Supplemental Text and Figures:

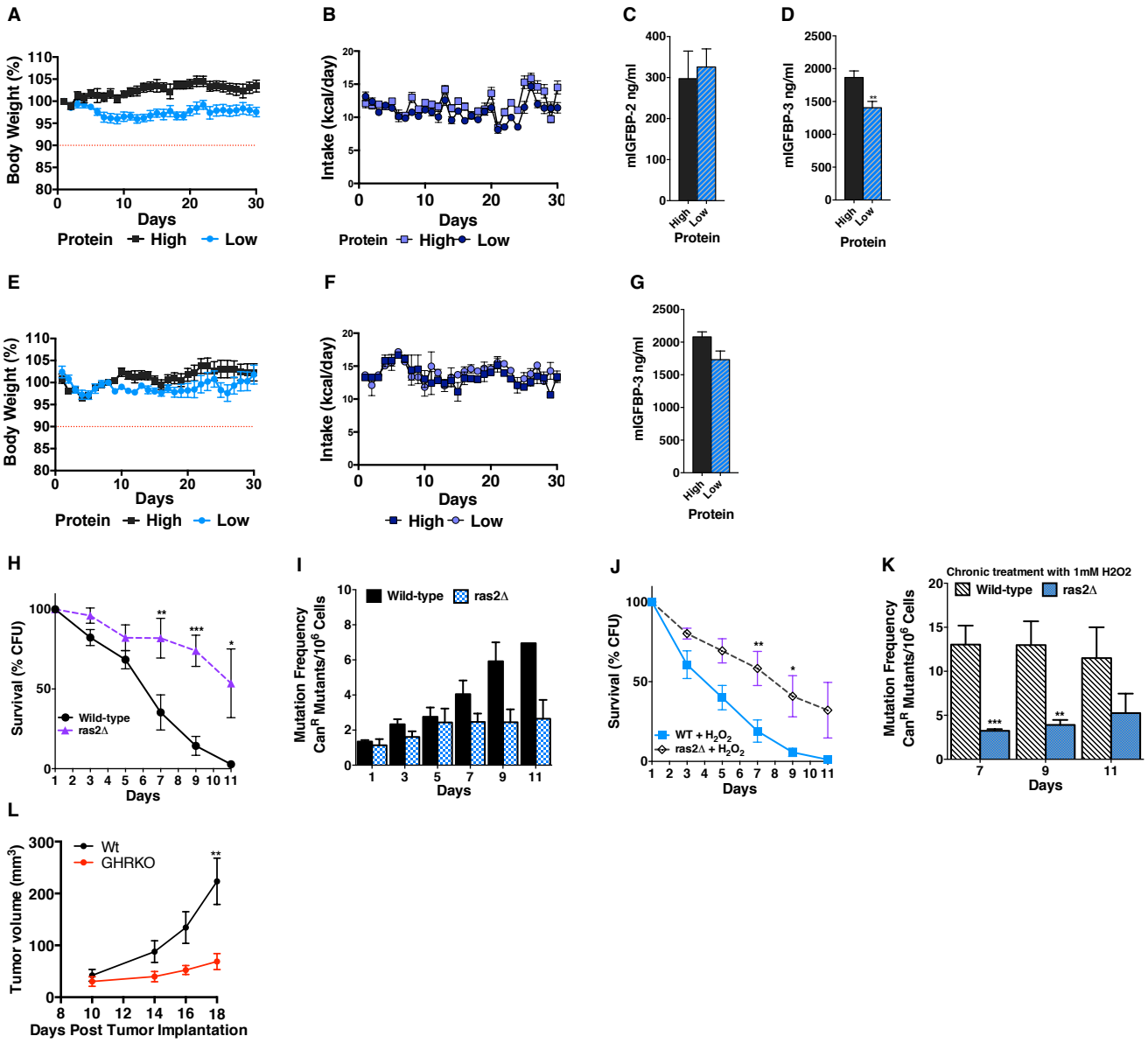

Fig. S1

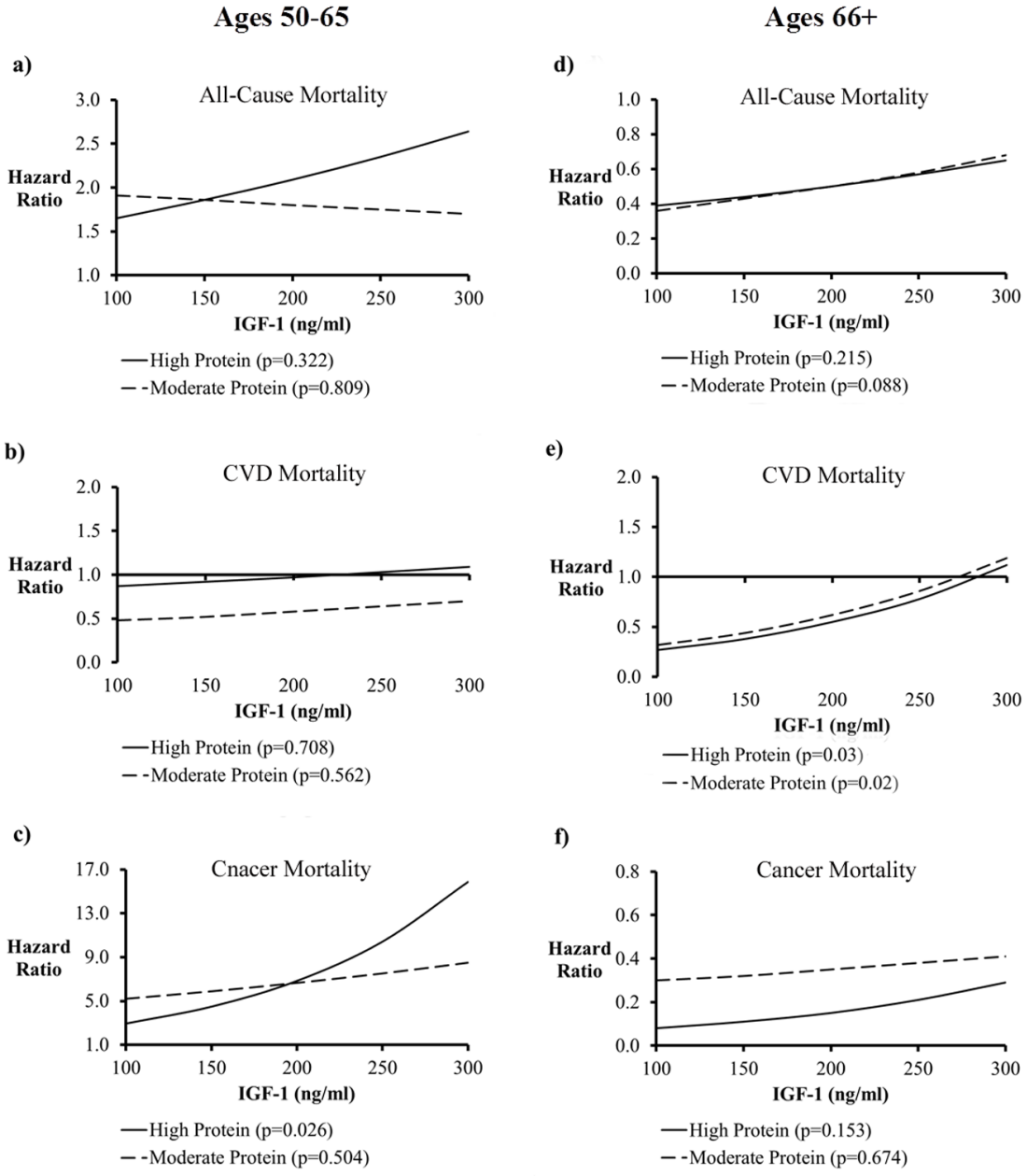
Ages 50-65
Ages 66+
a)
All-Cause Mortality
Hazard Ratio
3.0
2.5
2.0
1.5
1.0
100
150
200
250
300
IGF-1 (ng/ml)
High Protein (p=0.322)
Moderate Protein (p=0.809)
d)
All-Cause Mortality
Hazard Ratio
1.0
0.8
0.6
0.4
0.2
0.0
100
150
200
250
300
IGF-1 (ng/ml)
High Protein (p=0.215)
Moderate Protein (p=0.088)
b)
CVD Mortality
Hazard Ratio
2.0
1.5
1.0
0.5
0.0
100
150
200
250
300
IGF-1 (ng/ml)
High Protein (p=0.708)
Moderate Protein (p=0.562)
e)
CVD Mortality
Hazard Ratio
2.0
1.5
1.0
0.5
0.0
100
150
200
250
300
IGF-1 (ng/ml)
High Protein (p=0.03)
Moderate Protein (p=0.02)
c)
Cnacer Mortality
Hazard Ratio
17.0
13.0
9.0
5.0
1.0
100
150
200
250
300
IGF-1 (ng/ml)
High Protein (p=0.026)
Moderate Protein (p=0.504)
f)
Cancer Mortality
Hazard Ratio
0.8
0.6
0.4
0.2
0.0
100
150
200
250
300
IGF-1 (ng/ml)
High Protein (p=0.153)
Moderate Protein (p=0.674)

