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Effect of Ames dwarfism and caloric restriction on spontaneous mutation frequency in different mouse tissues

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Abstract

Genetic instability has been implicated as a causal factor in cancer and aging. Caloric restriction (CR) and suppression of the somatotroph axis significantly increase life span in the mouse and reduces multiple symptoms of aging, including cancer. To test if *in vivo* spontaneous mutation frequency is reduced by such mechanisms, we crossed long-lived Ames dwarf mice with a C57BL/6J line harboring multiple copies of the *lacZ* mutation reporter gene as part of a plasmid that can be recovered from tissues and organs into *E. coli* to measure mutant frequencies. Four cohorts were studied: (1) *ad lib* wild-type; (2) CR wild-type; (3) *ad lib* dwarf; and (4) CR dwarf. While both CR wild-type and *ad lib* dwarf mice lived significantly longer than the *ad lib* wild-type mice, under CR conditions dwarf mice did not live any longer than *ad lib* wild-type mice. While this may be due to an as yet unknown adverse effect of the C57Bl/6 background, it did not prevent an effect on spontaneous mutation frequencies at the *lacZ* locus, which were assessed in liver, kidney and small intestine of 7- and 15-month old mice of all four cohorts. A lower mutant frequency in the *ad lib* dwarf background was observed in liver and kidney at 7 and 15 months of age and in small intestine at 15 months of age as compared to the *ad lib* wild-type. CR also significantly reduced spontaneous mutant frequency in kidney and small intestine, but not in liver. In a separate cohort of *lacZ*-C57BL/6J mice CR was also found to significantly reduce spontaneous mutant frequency in liver and small intestine, across three age levels. These results indicate that two major pro-longevity interventions in the mouse are associated with a reduced mutation frequency. This could be responsible, at least in part, for the enhanced longevity associated with Ames dwarfism and CR.

1. Introduction

A gradual loss of genome integrity has long been implicated in aging and may play an especially prominent role in aging-related diseases, such as cancer (DePinho, 2000; Vijg, 2000). Our laboratory has previously demonstrated that genomic mutations increase with age in an organ and tissue-dependent manner (Dollé et al., 1997; Dollé et al., 2000; Vijg et al., 2002; Vijg, 2002). While these results demonstrated that mutations increase with chronological age, they did not establish that spontaneous mutations accumulate as a function of biological age. One

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strategy for testing such a potential causal relationship is to compare spontaneous mutation frequencies between normal mice and mice of increased longevity as a possible consequence of delayed aging. There are currently two major interventions that have been convincingly demonstrated in many laboratories to increase life span of mice and delay many symptoms of aging: (1) limiting food, i.e., caloric restriction (CR) and (2) inhibiting the growth hormone/pituitary axis (Bartke, 2005). To test for a possible major role of genomic instability as a cause of aging it would be important to demonstrate that in such mouse models of extended life span, mutation accumulation is retarded together with symptoms of aging.

The oldest model for extending life span is the reduction of caloric intake (Masoro, 2005). Caloric restriction typically refers to a diet in which calories are limited by 30–40 % compared with animals fed *ad lib*. Caloric restriction extends life span in multiple species, including rodents, flies and worms, but its mechanism of action remains unclear. One hypothesis is that CR affects basal metabolism, slowing the production of toxic reactive oxygen species, which in turn may slow aging. There is evidence that CR reduces spontaneous mutation frequency at the Hprt locus in T lymphocytes in mice (Dempsey et al., 1993) and rats (Aidoo et al., 2003). Evidence has also been obtained that CR reduces the number of mutations in T lymphocytes of rats induced by environmental mutagens (Casciano et al., 1996). However, the Hprt assay does not allow the direct analysis of different organs and tissues. In one study of caloric restriction in 6 and 12 month old mice harboring the *lacI* reporter gene as part of a bacteriophage lambda vector, no CR-related changes in mutant frequency were observed in liver (Stuart et al., 2000).

Ames dwarf mice are homozygous for a mutation in the *Prop-1* gene and live 50–70% longer than wild-type mice (Brown-Borg et al., 1996). The Prop-1 mutants are deficient in growth hormone, thyroid stimulating hormone and prolactin, and show reduced levels of plasma insulin, IGF-1 and glucose. Like mice subjected to CR, Ames dwarf mice show a delayed occurrence of neoplastic lesions compared with their normal siblings, which may contribute significantly to the extended life span in these mice (Ikeno et al., 2003). It has been hypothesized that the beneficial effects of the Prop-1 mutation are due to the reduction in plasma IGF-1, which may also underlie the beneficial effects of caloric restriction (Bartke, 2002).

To study the effects of both CR and Ames dwarfism on spontaneous mutation frequencies in different tissues we crossed Ames mutant mice with mice harboring *lacZ* reporter genes, and measured mutant frequencies in liver, kidney and small intestine of 7 and 15 month old animals of the following 4 cohorts: (1) *ad lib* wild-type mice; (2) CR wild-type mice; (3) *ad lib* dwarf mice; and (4) CR dwarf mice. The results indicate a generally strong effect of the Ames dwarf mutation in reducing spontaneous mutations at the *lacZ* locus with a somewhat smaller effect of CR. The latter was confirmed in an independent *lacZ* C57BL/6J cohort for studying CR alone. These results point towards a possible role of reduced mutagenesis in increasing life span.

