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Adult-onset calorie restriction and fasting delay spontaneous tumorigenesis in p53-deficient mice

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Heterozygous p53-deficient ($p53^{+/-}$) mice, a potential model for human Li-Fraumeni Syndrome, have one functional allele of the p53 tumor suppressor gene. These mice are prone to spontaneous neoplasms, most commonly sarcoma and lymphoma; the median time to death of p53+/– mice is 18 months. We have shown previously that juvenile-onset calorie restriction (CR) to 60% of *ad libitum* (AL) intake delays tumor development in young p53-null (–/–) mice by a p53-independent and insulin-like growth factor 1 (IGF-1)-related mechanism. To determine whether CR is effective when started in adult p53-deficient mice, and to compare chronic CR with an intermittent fasting regimen, male p53+/– mice (7–10 months old, 31–32 mice/group) were randomly assigned to the following regimens: (i) AL (AIN-76A diet), (ii) CR to 60% of AL intake or (iii) 1 day/week fast. Food availability on non-fasting days was controlled to prevent compensatory over feeding. Relative to the AL group, CR significantly delayed ($P = 0.001$) the onset of tumors in adult mice, whereas the 1 day/week fast caused a moderate delay ($P = 0.039$). Substantial variation in longevity and maximum body weight within treatments was not correlated with variation in growth characteristics of individual mice. In a separate group of p53+/– mice treated for 4 weeks (n = five mice per treatment), plasma IGF-1 levels in CR versus AL mice were reduced by 20% ($P < 0.01$) and leptin levels were reduced by 71% ($P < 0.01$); fasted mice had intermediate levels of leptin and IGF-1. Our findings that CR or a 1 day/week fast suppressed carcinogenesis—even when started late in life in mice predestined to develop tumors due to decreased p53 gene dosage—support efforts to identify suitable interventions influencing energy balance in humans as a tool for cancer prevention.

Introduction

Future progress in mechanism-based cancer prevention research may be facilitated by the use of animal models displaying specific genetic susceptibilities for cancer, such as p53-deficient mice (1). These mice are highly relevant to the study of human cancer and its prevention for two reasons. First, mutation of the p53 tumor suppressor gene is the most frequently observed genetic lesion in human cancer; over half of all human tumors examined to date have identifiable point mutations or deletions in p53 (2). Secondly, germline inactivation of one allele of p53 is a hallmark of Li-Fraumeni Syndrome, a familial cancer syndrome. Mice lacking one (p53+/–) or both (p53–/–) alleles of the p53 gene are viable but display the early onset of a variety of cancers, notably sarcomas and lymphomas (3,4). Median tumor-free survival times vary by p53 genotype from ~6 months in p53–/– mice to ~18 months in p53+/– mice and 26 months in p53+/+ mice. These results suggest that p53+/– mice could be a good model for humans susceptible to heritable forms of cancer resulting from decreased p53 gene dosage, such as individuals with Li-Fraumeni Syndrome (5). A recent review (1) has discussed the utility of p53-deficient mice for studying carcinogenesis and cancer prevention.

Calorie restriction (CR) is arguably the best-documented and most potent experimental manipulation for decreasing tumor development in rodents (6) and increasing longevity in diverse organisms (7). The mechanisms underlying the antitumorigenic effects of CR have not yet been elucidated (8,9). Nevertheless, there is great interest in translating the anticarcinogenic effects of CR into prevention strategies. It is hoped that further characterization of the mechanisms underlying the anticancer effects of CR will lead to new targets for cancer prevention (10). Using p53–/– mice as our model system, we have reported that CR [restricted to 60% of *ad libitum* (AL) calorie intake, begun after weaning and continued for the duration of the study] increases the latency of spontaneous tumor development (mostly lymphomas) by ~75%. This level of CR also decreases serum insulin-like growth factor (IGF)-1 and leptin levels, increases urinary corticosterone levels, significantly slows thymocyte cell cycle traverse and selectively induces apoptosis in immature (CD4/CD8+/+) thymocytes (1,11,12). Reductions in circulating IGF-1 levels appear to mediate many of the anticancer effects of CR (13,14).