Fig. S2

Supplemental Figure Legends:

Figure S1. Figure S1A-G, related to Figure 3A-D, F-I. Figure S1H-K, related to Figure 3L-P Figure S1L, related to Figure 3E. (A) 30-day body weight of 18-week-old male C57BL/6 mice fed isocaloric diets varying in protein content either high (18%) or low (4%). **(B)** 30-day food intake in kcal/day of 18-week-old male C57BL/6 mice fed isocaloric diets varying in protein content either high (18%) or low (4%). **(C)** IGFBP-2 at day 16 in male (18wk) C57BL/6 mice fed either a high protein (n=10) or low protein (n=10) diet. **(D)** IGFBP-3 at day 16 in male (18wk) C57BL/6 mice fed either a high protein (n=10) or low protein (n=10) diet. **(E)** 30-day body weight of 12-week-old female BALB/c mice fed isocaloric diets varying in protein content either high (18%) or low (7%). **(F)** 30-day food intake in kcal/day of 12-week-old female BALB/c mice fed isocaloric diets varying in protein content either high (18%) or low (7%). **(G)** IGFBP-3 at day 16 in 12-week-old female BALB/c mice fed either a high protein (n=10) or low protein (n=10) diet.

(H) Yeast chronological survival and **(I)** attenuated age-dependent genomic instability shown as mutation frequency in the CAN1 gene (measured as Can^r mutants/10^6 cells) in wild-type (DBY746) compared to ras2Δ mutants. **(J)** Chronological survival of wild-type and ras2Δ mutants chronically treated with 1mM H_2O_2. **(K)** Lack of Ras2 protects against oxidative stress-induced genomic instability (mutation frequency Can^r). **(L)** Tumor volume progression of B16 melanoma in 10-month old female GHRKO mice (n=5) *vs* age-matched wild-type controls (Wt; n=13). In all graphs, data points represent the mean of the biological replicates ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001; t-test; ANOVA.

Figure S2. IGF-1 Moderates the Association between Protein Consumption and Mortality. Related to Table 1. Based on results from Cox Proportional Hazard Models of the interaction between protein and IGF-1 on mortality, predicted Hazard Ratios were calculated by IGF-1 for both moderate and high protein groups relative to the low protein group. No significant interactions between protein and IGF-1 were found for all-cause (a) or CVD mortality (b) in the 50-65 year old age group. However, the interaction for IGF-1 and high vs low protein was significant (p=.026) for cancer mortality (c) for subjects ages 50-65. Results show that for every 10 ng/ml increase in IGF-1 the mortality risk of cancer increases for the high protein group relative to the low protein group by 9% ($HR_{\text{high protein x IGF-1}}$: 1.09; 95% CI: 1.01-1.17). The interaction between protein and IGF-1 was significant for respondents ages 66+ only for CVD mortality. Those with high or moderate protein diets had a reduced risk of CVD if IGF-1 was also low; however, as IGF-1 increased there was no benefit.