2. Materials and Methods

2.1. Mouse breeding and maintenance and tissue collection

C57BL/6J *lacZ*-plasmid transgenic mice, homozygous for two *lacZ*-plasmid clusters, one on chromosome 3 (A) and one on chromosome 4 (B) (Dollé et al., 1997) were crossed to heterozygous Ames dwarf ($Prop1^{df}/Prop1^{+}$, $lacZ^{-/-,-/-}$) mice in a heterogeneous background (Brown-Borg et al., 1996). The resulting F1 mice were further crossed for obtaining the 4 study cohorts as described in the Results section and shown in Fig. 1. The *Prop-1* mutation or the *lacZ* cluster was identified from tail DNA using PCR-based tests described previously (Dollé et al., 2001; Garcia et al., 2007). Animals were sacrificed by CO_2 inhalation followed by cervical dislocation and tissues excised and snap-frozen in liquid nitrogen for DNA isolation.

In addition to the dwarf-*lacZ* hybrids, one CR and one *ad lib* cohort of homozygous C57Bl/6J *lacZ*- pUR288-plasmid transgenic mice were also initiated. The animals were maintained either fed *ad libitum* (*ad lib*) or under CR conditions in the Animal Facility of the University of Texas Health Science Center as described previously (Ikeno et al., 2005). Mice were kept pathogen-free in micro-isolator units on Tek FRESH ultra laboratory bedding (Harlan Tekland, Madison, WI) and all procedures followed the guidelines approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and South Texas Veterans Health Care System. At 21 days after birth, animals were weaned and housed 4 animals/cage. *Ad lib* diet was provided for all animals until 2 months of age when the caloric restriction started. After 2 months, wild-type animals were fed 60 % of the *ad lib* intake until sacrificed. Since Ames dwarf mice were more susceptible to an early death, the animals were first fed 80 % of the Ames dwarf *ad lib* intake for a month and after this period they were switched to a 60 % calorie restricted diet. Measurement of food intake was done weekly on 10 % of the *ad lib* fed control animals matched for age as described previously (Ikeno et al., 2005). For the dwarf-*lacZ* hybrids, separate cohorts of 28 animals each were established to determine their survival; date of death was recorded when the mice died spontaneously. All studies were done exclusively with male animals.

2.2. Plasmid rescue and mutant frequency determination

DNA was extracted by routine phenol/chloroform extractions. Detailed protocols for plasmid rescue and mutant frequency determinations for this model are provided elsewhere (Garcia et al., 2007). Briefly, 10 to 20 μg genomic DNA was digested with *Hin*d III for 1 h in the presence of magnetic beads (Dynal) precoated with *lacZ*-lacI fusion protein. The beads were washed three times to remove the unbound mouse genomic DNA. Plasmids were subsequently eluted from the beads by IPTG. After circularization of the plasmids with T4 DNA ligase they were ethanol-precipitated and used to electrotransform *E.coli* C (Δ*lacZ*, *galE*$^{-}$) cells. One thousandth of the transformed cells was plated on the titer plate (with X-gal) and the remainder on the selective plate (with p-gal). The plates were incubated for 15 h at 37 °C. Mutant frequencies were determined as the number of colonies on the selective plates versus the number of colonies on the titer plate (times the dilution factor of 1000). Each mutant frequency is based on at least 300,000 recovered plasmids. The background mutant frequency of this system is about 1×10^{-5}, as determined in *E. coli*, and consists mostly of false positive size-change mutants at *Hin*d III star-activity sites (Dollé et al., 1999).

2.3. Statistical analysis

Survival curves were compared using the log-rank test. The number of mutations was analyzed for effects of age, genotype and diet using a generalized linear model analysis (McCullagh et al., 1989). The logarithm was used as the link function and the distribution was negative binomial. The ratios of mutant frequencies were used to compare experimental groups (antilogs of differences in the logarithm of the mutant frequencies). A full model was used, that is, a model that included main effects, two-factor interactions, and the three-factor interaction. The estimated dispersion parameters (liver, kidney and small intestine) were significantly different from zero. All calculations were carried out using PROC GENMOD of SAS v9.1. Statistical significance was $P \leq 0.05$.

3. Results

The breeding scheme for crossing the *lacZ*-plasmid reporter construct into the Ames background is shown in Fig. 1. While Ames dwarf mice have a heterogeneous genetic background, the *lacZ*-plasmid animals were in C57BL/6J, with the transgene cluster at two chromosomal locations, one on chromosome 3 and the other on chromosome 4. We initially decided to cross the Ames dwarf mutation as far as possible into the *lacZ*-C57BL/6J

background. This would have had the advantage of simultaneously homogenizing the Ames background into mostly C57BL/6J, thereby reducing heterogeneity, and generating homozygosity for the *lacZ* loci, thus increasing the number of *lacZ*-plasmids that can be recovered for mutation analysis. However, with an increased contribution of the C57BL/6J background, mortality of homozygous dwarf mice between weaning and adulthood was found to rise appreciably. Although unknown to us at the time, similar results for Ames dwarf mice were obtained by Nasonkin et al. who found that a fraction of pups with a higher contribution of the C57BL/6J background display lethargy and die precipitously between weaning and adulthood (Nasonkin et al., 2004). For this reason we decided to cross the F5 $Prop1^{df}/Prop1^{+}$,$lacZ^{A/A}/lacZ^{B/B}$ back to the original heterozygous dwarf animals ($Prop1^{df}/Prop1^{+}$, $lacZ^{-/-,-/-}$). This reduction in C57BL/6J background proved to be sufficient to reduce the early mortality to a normal level.