Substantial efforts have also been made to determine how changes in energy balance throughout life influence carcinogenesis, as well as to define the level and pattern of CR required for beneficial effects on health (6). The incidence of obesity is increasing in much of the world, particularly in the US and other developed countries (15), and there is growing evidence that it is an important risk factor for several cancers (16). Thus, CR may have direct applications to cancer prevention strategies in human populations, and more work is needed to determine how the degree and pattern of CR influences carcinogenesis and other health outcomes.

While juvenile-onset CR almost uniformly increases longevity and reduces the initiation and progression of cancer, studies of adult-onset CR and fasting have had mixed results. Intermittent CR sometimes increases longevity (17) and some-

Abbreviations: AL, *ad libitum*; CR, calorie restricted; IGF-1, insulin-like growth factor-1; p53+/–, heterozygous p53-deficient.

times does not (18,19). Some of the negative results of intermittent CR experiments can be explained by lack of control over compensatory feeding. However, at least one report in rats failed to detect a protective effect of well-controlled cyclical CR on tumor incidence in DMBA-treated rats (19). Because fasting is a common practice in human populations, further work to determine its physiological and health effects is warranted. Similarly, there are a modest number of studies concerning adult-onset CR and not all of these results in increased longevity (6,20). A recent paper (21) reports microarray expression profiles of mice subjected to calorie restriction when young (7 months old) versus old (27 months old) for short (4 week) and long (7–27 month) time periods. Both short-term CR and late-onset CR reproduce some but not all the changes in gene expression associated with earlier onset and long duration CR.

In this paper we report the consequences of adult-onset CR (restricted to 60% of AL calorie intake) and a 1 day/week fasting regimen on tumorigenesis in p53+/– mice. Both adult-onset CR and our fasting regimen increased tumor-free survival in p53+/– mice. The data demonstrate that even when initiated well into adulthood, CR suppresses tumorigenesis in mice genetically susceptible to spontaneous tumor formation and suggest that a less severe and possibly more easily adopted dietary restriction regimen, in the form of a 1 day/week fast, may also be effective.

Materials and methods

Animals and diets

A group of 94 male p53+/– mice were produced and maintained at the MD Anderson Cancer Center Science Park Mouse Production Facility (Bastrop, TX). These mice were held on a 12:12 light:dark cycle at 24°C in polycarbonate cages (four mice per cage) with hardwood bedding and were fed Purina Rodent Chow (Richmond, IN) AL. When the median age of these mice reached 9 months (range 6.5–10.5) they were shipped to the NCI at Frederick (Frederick, MD), where the experiment was conducted. Further details of our animal husbandry methods are presented in refs (4) and (11).

On arrival mice were singly housed in quarantine for 6 weeks, where they received AIN-76A diet (Bio-Serve, Frenchtown, NJ) AL. The mice (average age 10.5 months) were then randomized to one of three treatments: (i) AL (controls fed AIN-76A diet AL), (ii) CR (individuals fed 60% of the average amount consumed by AL mice in the previous week) and (iii) a 1 day/week fasting regimen. All mice received distilled water AL. CR mice received a modified version of the AIN-76A diet formulated so that the reduction in energy intake was entirely due to a reduction in carbohydrates; intake of all other nutrients was equivalent to those in the control group (4). Fasting mice were restricted to eating the same average daily intake of AIN-76A diet as AL animals for 6 days/week; on the seventh day (Monday) they were deprived of food. This resulted in an average intake for the fasted group equal to 86% of the AL group and prevented compensatory over feeding following the fast day. Body weight and food consumption data were recorded weekly and dead or moribund mice were necropsied.

