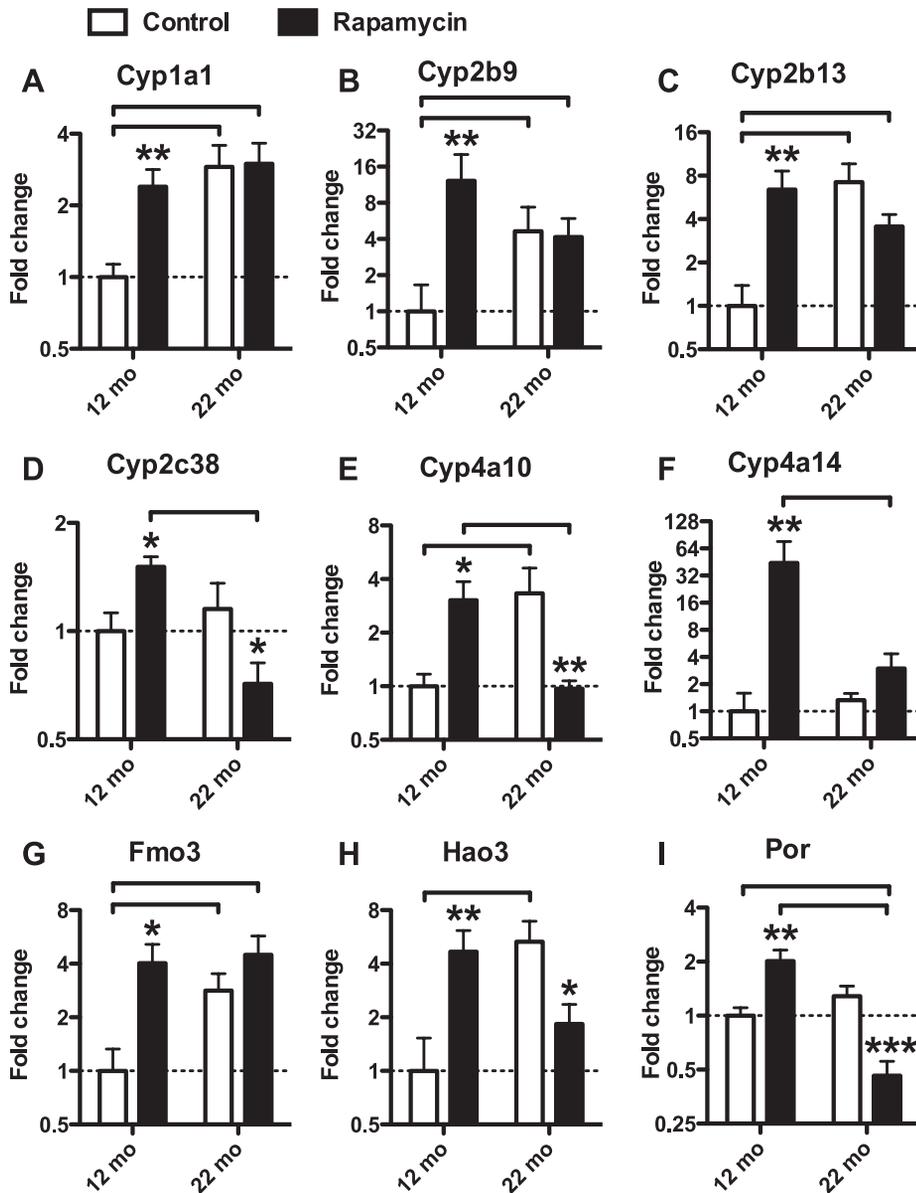


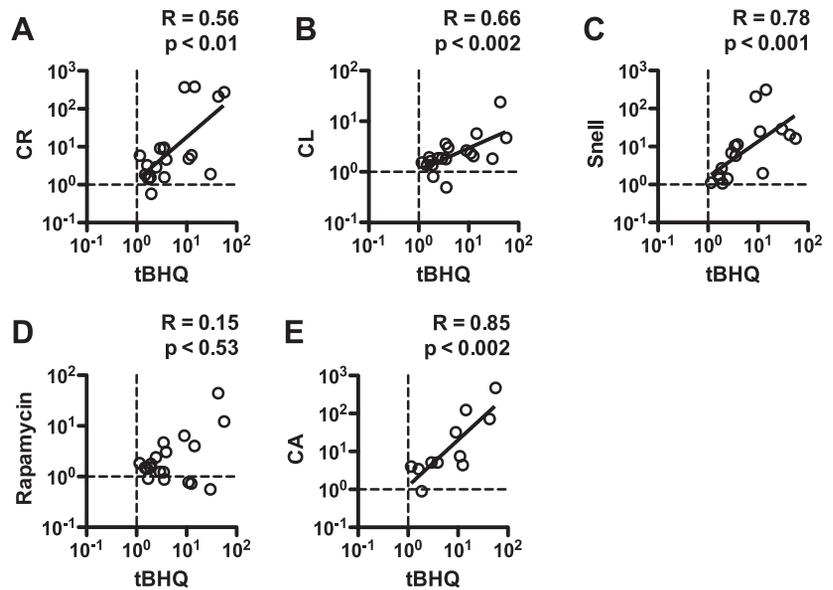
Fig. 3. Rapamycin-dependent elevation of many xenobiotic genes is reversed later in life. Mice were given food containing rapamycin starting at 9 mo of age and euthanized at 12 or 22 mo of age. Bars indicate means  $\pm$  SE for rapamycin-treated and age-matched control mice, in each case normalized to the average level of mRNA in 12-mo-old control animals;  $n = 6$  mice per treatment. Asterisks over rapamycin bars indicate significant differences from age-matched controls (2-tailed  $t$ -test,  $*P < 0.05$ ,  $**P < 0.01$ ). Horizontal lines indicate significant differences vs. 12-mo-old control mice or effects of age, as indicated.

Phase I XME genes are similarly expressed in genetic, dietary, and pharmacological interventions that extend lifespan. For a more comprehensive test of similarities among these various models, we calculated the correlation coefficients for each of the systems studied in our laboratory, together with our data on GHRKO mice (see Fig. 7) and published data for Little mice and CA-treated mice. Figure 5 tabulates these correlation coefficients and shows the expected high correlation ( $R > 0.7$ ) among the mutants with blocks in the GH/IGF-I pathway, i.e., Snell, GHRKO, and Little mice. The two dietary interventions, CR and CL, are well correlated ( $R > 0.5$ ) with one another and with each of the GH/IGF-I mutant mice, and the same is true for mRNAs in mice subjected to short-term exposure to CA (1) or tBHQ. The exception is rapamycin, for which there are significant correlations with CR, CL, and CA-treated mice, but less agreement with mRNA patterns in the tBHQ-treated mice, or with the Snell, GHRKO, and Little mice.

Figure 7 assembles data for each of these eight model systems, listing in each case the ratio of mRNA levels in the