Using this strategy we established four cohorts: (1) *ad lib* wild-type; (2) CR wild-type, (3) *ad lib* dwarf and (4) CR dwarf. The survival of animals under all four conditions was determined in parallel (Fig. 2). As expected, calorie restriction extended the longevity of wild-type mice compared to that in *ad lib* wild-type mice (P=0.002), and *ad lib* Ames dwarf mice outlived *ad lib* wild-type animals (p=6.35e-06). However, we did not observe the further increase in longevity for the Ames dwarf mice under CR as reported previously (Bartke et al., 2001). Indeed, CR appeared to obliterate the gain in life-span of the dwarf cohort! Although we were careful to gradually calorie restrict the dwarf mice (20 % for the first month and then the 40 % reduction of food intake that is commonly applied for CR by the animal facility in San Antonio) we did see excess mortality in the CR dwarf cohort. Reasoning that 40% CR might be too severe for these fragile dwarf mice we reduced this to 30% after 19 months. However, even after censoring the cohorts for mortality before that age, we did not see any appreciable improvement of the survival curve. Indeed, the earliest age at which censoring deaths improved survival of the CR dwarf cohort was 34 months. It was only at that late age that the survival curve began to parallel the ones for the *ad lib* dwarf and CR wild type cohorts (results not shown).

In view of the high costs associated with this type of study we limited our comparison of spontaneous mutation frequency at the *lacZ* locus to two, relatively early time points. For each cohort, between 5 and 8 mice were sacrificed at 7 and 15 months of age. Tissues were frozen and DNA extracted following a schedule in which always all four conditions were handled side-by-side. For example, liver DNAs for one time point were extracted simultaneously for all four conditions. All samples were coded and mutant frequency determinations performed blindly. For mutant frequency determinations we selected liver, kidney and small intestine on the basis of the robust age-related increases previously observed in these organs (Dollé et al., 2006; Dollé et al., 1997; Dollé et al., 2000).

Spontaneous mutant frequencies for liver, kidney and small intestine of the *lacZ*-Ames dwarf hybrids at the two ages are shown in Fig. 3. All raw data are available from Supplementary Table 1–Supplementary Table 5. The *lacZ* mutant frequency data were subjected to statistical analysis by comparing ratios of mutant frequencies in dwarf versus wild type by diet, CR versus *ad lib* by genotype (Table 1) and *ad lib* dwarf versus CR wild type, CR dwarf versus combined CR wild type and *ad lib* dwarf, and *ad lib* wild type versus combined CR wild type and *ad lib* dwarf (Table 2).

As expected, all three organs experienced an increase of the spontaneous mutant frequency between 7 and 15 months (although for kidney this was not statistically significant). With the exception of small intestine at 7 months, there was a significantly lower mutant frequency in the dwarf background as compared to the *ad lib* wild-type (Table 1), as indicated by the ratio of mutant frequencies. Of note, while it was clear that overall there was a significant protective

effect of the Ames dwarf mutation against spontaneous mutations in the small intestine, 7 month-old Ames dwarf animals showed a higher mutant frequency (14.10×10^{-5}) than their wild-type littermate controls (10.55×10^{-5}). In our experience, the spontaneous mutant frequency at the *lacZ* locus in small intestine at this age level is generally around 15×10^{-5}. After re-confirming the genotypes we decided to re-extract DNA from the remaining tissues and repeat the experiment. Since essentially the same results were obtained we conclude that these observations were part of the natural variation, which is not unusual for an end point such as mutation frequency. It is also not impossible that animal-to-animal variation is higher for animals in a mixed genetic background, although that was not obvious in this case (compare Fig. 3 and Fig. 4).

For the CR diet, there was a lower mutant frequency in Ames dwarf mice compared to wild-type in kidney and small intestine at 7 months and small intestine at 15 months (indicated by ratios less than 1.00; Table 1, left columns). This can also be concluded from the ratios CR versus *ad lib* in the dwarf. In addition to the Ames dwarf mutation, CR in this mixed background significantly reduced spontaneous mutant frequency in kidney at 7 months and small intestine at 7 and 15 months (Table 1; right columns). In wild-type mice, CR reduced mutant frequency compared to *ad lib* in all tissues and at both ages except for small intestine at 7 months (Table 1).

In all tissues and at both ages, there was no significant difference in mutant frequency between *ad lib* Ames dwarf mice and CR wild-type mice (ratios not significantly different from 1.00; Table 2, left columns). For comparison with other groups we then combined the *ad lib* Ames dwarf mice and CR wild-type mice. The mutant frequency in CR Ames dwarf mice was significantly lower than in the combined *ad lib* Ames dwarf and CR wild-type mice in kidney and small intestine at 7 months and in small intestine at 15 months. Mutant frequency was higher in *ad lib* wild type than in combined *ad lib* Ames dwarf and CR wild-type mice in all tissues at both ages except small intestine at 7 months (Table 2).

Except for the results for small intestine at 7 months, the mutant frequency results are consistent across the different tissues and the two ages. Except for CR Ames dwarf mice, the longevity and mutant frequency results overall are consistent; *ad lib* wild type mice have the highest mutant frequency and the poorest survival while *ad lib* Ames dwarf and CR wild type have similar mutant frequencies and survival.