In a separate experiment, adult (8–9 months old) p53+/– mice were randomly assigned to the same control, CR and the 1 day/week fasting regimens (n = 4–5/group) for 4 weeks. This time point, well before the appearance of tumors, was chosen to avoid the possibility that the plasma markers of interest would be influenced by pathological changes associated with tumor development. Growth data from previous studies (4,11) with the CR regimen used in this study suggest that 4 weeks is sufficient time for the animals' body weight to stabilize. Plasma samples were collected on a Thursday morning (3 days post-fast) and plasma IGF-1 (rodent IGF-1 RIA, Diagnostic System Laboratories, Webster, TX) and leptin (Mouse leptin RIA, Linco, St Louis, MO) levels were measured.

Statistical analysis

Body mass and food consumption were analyzed using analysis of variance, tumor types were compared by χ^2 tests, and differences in survival were analyzed using Kaplan–Meier survival analysis to estimate mean longevity and a Cox proportional hazards model to compare the two treatments to the controls. Animals dying of non-cancer causes were treated as censored observations. We analyzed the relationship between individual variation in maximum body mass, rate of weight gain and longevity for animals in the control group using correlation analysis. Rate of weight gain (= growth rate) was determined using linear regression on body weight data from weeks 4 to 10 of the study. The slope of the regression equation is the rate of weight gained per week. Results for two outliers were excluded from this analysis.

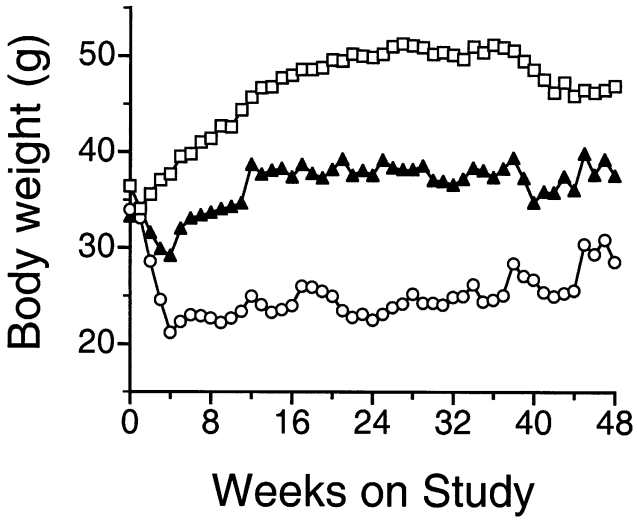

Fig. 1. Mean body weights of male p53+/– mice fed AIN-76A diet AL (squares), CR (circles) or subjected to a 1 day/week fast (triangles) (n = 31–32/group). The values represent the mean weekly body weights for each group through the first 48 weeks of study.

Results

Adult-onset CR and a 1 day/week fasting regimen significantly reduced weight gain in p53+/– mice (Figure 1). Mean body weight stabilized to ~50 g in AL mice, 27 g in CR mice and 38 g in fasted mice. Repeated measures ANOVA indicated that mean body weight for the mice from the different treatment groups differed overall ($P < 0.0001$). The body weights were directly proportional to the level of CR imposed with our diet regimens, and mice in the two treatment groups were notably sleeker and more active than mice in the AL group. Body weight showed more variability in the final 6 months of the study as more of the surviving mice developed tumors. Food consumption averaged ~30 g/week in AL mice. Based on this level of food consumption, CR animals received ~18 g/week of food and fasting animals received ~26 g/week.

Both CR and a 1 day/week fast increased longevity in adult p53+/– mice (Figure 2). Mean longevity (E_x) was 313 [standard deviation (SD) = 17] days for AL mice, 388 (SD = 23) days for CR mice and 357 (SD = 23) days for fasted mice: a 24% increase in the CR group compared with AL mice and a 14% increase in fasted mice. Cox proportional hazards analysis (one-tailed) indicated that CR potently lengthened tumor-free survival ($P = 0.001$). In addition, a 1 day/week fasting regimen also lengthened survival ($P = 0.039$), although to a lesser extent than CR.