Table S1: Sample Characteristics. Related to Table 1.

	Full-Samples N=(6,381)	Low Protein (N=437)	Moderate Protein (N=4,798)	High Protein (N=1,146)
Age	64.8 (10.0)	65.3 (10.1)	64.8 (10.0)	64.5 (9.8)
Female	55.4	56.3	55.0	57.0
White	85.1	81.6	86.5	80.3
Black	8.7	12.3	7.9	10.9
Hispanic	6.2	6.1	5.6	8.8
Education	11.5 (3.6)	11.1 (3.3)	11.6 (3.5)	11.0 (3.8)
Waist Circumference (cm)	96.9 (13.2)	96.5 (13.5)	96.8 (13.2)	97.5 (13.0)
Current Smoker	19.1	21.8	19.1	18.2
Former Smoker	38.0	39.8	37.9	37.8
History of MI	9.1	7.9	8.9	10.2
History of Cancer	7.3	11.7	7.5	5.0
History of Diabetes	10.9	2.6	10.3	17.0
Tried to lose weight	38.2	37.5	37.0	43.9
Changed diet (health reason)	23.1	15.0	22.4	29.3
% Calories from Animal Protein	10.6 (5.1)	4.1 (1.8)	9.5 (3.1)	18.3 (4.9)
% Calories from Protein	16.0 (4.8)	8.5 (1.4)	14.9 (2.6)	23.7 (4.1)
% Calories from Fat	33.0 (9.6)	31.9 (11.2)	33.6(9.3)	31.0 (10.2)
% Calories from Carbs	50.7 (11.6)	56.6 (13.5)	51.3 (11.0)	45.4 (11.4)
Total Calories	1,822.9 (832.2)	1965.6 (1072.8)	1862.5 (807.4)	1593.6 (796.2)
24-hr Recall More	1.6	1.8	1.4	2.1
24-hr Recall Usual	93.3	83.0	93.9	94.6
24-hr Recall Less	5.1	15.2	4.7	3.3
Died (All-Cause)	40.4	42.9	39.6	42.9
Died (CVD)	18.7	21.3	18.0	20.7
Died (Cancer)	9.9	9.8	10.1	9.0
Died (Diabetes)	1.0	0.2	0.9	2.0
Person Years	83,308	5,183	63,661	14,464

BMI: Body Mass Index; MI: Myocardial Infarction; 24-hr Recall (More/Usual/Less): Food reported during 24-hour recall is (more than/same as/less than) subject's normal diet; CVD: Cardiovascular disease

Table S2: Association between Protein Intake and Mortality (N=6,381). Related to Figure 1.

	Hazard Ratio (95% CI)			
	All-Cause Mortality	CVD Mortality	Cancer Mortality	Diabetes Mortality
Protein (% kcal)				
<10 (n=436)	*reference*	*reference*	*reference*	*Reference*
10-19.9 (n=4,800)	0.90 (0.74-1.11)	0.82 (0.61-1.11)	0.98 (0.66-1.45)	3.23 (1.02-10.20)
20+ (n=1,145)	0.93 (0.74-1.19)	0.88 (0.63-1.22)	0.89 (0.56-1.44)	5.51 (1.69-17.99)

Covariates: Age, sex, race/ethnicity, education, waist circumference, smoking, chronic conditions (diabetes, cancer, MI), trying to lose in last year weight, diet changed in last year, reported intake representative of typical diet, total calories

Table S3: The Influence of IGF-1 on the Association between Mortality and Protein Intake (N=2,253). Related to Table 1.