Since the observed CR effect was not as strong as the effect of the dwarf mutation, we decided to separately test for CR in a *lacZ*-C57BL/6 cohort, which would allow us to compare the effects of caloric restriction alone in a homogeneous genetic background. In this approach, animals were housed under caloric restriction or *ad lib* conditions and samples were collected at 7, 15 and 25 months of age. Mutant frequencies at the *lacZ* reporter locus of C57BL/6J mice by age and dietary condition are shown in Fig. 4 for liver and small intestine. Mutant frequency increased with age in both liver ($p=0.0001$, Fig. 4) and small intestine ($p=0.0016$; Fig 4). In liver, CR mice had lower mutant frequencies than *ad lib* mice at all ages but this difference approached statistical significance only at 15 months (Table 3). In small intestine, the mutant frequency in calorie-restricted mice was lower than *ad lib* controls at every age but the difference was statistically significant only at 25 months of age (Table 3). Hence, the results from this separate cohort essentially confirmed the results obtained for CR with the Ames dwarf mutant cohort.

4. Discussion

There is increasing evidence that life extension through caloric restriction, at least in rodents, exerts its beneficial effect in delaying multiple symptoms of aging by suppressing the growth

hormone/insulin-like growth factor 1 (GH/IGF1) somatotroph axis (Bartke, 2005). In turn, this may lead to the activation, possibly mediated by Forkhead box O (FoxO) transcription factors (Carter et al., 2007), of various cellular defense systems, including antioxidant defense and DNA repair (Brown-Borg et al., 2000). Indeed, recent results obtained with mice harboring defects in various pathways for repairing DNA damage, indicate that suppression of the GH/IGF1 axis is part of a DNA damage response involving metabolic changes that shift energy usage from growth and proliferation to protective maintenance (Niedernhofer et al., 2006; Vijg, 2006). Our present results are in keeping with this concept. Indeed, the overall pattern emerging from our present work is that both CR and the Ames dwarf mutation reduce somatic mutation accumulation in different tissues of the mouse.

The concept of a general reduction of cellular mutation load in mice subjected to CR is in keeping with results obtained by Dempsey et al. (Dempsey et al., 1993) and Aidoo et al (Aidoo et al., 2003), for somatic mutant frequency in T lymphocytes in mice and rats, respectively. Of note, Stuart et al., also using a mouse model harboring a mutational reporter gene, found no significant reduction in the spontaneous mutant frequency in liver of CR mice as compared with *ad lib* fed animals (Stuart et al., 2000). Interestingly, also in our present study, it is in liver where the reduction in spontaneous mutant frequency is weakest, either in the combined CR/dwarf cohort or the C57BL/6J cohort alone. Hence, it is possible that Stuart et al. lacked the power to detect a small difference that may actually have been present. The aforementioned HPRT assay is generally more sensitive than the fully neutral, transgenic reporter assays in detecting small differences, due to the relatively low background mutation frequency of this assay, which in turn is likely to be due to *in vivo* selection against cells with an inactivated HPRT gene (Vijg et al., 1998). It is therefore reasonable to interpret our present results as indicative for a relatively small but significant reduction in spontaneous mutation frequency. This raises the question of the underlying causes of such a reduction and its potential impact in explaining the increased longevity of these mice. As mentioned, while the mechanisms by which reduced GH/IGF1 signals can lead to increased longevity are not clear as yet, several studies have shown that this condition is associated with an upregulation of antioxidant defense capacity. It is known that factors that promote the generation of cellular reactive oxygen species (ROS) and/or impair anti-oxidative processes contribute to oxidative damage which accumulates with age and might be responsible for the progressive decline in physiological systems that occurs in virtually all organisms. There is abundant evidence that both in dwarf mice and mice kept under CR conditions antioxidant defense and stress responses are increased (Brown-Borg et al., 2000; Romanick et al., 2004). The importance of the antioxidant defense system in the maintenance of genome stability was demonstrated by Busuttil et al., who demonstrated greatly accelerated mutation accumulation in liver of SOD1-null mice (Busuttil et al., 2005). This indicates that oxidative stress is a causal factor of the increased incidence of liver cancer in this mutant mouse model (Elchuri et al., 2005). Hence, the lower spontaneous somatic mutation accumulation observed for the GH/IGF1-deficient mice in our present study could be a result of a decreased susceptibility to oxidative damage-induced mutagenesis. This effect is likely to be stronger in the dwarf mice than in CR because of the stronger reduction in GH/IGF1 in these animals and/or because the downregulation of the somatotroph axis under CR conditions only occurred after 2 months. However, the possibility cannot be ruled out that the mechanism by which the Ames mutation and caloric restriction affect longevity is different (Bartke et al., 2001).

Alternatively, the reduced mutant frequency in CR and dwarf mice could be due to decreased cell proliferative activity. CR in the mouse has been found associated with a reduced BrdU labeling index in all of six different cell types in five different organs analyzed, up until 13 months (Wolf et al., 1995). This would reduce the chance of replication errors as a source of spontaneous mutations. In addition, CR (in rats) has been associated with an increase in

spontaneous apoptosis in the liver (James et al., 1994). This is also expected to reduce mutation load of the tissue as a whole, due to the preventive removal of damaged cells.