Causes of death were similar among the three treatment groups (Table I). Most (77–88%) deaths were due to neoplasms, with histiocytic sarcomas the most common type. The remaining deaths were due to a variety of causes such as infection, polyarteritis or urogenital syndrome. None of the differences in cause of death between groups were statistically significant. Multiple tumor burden, arising from different tissues, was 40% (13/32) in the AL-fed mice. Relative to

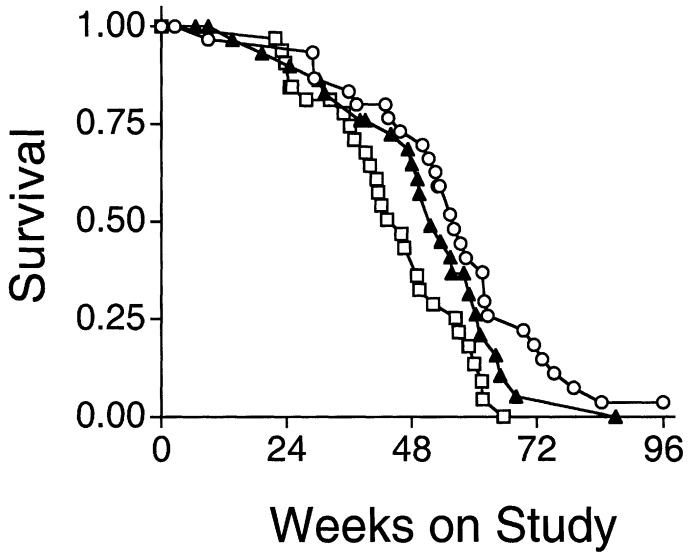

Fig. 2. Kaplan–Meier survival curves by week of study for male p53+/– mice fed AIN-76A diet AL (squares), CR (circles) or subjected to a 1 day/week fast (triangles) (n = 31–32/group).

Table I. Causes of death among p53+/– mice fed AL, CR to 60% of the AL group or fasted for 1 day/week

	AL (n = 32)	CR (n = 31)	1 day fast (n = 31)
Cause of death, number of mice (% of group)			
Neoplasm	28 (88)	27 (87)	24 (77)
Other/unknown	4 (12)	4 (13)	7 (23)
Cause of death, number of mice (% of group)			
Hematopoietic neoplasm (HN)			
Histiocytic sarcoma	13 (41)	13 (42)	10 (32)
Other HN[a]	4 (12)	2 (6)	5 (16)
Sarcoma[b]	4 (12)	4 (13)	6 (19)
Carcinoma[c]	6 (19)	7 (23)	2 (6)
Other neoplasm[d]	1 (3)	1 (3)	1 (3)
Other[e]/unknown	4 (12)	3 (10)	7 (23)
Terminal death[f]	0	1 (3)	0

[a]Lymphoblastic lymphoma; follicular center cell lymphoma; myelogenous leukemia.
[b]Hemangiosarcoma; osteosarcoma; sarcoma, subcutis/muscle.
[c]Alveolar carcinoma, lung; hepatocellular carcinoma; preputial gland carcinoma; squamous cell carcinoma, skin; squamous cell carcinoma, Zymbal's gland.
[d]Astrocyoma, brain; melanoma, eye; olfactory neuroblastoma.
[e]Infection/septicemia; male urogenital syndrome; polyarteritis nodosa; biliary/pancreatic cyst; choke (dilated esophagus).
[f]At 672 days on study (final genotyping confirmed p53+/– genotype).

the AL group, multiple tumor burden was lower in the CR group (7/31; 23%; P = 0.10) and fasted group (8/31; 26%; P = 0.16). Similarly, the proportion of mice in each group with malignant tumors that metastasized, relative to the AL group (24/32; 75%), was lower in the CR group (17/31; 55%; P = 0.08) and the fasted group (19/31; 61%; P = 0.18).