	Hazard Ratio (95% CI)			
	Ages 50-65 (N=1,125)		Ages 66+ (N=1,128)	
	Model 1	Model 2	Model 1	Model 1
All-Cause Mortality				
Low Protein	Reference	Reference	Reference	Reference
Moderate Protein	1.73 (0.91-3.30)	1.69 (0.90-3.20)	0.62 (0.41-0.94)	0.62 (0.40-0.94)
High Protein	2.83 (1.39-5.76)	2.71 (1.34-5.47)	0.59 (0.36-0.95)	0.59 (0.37-0.95)
IGF-1 (10 ng/ml)		1.01 (0.99-1.04)		1.00 (0.98-1.01)
CVD Mortality				
Low Protein	Reference	Reference	Reference	Reference
Moderate Protein	0.76 (0.33-1.74)	0.71 (0.33-1.54)	0.77 (0.43-1.38)	0.77 (0.43-1.37)
High Protein	1.29 (0.49-3.40)	1.03 (0.39-2.75)	0.69 (0.36-1.34)	0.70 (0.36-1.35)
IGF-1 (10 ng/ml)		1.04 (1.01-1.07)		0.99 (0.97-1.00)
Cancer Mortality				
Low Protein	Reference	Reference	Reference	Reference
Moderate Protein	6.91 (1.56-30.68)	6.91 (1.56-30.72)	0.41 (0.18-0.92)	0.39 (0.18-0.86)
High Protein	13.05 (2.77-61.40)	13.09 (2.80-61.13)	0.24 (0.08-0.68)	0.23 (0.08-0.67)
IGF-1 (10 ng/ml)		1.00 (0.96-1.03)		0.97 (0.94-0.99)

Model 1: (Baseline Model) Adjusted for age, sex, race/ethnicity, education, waist circumference, smoking, chronic conditions (diabetes, cancer, MI), trying to lose in last year weight, diet changed in last year, reported intake representative of typical diet, total calories.
Model 2: Adjusted for Covariates and IGF-1

Table S4: Hazard Ratios for the Interaction between Protein and IGF-1 on Mortality. Related to Table 1.

	Ages 50-65		Ages 66+	
	Hazard Ratio	P-Value	Hazard Ratio	P-Value
All-Cause				
Moderate	2.03	0.349	0.27	0.008
High	1.30	0.722	0.30	0.040
IGF-1	1.01	0.738	0.97	0.077
Moderate Protein x IGF-1	**0.99**	**0.809**	**1.03**	**0.088**
High Protein x IGF-1	**1.02**	**0.322**	**1.03**	**0.215**
CVD				
Moderate	0.39	0.379	0.16	0.008
High	0.77	0.802	0.13	0.020
IGF-1	1.02	0.520	0.93	0.007
Moderate Protein x IGF-1	**1.02**	**0.562**	**1.07**	**0.020**
High Protein x IGF-1	**1.01**	**0.708**	**1.07**	**0.031**
Cancer				
Moderate	4.08	0.264	0.25	0.201
High	1.27	0.858	0.04	0.018
IGF-1	0.96	0.194	0.96	0.243
Moderate Protein x IGF-1	**1.02**	**0.504**	**1.02**	**0.674**
High Protein x IGF-1	**1.09**	**0.026**	**1.06**	**0.153**

All models included age, sex, race/ethnicity, education, waist circumference, smoking, chronic conditions (diabetes, cancer, MI), trying to lose in last year weight, diet changed in last year, reported intake representative of typical diet, total calories.

Table S5: Influence of Animal and Vegetable Protein on the Associations between Mortality and Protein intake. Related to Table 1.