slow-aging mice to levels in the corresponding set of control mice. As a crude index of the extent of mRNA elevation, the figure also includes the geometric mean value for the set of ratios in each column. From this perspective, the elevation of phase I genes is strongest in the mutant and CR mice, which also show the largest increase in longevity, and smaller in the CL and rapamycin mice, where the longevity effect is significant but not as large as in dwarf or CR animals. Some mRNAs, including Cyp2b13, Cyp2b9, Cyp4a14, Fmo3, and Hao3, show strong elevations, at least threefold and as high as 7,500-fold, in each of the mouse models known to be long lived, in addition to mice treated with tBHQ or CA. Other mRNAs, however, such as Cyp1a2, show only minor and inconsistent changes. This analysis was performed with mRNA expression data generated by RT-PCR and does not reflect posttranslational effects or effects of protein turnover. For an initial evaluation, we measured levels of Cyp2b10 in liver of GHRKO mice and found a significant, fourfold elevation by immunoblot relative to littermate control mice, similar to the

Fig. 4. Acute exposure to *tert*-butylhydroquinone (tBHQ) mimics mRNA patterns seen in CR, CL, Snell, and cholic acid (CA)-treated mice but not rapamycin-treated mice. Scatterplots show mRNA levels for xenobiotic metabolism genes in liver of mice after 2-wk exposure to tBHQ, compared with CR (A), CL (B), Snell (C), rapamycin-treated (D), and CA-treated mice (E). Each symbol represents a different mRNA, plotted to show the ratio of the mean level in experimental mice to their respective controls. All mice were 12 mo old except for the published data on CA (1), which represent mice 2–4 mo of age. Each panel also shows the Spearman correlation coefficient *R* with its associated *P* value. Diagonal lines indicate least square fits. Note that the correlation of tBHQ effects with rapamycin is not statistically significant.



observed changes at the mRNA level (Steinbaugh and Miller, unpublished data).