In a separate group of p53+/– mice either fed AL or subjected to CR or a 1 day/week fast for 4 weeks (Table II), CR (relative to AL feeding) significantly reduced plasma leptin (4.8 versus 16.9 ng/ml; $P < 0.05$) and IGF-1 levels (412 versus 514 ng/ml; $P < 0.05$). In addition, plasma leptin (14.7 ng/ml) and IGF-1 (473 ng/ml) levels in fasted individuals were lower than those in the AL mice (Table II), although the

Table II. Effects of CR and a 1 day/week fasting regimen on mean (± SD) plasma leptin and IGF-1 levels

	Leptin[a] (ng/ml)	n	IGF-1[a] (ng/ml)	n
AL	16.9[a] (9.7)	5	514[a] (54)	5
CR	4.8[b] (3.9)	5	412[b] (54)	5
1 day fast	14.7[a] (7.4)	4	473[a] (121)	5

Means followed by different superscripts are significantly different ($P < 0.05$).

effects of fasting on leptin and IGF-1 were not statistically significant.

We also found substantial variation in the rate of weight gain, longevity and maximum body weight within each of the three treatment groups (Figure 3A and B). Overall, longevity and growth rate had a significant negative correlation (r = –0.27, P = 0.009, n = 92). However, the correlation between longevity and growth was not significant within the AL (r = –0.13, P =0.47, n =32), CR (r –0.13, P = 0.48, n = 29) or fasting (r = 0.24, P = 0.179, n = 31) treatments. Growth characteristics as well as growth rate differed among the three treatments. More than 80% of the mice in the AL and fasting treatments had significant and positive regression coefficients relating age and body size during weeks 4–10 of the study, whereas only 23% of the CR mice showed this pattern of regular growth. Maximum weight and longevity within treatments were not correlated in the complete data set (r = –0.10, P = 0.33), but there was some evidence for a positive association between maximum weight and longevity (Figure 3B). We found positive correlations between weight and longevity in all three treatments (AL: r = 0.21, P = 0.24; CR: r = 0.26, P = 0.17; fasting: r = 0.40, P =0.03), but the correlation was only significant in the fasting group. Lastly, similar results were obtained in analyses that included only individuals with cancer as the cause of death.

Discussion

p53+/– mice, with a single functional allele of the p53 tumor suppressor gene, are analogous to humans susceptible to heritable forms of cancer caused by decreased p53 gene dosage, such as individuals with Li-Fraumeni Syndrome. Male p53+/– mice spontaneously develop fatal tumors (most commonly sarcomas and hematopoietic neoplasms, which are also common in Li-Fraumeni patients) in middle age. The median time to death in these mice is ~18 months of age. We have shown previously that CR (restriction to 60% of AL calorie intake) started after weaning and continued throughout life, significantly delays tumor development in male p53–/– mice, all of which otherwise spontaneously develop tumors and die before 10 months of age (4,11).

To determine whether CR is effective in offsetting p53-deficiency even if started later in life, and to compare CR with an intermittent fasting regimen, we randomized a group of mature male p53+/– mice, which had been fed AL for the first 10.5 months of their life, into three groups. First, to either continue AL feeding; secondly, to switch to a CR regimen (60% of AL intake); or thirdly, to switch to a 1 day/week fasting regimen. The adult-onset CR mice, relative to the AL mice, displayed a 25% increase in mean longevity (P = 0.001). The 1 day/week fast with controlled re-feeding, resulting in a 14% decrease in weekly food intake relative to the AL mice,