	Hazard Ration *(95% CI)*					
	Ages 50-65 (N=3,039)			Ages 66+ (N=3,342)		
	Model 1	Model4	Model 5	Model 1	Model4	Model 5
All-Cause Mortality						
Moderate Protein	1.34 *(0.81-2.22)*	1.15 *(0.67-1.96)*	1.33 *(0.80-2.21)*	0.79 *(0.62-0.99)*	0.79 *(0.61-1.01)*	0.80 *(0.63-1.01)*
High Protein	1.74 *(1.02-2.97)*	1.18 *(0.60-2.31)*	1.73 *(0.96-2.96)*	0.72 *(0.55-0.94)*	0.72 *(0.50-1.02)*	0.73 *(0.56-0.96)*
% kcal Animal Protein		1.03 *(1.00-1.06)*			1.00 *(0.98-1.02)*	
% kcal Vegetable Protein			1.01 *(0.96-1.06)*			0.98 *(0.95-1.01)*
CVD Mortality						
Moderate Protein	0.79 *(0.40-1.54)*	0.61 *(0.29-1.29)*	0.77 *(0.40-1.51)*	0.80 *(0.57-1.12)*	0.80 *(0.56-1.14)*	0.82 *(0.58-1.14)*
High Protein	1.03 *(0.51-2.09)*	0.55 *(0.19-1.62)*	1.02 *(0.50-2.06)*	0.78 *(0.54-1.14)*	0.77 *(0.48-1.25)*	0.79 *(0.54-1.16)*
% kcal Animal Protein		1.04 *(0.99-1.11)*			1.00 *(0.98-1.02)*	
% kcal Vegetable Protein			1.03 *(0.96-1.10)*			0.98 *(0.94-1.02)*
Cancer Mortality						
Moderate Protein	3.06 *(1.49-6.25)*	2.71 *(1.24-5.91)*	3.03 *(1.48-6.19)*	0.67 *(0.43-1.06)*	0.66 *(0.40-1.07)*	0.68 *(0.43-1.09)*
High Protein	4.33 *(1.96-9.56)*	3.19 *(1.21-8.35)*	4.30 *(1.93-9.59)*	0.40 *(0.23-0.71)*	0.38 *(0.17-0.82)*	0.41 *(0.23-0.72)*
% kcal Animal Protein		1.02 *(0.97-1.07)*			1.00 *(0.97-1.04)*	
% kcal Vegetable Protein			1.01 *(0.91-1.12)*			0.99 *(0.92-1.06)*
Diabetes Mortality						
Moderate Protein	3.43 *(0.69-17.02)*	2.99 *(0.58-15.31)*	3.64 *(0.76-17.55)*	5.38 *(0.95-30.49)*	6.20 *(0.35-37.01)*	5.50 *(0.96-31.50)*
High Protein	3.93 *(0.73-21.07)*	2.77 *(0.24-31.73)*	3.97 *(0.75-21.11)*	10.64 *(1.85-61.31)*	15.16 *(1.93-118.9)*	10.71 *(1.87-61.28)*
% kcal Animal Protein		1.02 *(0.92-1.14)*			0.98 *(0.90-1.06)*	
% kcal Vegetable Protein			0.90 *(0.75-1.07)*			0.98 *(0.84-1.14)*

Model 1: (Baseline Model) Adjusted for age, sex, race/ethnicity, education, waist circumference, smoking, chronic conditions (diabetes, cancer, MI), trying to lose in last year weight, diet changed in last year, reported intake representative of typical diet, total calories.
Model 4: Adjusted for Covariates and % kcals from Animal Protein
Model 5: Adjusted for Covariates and % kcals from Vegetable Protein

Reference=Low Protein
Ages 50-65: Low Protein (N=219), Moderate Protein (N=2,277), High Protein (N=543)
Ages 66+: Low Protein (N=218), Moderate Protein (N=2,521), High Protein (N=603)

Table S6: Adjusted mean HbA1c, Diabetes Prevalence, and mean BMI by Age and Protein Intake. Related to Figure 2.

	HbA1c	Diabetes (%)	BMI
Ages 50-65			
Low Protein Intake	5.52	2.8	27.65
Moderate Protein Intake	5.65	9.8	27.93
High Protein Intake	5.90	10.7	27.98
P-Value	<.001	<.001	.834
Ages 66+			
Low Protein Intake	5.52	5.0	26.19
Moderate Protein Intake	5.81	11.6	26.70
High Protein Intake	6.03	20.4	26.50
P-Value	<.001	<.001	.401

Estimated from models controlling for age, sex, race/ethnicity, education, smoking, diseases, total caloric intake, and dieting

Table S7: Associations between Diabetes Mortality and Protein Intake Among Participants with No Diabetes at Baseline. Related to Table 1.