DISCUSSION

XMEs have been reported to be transcriptionally upregulated in long-lived dwarf mice and mice on long-term CR (41). In this study, we show that rapamycin, tBHQ, and litter crowding also lead to transcriptional activation of phase I XMEs in mice, consistent with the patterns previously reported

in CR and long-lived dwarf models. Whether xenobiotic metabolism activity contributes to extension of lifespan in mammals is unknown, but microarray studies in *Caenorhabditis elegans* support such a hypothesis (28). On the basis of our data, we propose that elevation of phase I XMEs is a common signature of delayed or decelerated aging in mice, whether based on dietary, pharmacological, developmental or genetic changes, and we suggest that activation of this and related detoxification systems may contribute to slower aging in these animal models.

Long-term CR elevates XME expression in mouse liver tissue, depending on the age of the mice and the duration of

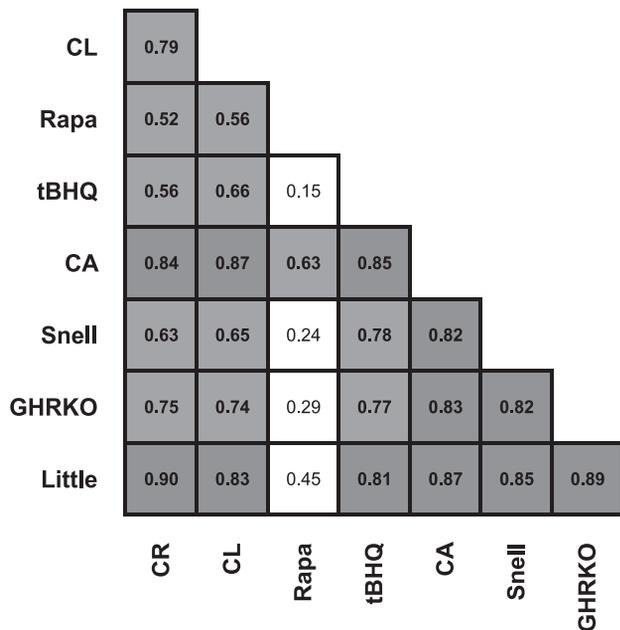


Fig. 5. Correlation matrix of hepatic xenobiotic metabolism gene expression. Each box shows the Spearman correlation coefficient *R* for the populations of mice in the corresponding row and column header. Values of *R* > 0.5 are statistically significant at *P* < 0.05. Dark gray shading denotes an *R* value > 0.8, light gray denotes an *R* value ranging from 0.5 to 0.8. Note that no significant relationship was observed for comparisons of rapamycin and tBHQ, Snell, GHRKO, or Little mice. CA and Little data were obtained from a previously published study (1).

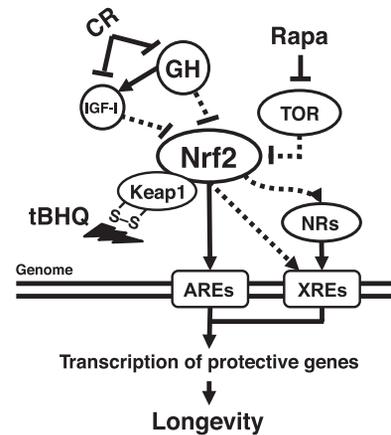


Fig. 6. Model for Nrf2-mediated regulation of aging. Nrf2 is a stress-responsive transcription factor that regulates expression of multiple protective genes, including XMEs. Activation of XMEs may protect cells from damage and could, in principle, help delay aging. Nrf2 is negatively regulated by Keap1 protein, which promotes rapid turnover of Nrf2 by the proteasome. Keap1 contains multiple cysteine residues, which are oxidized under stressful conditions, or by chemical Nrf2 activators, such as tBHQ, resulting in conformational changes that release bound Nrf2. Nrf2 can then translocate to the nucleus and induce transcription of XMEs through antioxidant response elements (ARE) and may also interact with nuclear receptors (NR) or xenobiotic response elements (XRE). Nrf2 may also be repressed by GH/IGF-I signaling or the target of rapamycin (TOR) pathway. CR and rapamycin treatment may enhance Nrf2 activity through attenuation of these signaling pathways.