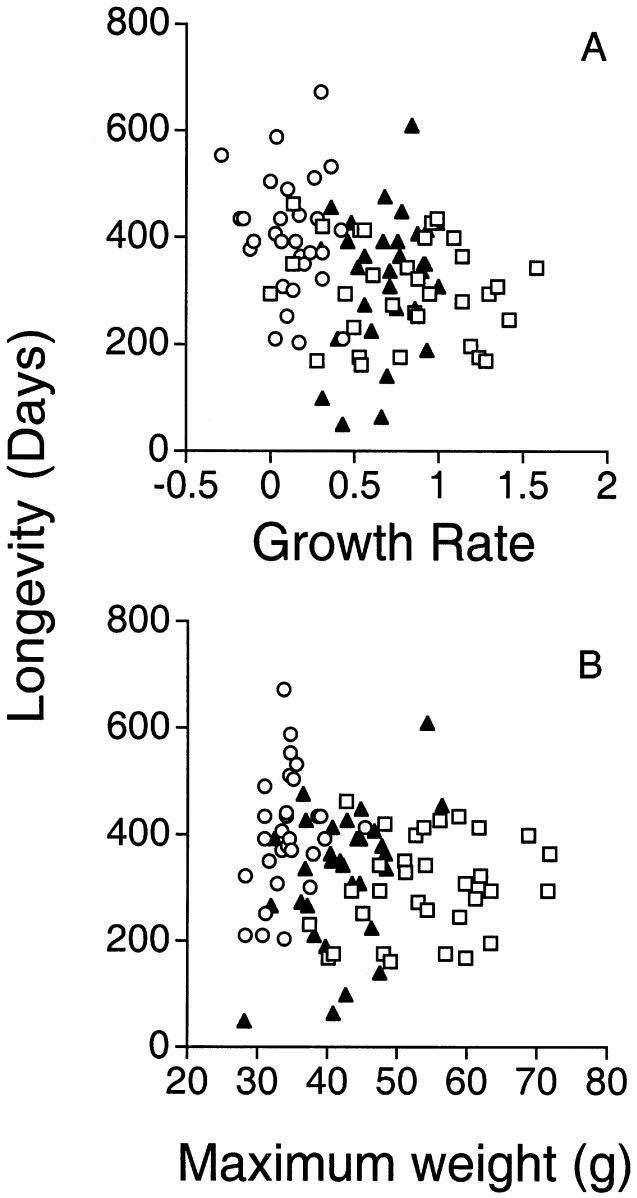

Fig. 3. Scatter plots for male p53+/– mice fed AIN-76A diet AL (squares), CR (circles) or subjected to a 1 day/week fast (triangles) displaying the relationships between: (**A**) growth rate (g/mouse/week) and longevity (days on study); and (**B**) maximum body weight (highest weight achieved for each mouse over the course of the study, in grams) and longevity (days on study). Growth rate was determined using linear regression on body weight data from weeks 4 to 10 on study.

also moderately increased longevity in this study ($P = 0.037$). These findings are relevant to humans for two reasons: (i) CR or interventions mimicking CR that occur during growth and maturation could have adverse consequences for development and (ii) older adults are more likely to adopt healthy behaviors. Evidence that adult-onset CR—even at modest levels of restriction such as a 1 day/week fast—may help prevent cancer will lend further support to population-level interventions aimed at altering energy balance. Modest changes in energy intake and body weight have significant benefits for cardiovascular disease and diabetes (22). Achieving a moderate reduction in energy intake in middle-aged adults, such as through a 1 day/week fasting regimen, could be a much less daunting task than a 40% reduction in intake from adolescence onward.

Our findings are consistent with previous reports of the protective effects of adult-onset CR on experimental tumor development in mice (20,23–26). The present study extends this past work by demonstrating that adult-onset CR is effective at suppressing tumorigenesis in mice genetically predestined to tumor development due to decreased p53 gene dosage. Past studies on the effects of fasting in mice and rats have reported mixed results (6). Some of this variation may be explained by differences in the extent to which various authors have controlled for compensatory feeding. However, it seems probable that there are also differences among genotypes in the relationships between food intake, growth characteristics and longevity (25,26). Overall, our results and past work support the view that the response to CR follows a dose–response relationship (stronger effects on growth, longevity and tumor suppression with increasing severity of restriction) rather than an abrupt threshold (6).

The observation that plasma levels of leptin and IGF-1 were significantly lower in CR mice relative to AL mice and were intermediate in mice undergoing the 1 day/week fasting regimen is also consistent with the notion of a dose–response relationship between energy balance and cancer. IGF-1 is a 70 amino acid polypeptide that shares ~50% homology with insulin (9), while leptin is the product of the *ob* gene, which is known to regulate appetite and energy expenditure (27). Reductions in leptin and IGF-1 levels have been observed in other studies of early-onset CR (27,28). In addition, the enhancement of apoptosis and suppression of proliferation in altered hepatic foci in rats in response to short-term fasting has been associated with decreased liver IGF-1 mRNA expression (29). Furthermore, IGF-1 infusion in young CR mice, in amounts which restored serum IGF-1 to AL levels in CR mice, has been shown to increase the rate of tumor progression independent of body weight in p53+/– mice treated with the bladder carcinogen *p*-cresidine (14). Higher plasma levels of IGF-1 have been linked to increased risk of several cancers in humans (9), while increased plasma leptin levels are associated with prostate cancer progression (30).