One thing that is not clear from our studies is how to reconcile the observed synergistic effect of dwarfism and CR on spontaneous mutant frequency. Indeed, while this effect confirms previous work of a synergistic effect of CR and the Ames dwarf mutation on life span (Bartke et al., 2001), this was not reproduced in this present study (Fig. 2A). Hence, formally there is no association between the observed reduced mutant frequency and the life span effect. However, we believe that a more likely explanation is that the increased mortality in the CR dwarf cohort is independent of the effect on mutant frequency. Most likely, the increased deaths are due to the C57BL/6 background parts of which still remained in the hybrid animals that made up the cohort. It is possible that the C57BL/6J background under CR conditions, together with the naturally rather frail dwarf mice lead to increased mortality during the entire life span. The observed late longevity effect (i.e., after 34 months) in the CR dwarfs may involve the survivors, possibly those with minimal adverse C57BL/6J background. It is possible that a relatively low IGF-1 serum level in this mouse strain as compared to others (J.N.; unpublished) increases their frailty, especially in combination with other stressors such as CR or dwarfism. Of note, mice carrying mutations in the Ercc1 DNA repair gene show premature aging and a short life span, but also a reduced activity of IGF-1 signals (Niedernhofer et al., 2006). Other likely explanations for differential effects of CR on longevity of Ames dwarf mice in this as compared to the earlier study (Bartke et al., 2001) include differences in husbandry (particularly in age of weaning) and details of the CR protocol (40% until the age of 19 months vs. 30% throughout). Whatever the explanation for the increased mortality of the CR dwarf cohort, their survival benefit does appear to manifest as reduced spontaneous genome instability, albeit not as increased life span.

What could be the possible consequences of a reduced spontaneous mutation frequency in dwarf mice or mice subjected to CR? While it has been hypothesized that somatic mutation accumulation could be responsible for aging-related cell and tissue degeneration in general (Vijg, 2002), the most likely effect of the reduction in spontaneous mutation frequency in the CR and dwarf mice would be reduced cancer. Indeed, cancer is the phenotype most often found to be delayed and even reduced in the spectrum of aging-related pathology. Ikeno et al demonstrated that Ames dwarf mice show a delayed occurrence of neoplastic lesions compared with their normal siblings, a difference that likely contributed importantly to the extended life span in these mice (Ikeno et al., 2003). However, genome instability has also been implicated in other age-related diseases and the possibility cannot be ruled out that reduced spontaneous mutation rates also explain, in part, the delayed appearance of other forms of pathology in dwarf mice or CR conditions.

In summary, the results of this study provide evidence for delayed spontaneous mutation accumulation in dwarf mice and in mice subjected to caloric restriction. The protective effect was stronger in dwarf mice than in CR but seems to be present in multiple tissues. The magnitude of the effect was small, i.e., not more than an approximate 20% reduction in mutation frequency was observed in most cases, i.e., three tissues at two age levels. However, even such a small reduction in spontaneous mutagenesis could explain the reduction in spontaneous tumors that is a hallmark of both CR and dwarf mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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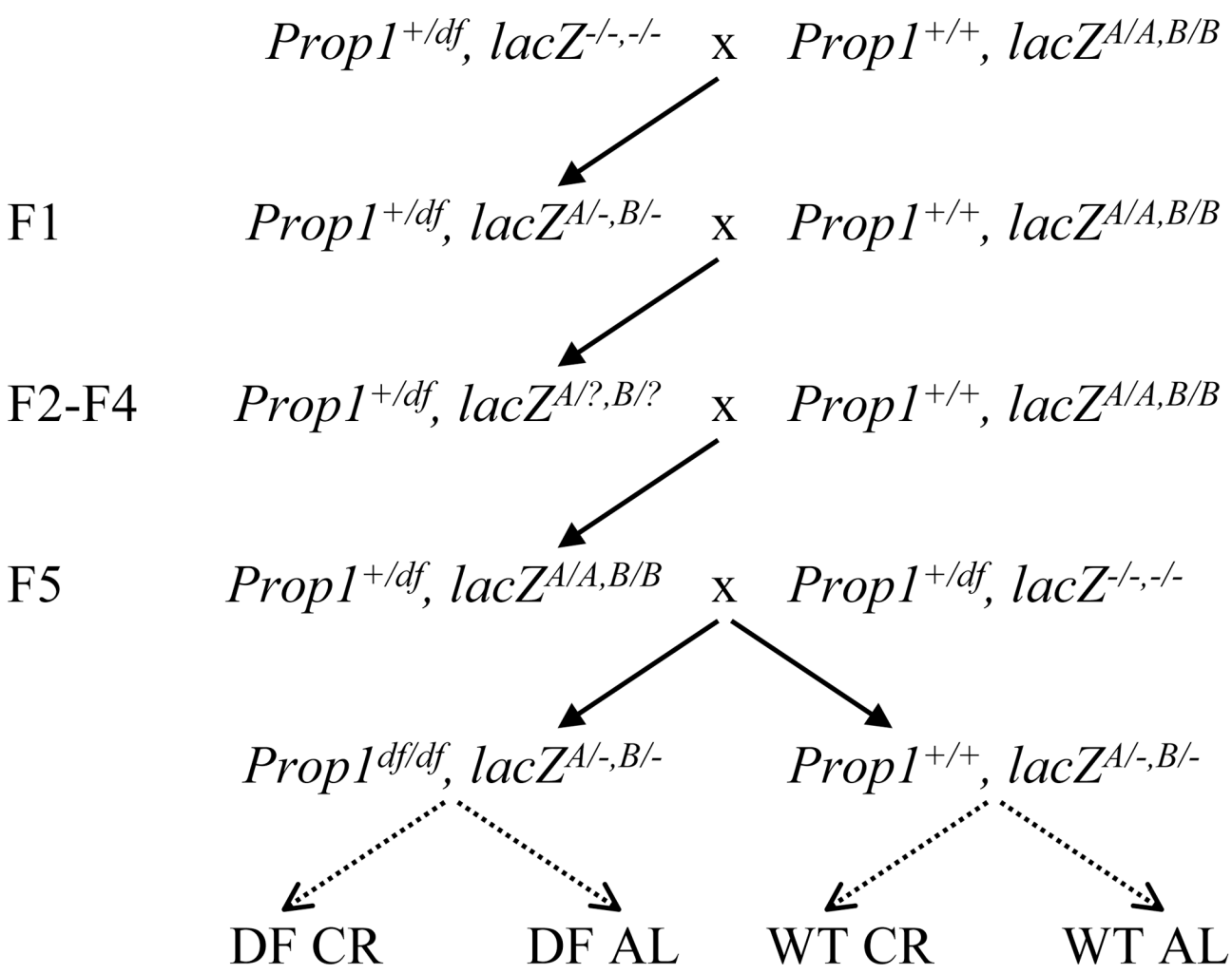