	CR	CL	Rapa	tBHQ	CA	Little	Snell	GHRKO
Cyp1a1	2.9	1.9	2.4	2.4	–	1.7	1.4	1.2
Cyp1a2	1.4	1.6	0.9	1.7	–	1.1	1.4	0.9
Cyp2a4	9.0	1.8	1.2	2.9	5.1	3.4	6.8	3.5
Cyp2a5	9.4	1.8	1.2	3.5	–	–	–	–
Cyp2b10	6.0	2.1	0.7	12	4.4	21	2.0	8.8
Cyp2b13	368	2.6	6.4	9.0	32	7715	205	201
Cyp2b9	276	4.7	12	57	473	4127	16	61
Cyp2c37	1.6	0.5	0.9	3.6	–	2.6	5.7	0.8
Cyp2c38	1.6	1.4	1.5	1.9	0.9	2.0	2.7	2.2
Cyp3a11	0.6	0.8	1.8	1.9	–	1.0	1.1	0.9
Cyp3a41	1.8	1.4	1.5	1.5	–	–	–	0.7
Cyp4a10	4.6	3.0	3.0	3.9	5.1	6.8	11	3.1
Cyp4a14	210	24	44	43	72	26	20	24
Fmo3	379	5.7	4.0	14	124	128	314	11
Fmo4	3.3	1.9	1.4	1.6	3.4	3.0	1.8	1.9
Hao3	9.1	3.6	4.7	3.5	–	–	11	17
Mt1	4.9	2.3	0.8	11	7.5	4.4	25	36
Mt2	1.9	1.8	0.6	30	–	–	29	51
Por	5.8	1.5	1.8	1.1	4.0	2.4	1.1	1.4
Geo Mean	8.4	2.2	2.1	5.1	13	12	7.8	5.6

Fig. 7. Calculated values are means of gene expression as a ratio of experimental mice to age-matched control mice, normalized to  $\beta$ -actin levels. CR, calorie restricted; CL, crowded litter; Rapa, rapamycin treated; tBHQ, *tert*-butylhydroquinone treated; CA, cholic acid treated; GHRKO, GH receptor knockout. Degrees of shading indicate increasing levels of gene expression. Boldface indicates  $P < 0.05$ .

restriction (41, 42). CR extends lifespan and decreases the risk of cancer and other age-associated pathologies in rodents (26, 48). Physiological and biological studies have produced a large and growing list of differences between CR and control mice. Mice on CR have elevated antioxidant defenses (24, 49) and are resistant to multiple hepatotoxins (18, 49). The extent to which such changes contribute to increased lifespan in each case is uncertain, and the mechanism of slowed aging in CR rodents is still an area of active investigation. Whether CR can extend lifespan and delay aging in humans has not been evaluated, but an ongoing study of monkeys on CR suggests that results generated in rodents and other model organisms may also be applicable to primates (13).

Short-term CR during weaning by increasing the number of pups nursed by an individual mother produces “crowded litter” (CL) mice that are long lived (39), presumably because of restricted nutrient availability at this early period in postnatal life. The mechanism behind CL-mediated lifespan extension is not understood but presumably reflects stable epigenetic changes in gene expression in one or more cell types. Early, transient alterations in hormone levels can have strong, long-lasting effects on aging and lifespan. For example, short-term, intensive exposure of Ames mice to GH starting at 2 wk of age reverses both their extended lifespan and cellular stress resistance (35). Circulating IGF-I is reduced, at least at weaning, in CL mice (18), as it is in young CR mice (38), suggesting that reduced GH/IGF-I levels in early life may produce long-lasting changes that postpone aging and extend lifespan in both CL and CR mice.

The observation that diet-based interventions associated with lifespan extension upregulate phase I XMEs led us to ask whether such changes would be seen in mice in which extended longevity was produced pharmacologically. Research conducted by the National Institute on Aging Interventions

Testing Program (ITP) (31) has shown that rapamycin extends mean and maximal lifespan in mice, whether started at 9 or 20 mo of age (19, 30). Our data show that phase I gene expression is also upregulated, after 3 mo of rapamycin treatment initiated at 9 mo of age, in a pattern similar to that observed in CR and CL mice. Surprisingly, however, longer exposure to rapamycin (i.e., from 9 to 22 mo of age) resulted in a significant repression of many of these mRNAs, in some cases to levels significantly below those seen in 12-mo-old control animals. Thus, at least some of the changes in gene expression induced by rapamycin are transient, and it is not clear whether the health benefits seen in rapamycin-treated mice are due to its initial effects (such as elevation of protective enzyme levels), to compensatory down-regulation of these same genes, or to more complex feedback circuits. The transience of the effects seen in rapamycin-treated mice was not observed in aged CL mice; in the CL system, genes elevated in 12-mo-old mice remained high at least through 22 mo of age.