Three distinct functions of IGF-1 make it a likely factor in controlling tumor development in response to CR and fasting: (i) control of long bone growth or stature; (ii) regulation of anabolic signals to organs and tissues; and (iii) stimulation of mitogenesis and/or inhibition of apoptosis in a plethora of normal and neoplastic cell types (9). We, and others, are currently testing the hypothesis that the reduction in IGF-1 levels is a key mediator of many of the effects of CR on cell proliferation, apoptosis, immune response and gene expression. The interactions between IGF-1, leptin and other hormones, such as glucocorticoids, are also being explored. The finding in the present study that IGF-1 levels are reduced by adult-onset CR suggests that similar mechanisms are responsible for the effects of CR on longevity in adult and juvenile mice. Future studies might profitably explore the influence of IGF-1 treatment on tumor initiation and progression in adult mice, in mice not subject to CR, and in mice treated with other perturbations of energy balance such as fasting or exercise. Such experiments may help link results concerning animal models to patterns of cancer incidence and progression in human populations.

We found substantial variation in the rate of weight gain, maximum body weight attained and longevity within each of

the three treatment groups (Figure 3A and B). Longevity ranged from 161 to 462 days in AL mice, from 63 to 672 days in CR mice and from 49 to 609 days in the 1 day/week fasting group. Overall, survival and growth rate had a significant negative correlation ($r = –0.27$, $P = 0.009$, $n = 92$). This reflects the fact that the mice subjected to the CR and 1 day/week fasting regimens grew, on average, more slowly but lived longer than AL animals. However, there was no evidence for a correlation between survival and growth rate within treatments despite substantial variation in growth and longevity. Furthermore, maximum weight and longevity were not significantly correlated ($r = –0.10$, $P = 0.33$, $n = 92$). Thus, variation in growth characteristics does not account for within-treatment variation in longevity.

Results of past work have been mixed with some studies reporting that little of the residual variation in longevity found in studies of CR can be explained by individual differences in growth characteristics (6,31). Other studies have reported positive correlations between adult body weight and survival in CR mice (32). Exploration of different components of energy balance, including metabolic rate, physical activity and growth characteristics over the whole life course, and as mentioned above, possible hormonal mediators such as IGF-1, leptin and glucocorticoids, may explain some of this residual variation in longevity. For example, Ingram *et al*. (26) report significant associations between longevity of 29-month-old male C57BL/6J mice and measures of physical activity (among other variables). However, regression models suggest that $<50\%$ of the variation in longevity was explained by the physiological and behavioral variables they selected. Thus, much residual variation in longevity found in genetically similar cohorts might represent stochastic (arising from chance) environmental variation at the organism, cellular or molecular levels (33), rather than the predictable consequences of physiological differences at study onset. This implies that there may be limits to the extent to which we can explain individual variation in cancer susceptibility.

In conclusion, despite a long history of studies of CR and longevity, we still do not have a complete understanding of how energy balance (including the pattern of food intake), physical activity, body composition and routine energy expenditures interact to influence tumorigenesis over the entire life course. Progress in this field may be hastened by the use of animal models displaying specific and highly relevant genetic susceptibilities for cancer, such as p53+/– mice, which are allowing increasingly detailed analysis of factors influencing tumorigenesis. This present paper demonstrates that adult-onset CR and a 1 day/week fast can increase longevity in mice genetically predestined to develop fatal tumors due to p53 deficiency. This is encouraging both from the perspective of efforts to influence energy balance in adults through programs designed to change behavior and from the perspective of those aiming to better understand and translate the anticancer effects of CR into mechanism-based cancer prevention strategies.

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