Figure 1.

Breeding scheme. Homozygous C57BL/6J *lacZ*-plasmid transgenic mice (*Prop1*$^{+}$/*Prop1*$^{+}$, *lacZ*$^{A/A,B/B}$), carrying two integration sites, one on chromosome 3 (A) and one on chromosome 4 (B) were crossed to heterozygous Ames dwarf (*Prop1*^{df}/*Prop1*$^{+}$, *lacZ*$^{-/-,-/-}$) mice. The heterozygous Ames dwarf F1 (*Prop1*^{df}/*Prop1*$^{+}$, *lacZ*$^{A/-,B/-}$) were backcrossed with homozygous C57BL/6J *lacZ*-plasmid transgenic mice (*Prop1*$^{+}$/*Prop1*$^{+}$, *lacZ*$^{A/A,B/B}$) up to F5 generation. The F5 heterozygous Ames were backcrossed to the original Ames background and the offspring were used in the experiment. Both homozygous dwarf (DF; *Prop1*^{df}/*Prop1*^{df}, *lacZ*$^{A/-,B/-}$) and wild-type (WT; *Prop1*$^{+}$/*Prop1*$^{+}$, *lacZ*$^{A/-,B/-}$) animals were *ad lib* (AL) fed or calorie-restricted (CR).

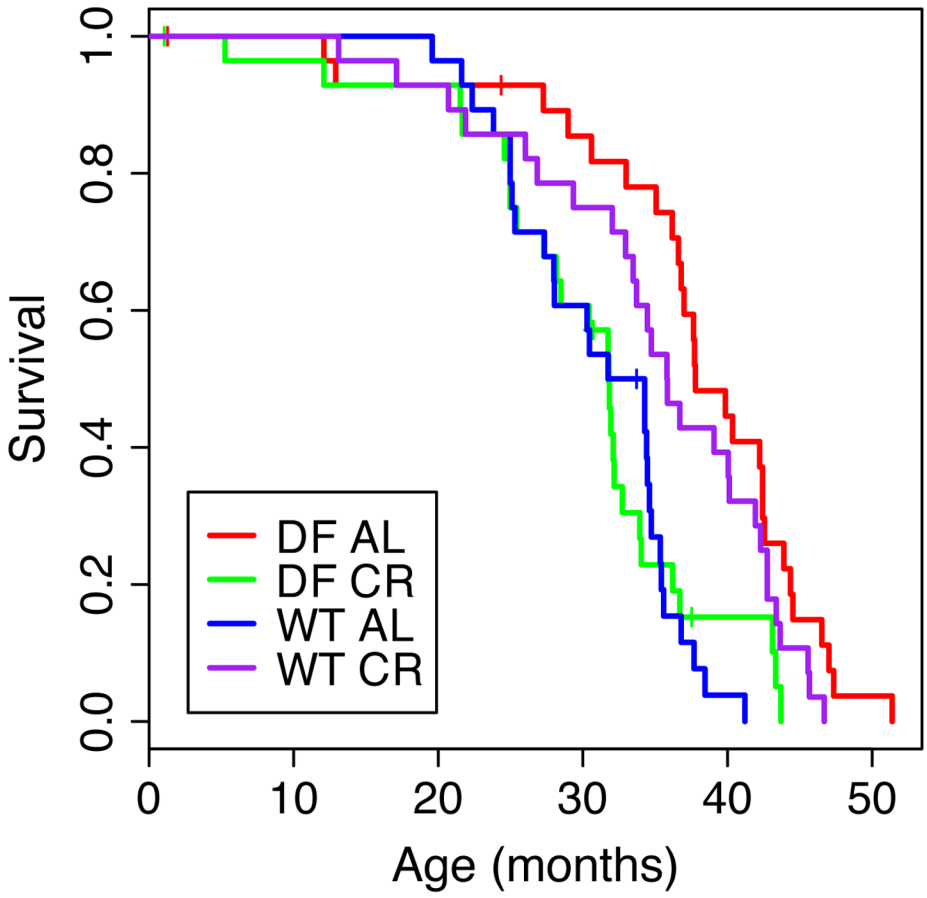

Figure 2.
Survival plots of Ames dwarf (DF) and wild-type (WT) mice fed *ad lib* (AL) or calorie restricted (CR).