Nuclear receptors are the best-characterized regulators of phase I XMEs. A study utilizing Little mice crossed with constitutive androstane receptor (CAR)/pregnane X receptor (PXR) or farnesoid X receptor (FXR) knockouts showed that FXR, but not CAR or PXR, is the nuclear receptor that primarily regulates phase I XME expression in Little mice (1). Mechanisms for regulation of xenobiotic metabolism have not been evaluated in other mouse models, but there is circumstantial evidence to suggest alteration of phase I gene expression by additional factors. Peroxisome proliferator-activated receptor (PPAR) isoforms, for example, are elevated in Ames and GHRKO liver tissue (27), and multiple Nrf2-regulated genes show increased transcription in liver of Ames, Snell, and CR mice (23, 36, 40).

Nrf2 is a stress-responsive transcription factor that also regulates XME gene expression (4, 21) (Fig. 6). During unstressed conditions, Nrf2 is sequestered in the cytosol by

Keap1, which maintains Nrf2 in an inactive state, subject to rapid degradation by the proteasome. In response to oxidative stress, Nrf2/Keap1 binding is disrupted, and Nrf2 translocates to the nucleus, where it acts as a transcription factor to modulate expression of well over 100 genes. Studies on the *C. elegans* Nrf2 ortholog *skn-1* have shown that overexpression of SKN-1 can increase worm lifespan and that *skn-1* is necessary for CR-mediated lifespan extension (9, 45). Mutation of *Keap1*, which increases Nrf2 activity, has been reported to extend the lifespan of male *Drosophila melanogaster* fruit flies (43). Whether Nrf2 contributes to the rate of aging in mammals remains uncertain (25, 37). *Nrf2*<sup>-/-</sup> mice on long-term CR were reported to have increased susceptibility to cancer but show no difference in lifespan relative to wild-type mice on CR (36). Hepatocyte-specific deletion of *Keap1* increases Nrf2 nuclear localization and XME mRNA expression in a similar pattern to the one we have seen in our long-lived mice (33), and *Nrf2*<sup>-/-</sup> mice have lowered XME gene expression in the liver (3). Hepatic activation of Nrf2 is also associated with increased resistance to acetaminophen toxicity (33), which is also seen in CR mice (18). Conversely, *Nrf2*<sup>-/-</sup> mice are sensitive to chemical stress, suggesting that Nrf2 is a key mediator of stress resistance (15, 46). Nrf2 may also serve as a regulator of nuclear receptor activity (Fig. 6). *Nrf2*<sup>-/-</sup> mice have attenuated mRNA expression of CAR, PXR, and AhR nuclear receptors, and multiple nuclear receptor-regulated phase I XMEs (3).

Nrf2 can be activated by a chemically diverse group of pharmacological agents (20). Clinical studies of Nrf2 activators have reported potent chemopreventive effects, suggesting that regulation of Nrf2 activity may be a promising approach to delay cancer and possibly aging (22). Our data show that short-term exposure to tBHQ can upregulate phase I genes and that the pattern of genes activated is similar to that seen in CR and CL mice, in mice treated with CA (1), and in GH/IGF-I-deficient dwarf mice, although correlation with gene expression patterns in rapamycin-treated mice is less striking.

The results of this study support the hypothesis that expression of xenobiotic metabolism genes is associated with many models of extended longevity in mice. We postulate that elevation of XME gene expression, presumably through elevation of the corresponding proteins, protects the liver and perhaps other tissues from the damaging effects of ingested toxins and harmful endogenous molecules produced through ordinary metabolism and thus slows down the rate of age-dependent decline in tissue homeostasis. Providing further molecular detail and refinement of this idea will require additional investigation, and our data provide a strong justification for pursuing such questions. In addition, our results may prompt new work to determine whether chronic elevation of xenobiotic metabolism genes could slow aging and increase lifespan. This could potentially be accomplished through pharmacological interventions with Nrf2 activators or bile acid compounds, genetic models of elevated Nrf2 activity such as the tissue-specific *Keap1*<sup>-/-</sup> mouse, or upstream nuclear receptors that are known to regulate xenobiotic metabolism.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

#### AUTHOR CONTRIBUTIONS

Author contributions: M.J.S., L.Y.S., A.B., and R.A.M. conception and design of research; M.J.S. and L.Y.S. performed experiments; M.J.S. analyzed data; M.J.S. and R.A.M. interpreted results of experiments; M.J.S. prepared Figs.; M.J.S. drafted manuscript; M.J.S. and R.A.M. edited and revised manuscript; M.J.S. and R.A.M. approved final version of manuscript.

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