	Hazard Ratio (95% Confidence Intervals
High Protein (n=930)	73.52 (4.47-1209.7)
Moderate Protein (n=4,441)	22.93 (1.31-400.7)

Reference=Low Protein (n=449 in both age groups)

Cox Proportional Hazard Model: Adjusted for age, sex, race/ethnicity, education, waist circumference, smoking, other chronic conditions (cancer, MI), trying to lose in last year weight, diet changed in last year, reported intake representative of typical diet, total calories.

Results

Using Cox Proportional Hazard models we found no association between protein consumption and either all-cause, CVD, or cancer mortality (Table S2). However, high and moderate protein consumption were positively associated with diabetes-related mortality. One explanation is that diabetes may be more prevalent in these groups, possibly because of a switch to a higher protein, lower fat, and lower carbohydrate intake following a diabetes diagnosis.

Finally, high versus low protein consumption was found to be associated with an over ten-fold increase in the risk of diabetes mortality for subjects age 66 and over. However, the much higher prevalence of subjects with a history of diabetes in the high protein group and the small number of subjects dying of diabetes in the low protein group may account for this, thus emphasizing the need for additional studies to determine the role of protein intake on diabetes incidence and mortality (HR: 10.64; 95% CI: 1.85-61.31).

Supplemental Materials and Methods

IGF-1 in Human Data

Half of the subjects in NHANES III were randomly selected to take part in the morning examination, following a recommended nine hour fast. Of this sub-sample, 2,253 subjects included in our study complied and have measured fasting serum data for IGF-1. IGF-1 was measured by Diagnostic Systems Laboratories Inc., using standard a laboratory protocol and reported in ng/ml.

Potential Confounders in Human Data

Age, race/ethnicity, education, sex, disease status, smoking, dietary changes, and total calorie consumption were included in analyses as potential confounders. Age was reported in years and top-coded at 90 in the data set by NHANES to protect confidentiality of respondents. Dummy variables were created to classify subjects into three race/ethnicity categories: non-Hispanic whites, non-Hispanic blacks, and Hispanics, Education was indicated by years of schooling. Dummy variables were created for self-reported smoking status—never, former, and current. Subjects were also asked to report on their history of diseases, in questions phrased as, "Has a Doctor ever told you had…" and used to create three dummy variables for presence of cancer, myocardial infarction, and diabetes history. Recent changes in dietary intake were assessed using responses to three questions—1) "During the past 12 months, have you tried to lose weight?"; 2) "During the past 12 months, have you changed what you eat because of any medical reason or health condition?"; and 3) (Following the 24-hour dietary recall) "Compare food consumed yesterday to usual". Waist circumference, which is preferred to BMI as an indicator of adiposity, was measured to the nearest 0.1 cm starting on the right side of the body at the iliac crest.

Protein restriction in mice

AIN-93G standard chow was used as the casein-based high protein reference diet (18% kcal from protein and low protein diet 1,O was used as the casein-based low protein diet (4% kcal from protein). Additional diets with contents ranging from 4%-18% kcal from protein were created using either the AIN-93G purified diet or the Soy protein diet (93G, G) as reference standards (Harlan Laboratories, WI). Diets were isocaloric and changes in kcal from fat or carbohydrates occurred in proportion to changes in kcal from protein. Daily intake measurements began 1 week before commencing the experiment in order to establish a baseline intake amount. All animals were fed daily for the duration of

the experiment, and were provided with chow in excess of 50% of their baseline intake in order to allow ad lib, non-calorically restricted feeding. Before tumor implantation BALB/c mice were assigned to one of the 2 different kcal from protein groups and were pre-fed for 1 week. Feeding of these mice was continued throughout the course of the experiment the same as described above. To determine the effect of low protein on old mice, 24-month-old C57BL/6 mice were placed either in an 18% or 4% kcal from animal protein group and fed a continuous diet as described above. Body weights and intake were determined daily. Animals had access to water at all times.