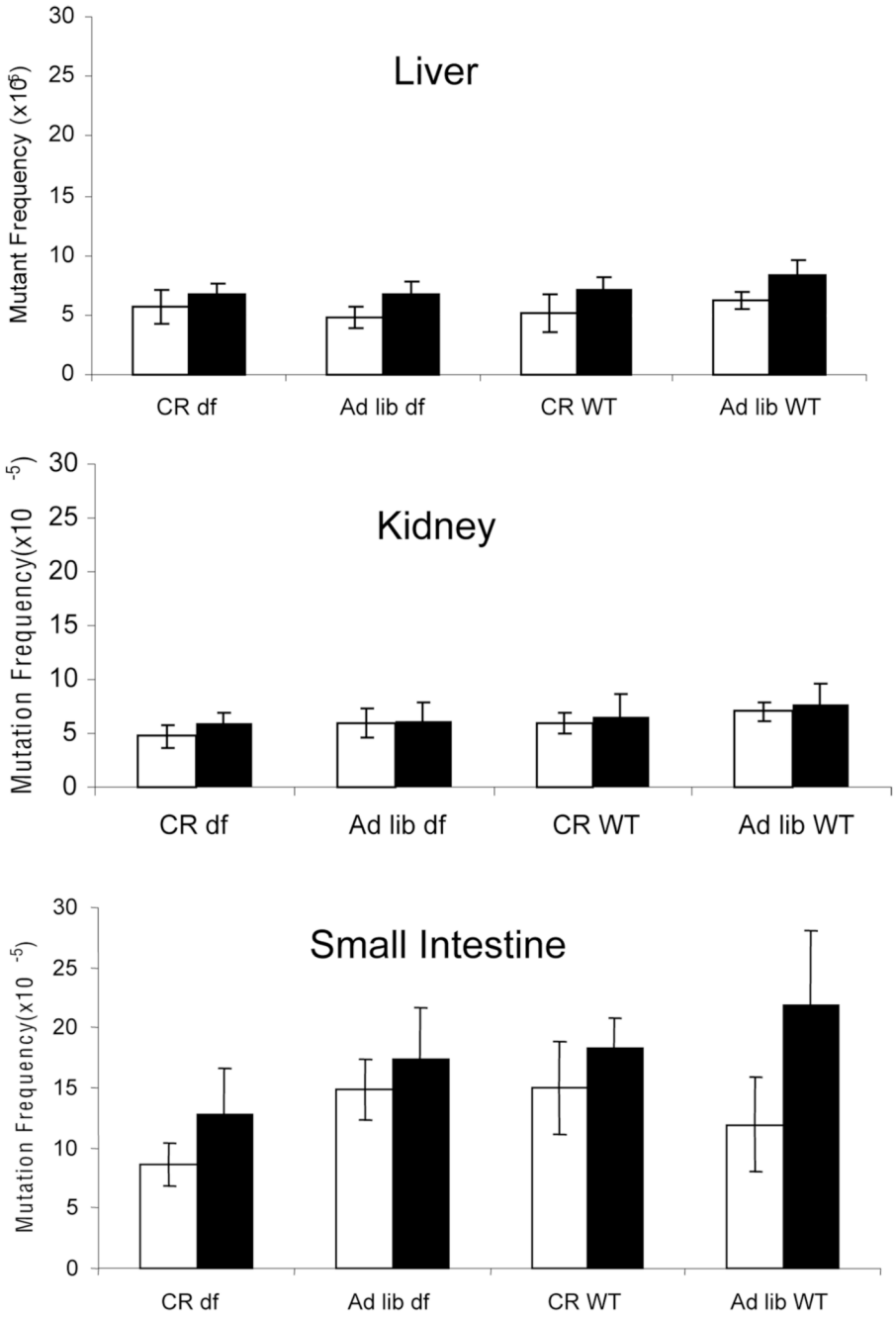

Figure 3.

Mutant frequency at the *lacZ* reporter locus of Ames dwarf mice and their wild-type littermate controls at 7-month old (white bars) and 15-month old (black bars) animals after caloric restriction or fed *ad lib*. Error bars represent 95% confidence intervals.