Serum mIGF-1, mIGFBP-1, mIGFBP-2, and mIGFBP-3 measurements in mice

Mice were anesthetized with 3% inhalant isoflurane, warmed gently to dilate the veins, and blood was collected from the tail vein to obtain serum weekly. Serum mIGF-1, mIGFBP-1, mIGFBP-2, and mIGFBP-3 assays were performed as previously described (Hwang et al., 2008) using an in-house ELISA assay using recombinant mouse IGF-1, IGFBP-1, mIGFBP-2, or mIGFBP-3 protein and polyclonal antibodies from R&D systems (Minneapolis, MN).

Statistical analysis for mouse data

IGF-1 comparisons between groups were performed using Student's t test, IGFBP-1, IGFBP-2, and IGFBP-3 group comparisons were performed by Student's *t* test and ANOVA, and tumor volume progression group comparisons were performed with two-way ANOVA using GraphPad Prism v.6. All statistical analyses were two-sided and P values <0.05 were considered significant. Error bars represent SEM.

Yeast survival and mutation frequency measurement

Cells of the widely used DBY746 yeast strain (MATα leu2-3,112 his3-Δ1 trp1-289, ura3-52 GAL+) were made prototrophic by transformation with the corresponding plasmid, inoculated onto 1 ml of complete synthetic medium (SDC) and grown overnight at 30 degrees Celsius on an orbital shaker at 200 RPM.

This starter culture was then split (1:100) onto fresh synthetic SDC media containing 0.5 X, 1X or 2 X of the standard amino-acid concentration (Hu et al., 2013) at a 5:1 flask volume to medium volume ratio and put back in the incubator at the very same conditions. Aliquots of each culture were harvested every other day and proper dilutions plated onto rich YPD plates. Colony forming units (C.F.U.) were counted after two days of growth. Percentage of survival was assessed considering the CFU at day 1 as 100% of survival. All experiments were made in triplicate and standard deviation is shown. For mutation frequency calculation, 10^7 cells were collected, at each survival time point, washed with water and plated onto synthetic complete (SDC) medium lacking arginine and supplemented with 60 microgram/ml L-canavanine sulphate (Can). Can resistant colonies were measured after two to three days of growth at 30 degrees Celsius and expressed as the number of Can resistant clones out of 10^6 viable CFU. All data points represent the mean of biological replicate samples. Error bars represent SEM; p values were determined using Student *t* tests or 2-way ANOVA using GraphPad Prism v.6.

Ras2 experiment growth conditions

Yeast chronological life span was monitored in expired SDC medium by measuring colony-forming units (CFUs) every 48 h. The number of CFUs at day 1 was considered to be the initial survival (100%) and was used to deter- mine the age-dependent mortality.

Ras2 experiment Can1 mutation frequency measurements

Spontaneous mutation frequency was evaluated by measuring the frequency of mutations of the CAN1 (YEL063) gene. In brief, overnight inoculations were diluted in liquid SDC medium and incubated at 30°C. The cells' viability was measured every 2 d starting at day 1 by plating appropriate dilutions onto yeast extract peptone dextrose (YPD) medium plates and counting the CFUs. To identify the canavanine-resistant mutants (Can^r) in the liquid culture, an appropriate number of cells (starting amount of 2 $\times 10^7$ cells) was harvested by centrifugation, washed once with sterile water, and plated on selective medium (SDC-Arg supplemented with 60 μg/ml l-canavanine sulfate). Mutant colonies were counted after 3–4 d. The mutation frequency was expressed as the ratio of Can^r to total viable cells. All data points represent the mean of biological replicate samples. Error bars represent SEM; p values were determined using 2-way ANOVA using GraphPad Prism v.6.