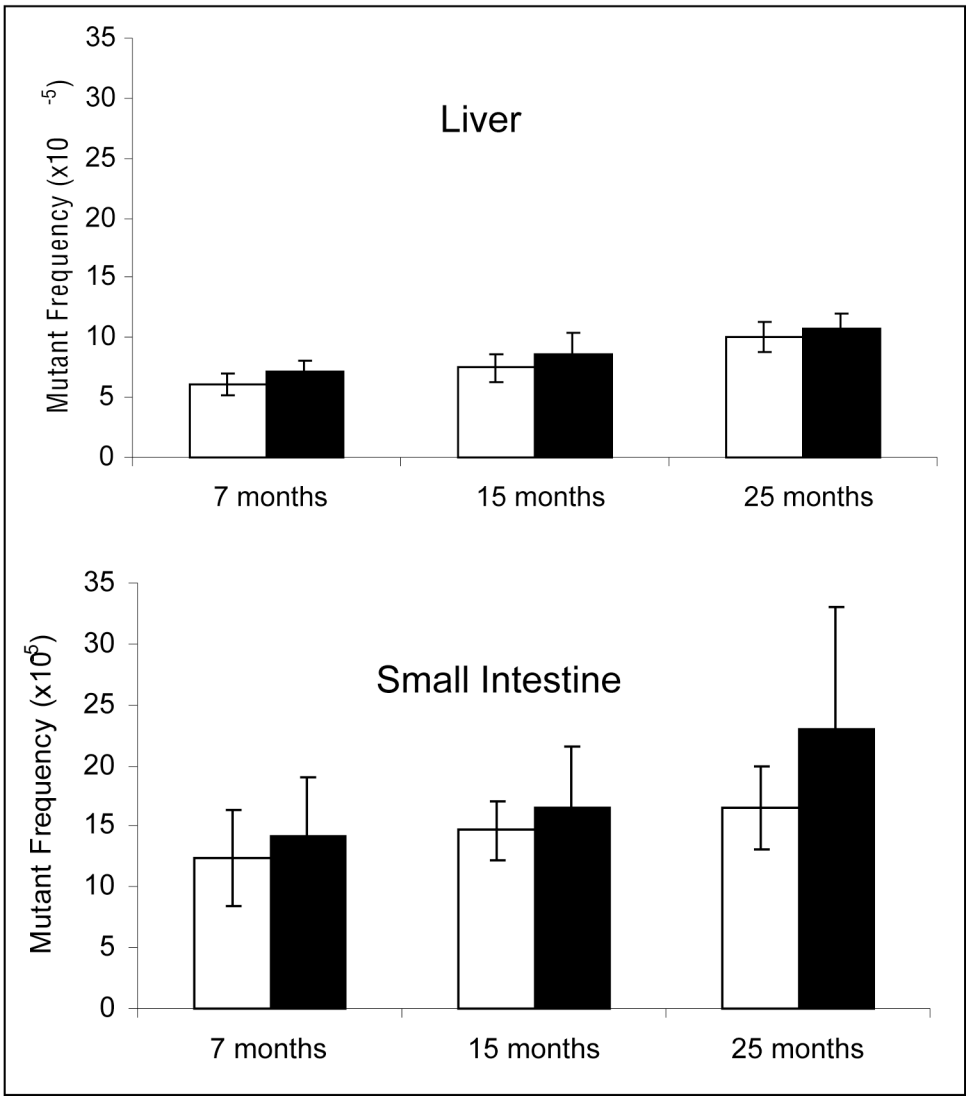

Figure 4.
Mutant frequency at the *lacZ* reporter locus of C57BL/6J mice at 7-, 15- and 25-months of age after caloric restriction (white bars) or fed *ad lib* (black bars). Error bars represent 95% confidence intervals.

Table 1

Ratio of mutant frequency observed in Ames dwarf mice to mutant frequency observed in wild-type mice by dietary condition, tissue and age; and ratio of mutant frequency observed in CR mice to mutant frequency observed in *Ad lib* mice by genotype, tissue and age

Tissue	Age (months)	Ratio Ames dwarf / Wild-type		Ratio CR / *Ad Lib*	
		Ad Lib	**CR**	**Ames dwarf**	**Wild-type**
Liver	7	**0.76** **	1.06	**1.20** †	**0.86** †
	15	**0.84** †	0.98	1.02	0.87
Kidney	7	**0.82** *	**0.79** *	**0.81** *	**0.83** †
	15	**0.77** **	0.93	0.99	**0.82** †
Intestine	7	**1.34** *	**0.57** **	**0.59** **	**1.37** **
	15	**0.79** †	**0.70** *	**0.74** *	0.83

Bold typeface indicates ratio different from 1.00 ($P \leq 0.10$)

** Ratio different from 1.00 ($P \leq 0.01$)

* Ratio different from 1.00 ($P \leq 0.05$)

† Ratio different from 1.00 ($P \leq 0.10$)

Table 2

Ratio of mutant frequency observed in *Ad lib* Ames dwarf mice to mutant frequency observed in CR wild-type mice by tissue and age; ratio of mutant frequency observed in CR Ames dwarf mice to mutant frequency observed in combined CR wild-type mice and *Ad lib* Ames dwarf mice by tissue and age; and ratio of mutant frequency observed in *Ad lib* wild-type mice to mutant frequency observed in combined CR wild-type mice and *Ad lib* Ames dwarf mice by tissue and age

Tissue	Age (months)	Ratio ***Ad Lib*** **Ames dwarf / CR Wild-type**	Ratio **CR Ames dwarf / (CR Wild-type and *Ad Lib* Ames dwarf)**	Ratio ***Ad Lib*** **Wild-type / (CR Wild-type and *Ad Lib* Ames dwarf)**
Liver	7	0.89	1.13	**1.23** **
	15	0.96	1.00	**1.17** †
Kidney	7	0.98	**0.80** **	**1.21** *
	15	0.94	0.97	**1.27** **
Intestine	7	0.97	**0.58** **	**0.74** **
	15	0.95	**0.72** **	**1.24** *

Bold typeface indicates ratio different from 1.00 ($P \leq 0.10$)

** Ratio different from 1.00 ($P \leq 0.01$)

* Ratio different from 1.00 ($P \leq 0.05$)

† Ratio different from 1.00 ($P \leq 0.10$)

Table 3

Ratio of mutant frequency observed in CR C57BL/6 mice to mutant frequency observed in *Ad lib* C57BL/6 mice by tissue and age

Tissue	Age (months)	Ratio CR C57BL/6 / *Ad Lib* C57BL/6
Liver	7	0.88
	15	**0.87** †
	25	0.96
Intestine	7	0.88
	15	0.88
	25	**0.72** *

Bold typeface indicates ratio different from 1.00 ($P \leq 0.10$)

** Ratio different from 1.00 ($P \leq 0.01$)

* Ratio different from 1.00 ($P \leq 0.05$)

† Ratio different from 1.00 ($P \leq 0.10$)