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Type 5 Adenylyl Cyclase Disruption Increases Longevity and Protects Against Stress

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SUMMARY

Mammalian models of longevity are related primarily to caloric restriction and alterations in metabolism. We examined mice in which type 5 adenylyl cyclase (*AC5*) is knocked out (*AC5* KO) and which are resistant to cardiac stress and have increased median lifespan of ~30%. *AC5* KO mice are protected from reduced bone density and susceptibility to fractures of aging. Old *AC5* KO mice are also protected from aging-induced cardiomyopathy, e.g., hypertrophy, apoptosis, fibrosis, and reduced cardiac function. Using a proteomic-based approach, we demonstrate a significant activation of the Raf/MEK/ERK signaling pathway and upregulation of cell protective molecules, including superoxide dismutase. Fibroblasts isolated from *AC5* KO mice exhibited ERK-dependent resistance to oxidative stress. These results suggest that AC is a fundamentally important mechanism regulating lifespan and stress resistance.

INTRODUCTION

Caloric restriction (CR) is a well-recognized mechanism mediating lifespan extension from yeast to mammals (Sinclair, 2005). Other mammalian models of longevity are related to CR through effects on metabolism, e.g., growth hormone (GH)/GH receptor (Bluher et al., 2003; Brown-Borg et al., 1996), and either those involved in the insulin signaling pathway or insulin-like growth factor receptors, and stimulation of foxo family transcription factors, resulting in prolongation of the maximum lifespan (Kenyon, 2005; Liang et al., 2003).

The current investigation reports a novel mammalian model of longevity based on interruption of beta-adrenergic receptor (β-AR) signaling at the level of AC, specifically disruption of the *AC5* gene, an AC isoform predominantly expressed in the heart and brain. AC is a key enzyme that catalyzes the synthesis of cAMP from ATP. cAMP activates protein kinase A (PKA; Walsh et al., 1968) and regulates the function of multiple proteins and transcriptional factors, such as CREB and ICER (Houslay and Kolch, 2000). AC also plays a pivotal role in β-AR signaling. At least nine membrane-bound isoforms of AC (AC1–AC9) exist, with diverse tissue distribution, biochemical properties, and specific catalytic activities (Hanoune and Defer, 2001). We generated a genetically engineered mouse model in which *AC5* is knocked out (*AC5* KO; Okumura et al., 2003b). Unexpectedly, *AC5* KO mice live significantly longer than control littermates. The hearts of these mice also demonstrate resistance to stresses, including pressure overload and catecholamine stimulation, in terms of maintenance of cardiac function and protection against apoptosis (Okumura et al., 2003c).

Another potentially important clinical correlation involves insights into cardioprotection for heart failure since there is considerable current interest in inhibition of β-AR signaling and AC for the treatment of heart failure (Bristow, 2000). In view of the parallel features of heart failure protection and longevity, it is interesting to speculate that the proteins in the β-AR signaling pathway may be involved in both longevity and stress resistance.

In this study, we demonstrate that *AC5* KO mice exhibit significantly extended median/maximum lifespan, retarded aging phenotypes in the heart and bone, and increased stress resistance. Using unbiased proteomic approaches, we found that *AC5* KO leads to upregulation of the Raf/MEK/ERK signaling pathway, which in turn mediates upregulation of superoxide dismutase (SOD), an important mechanism mediating lifespan extension and stress resistance.

RESULTS

AC5 KO Mice Exhibit Extended Lifespan

To determine the lifespan of *AC5* KO mice, we first conducted a retrospective study comparing the survival rate

of WT and *AC5* KO mice, which were all alive at 17 months (Figure 1 insert). Of these, only 16% of WT were still alive at 30 months of age, whereas 92% of *AC5* KO mice remained alive at this time. Next we conducted an anterospective study, which is summarized by the Kaplan-Meier survival analysis. This study revealed that the median lifespan was increased by 8 months (from 25 to 33 months), $p < 0.01$, and the maximum lifespan was extended by 4 months (from 33 to 37 months), $p < 0.02$, in *AC5* KO mice (Figure 1). Increased lifespan was similar in both males and females. For comparisons of *AC5* KO and WT, young mice (3–6 months) and old mice (20–30 months) were studied.

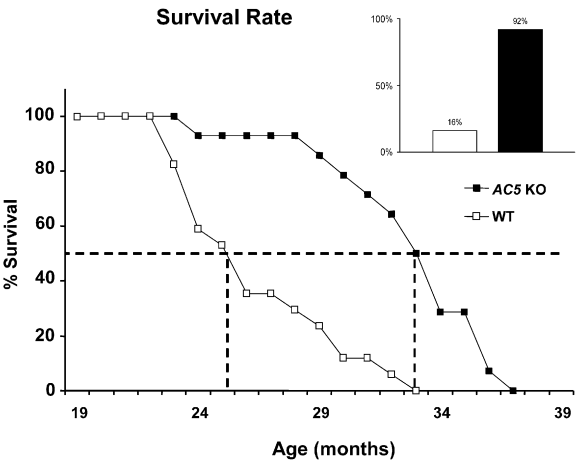

Figure 1. Extended Lifespan in *AC5* KO Mice

A retrospective study of WT (n = 25) and *AC5* KO mice (n = 13) demonstrated significant differences in longevity between WT and *AC5* KO, which were alive at 17 months (insert). At 30 months, only 16% of WT mice survived, whereas 92% of *AC5* KO mice were still alive. The Kaplan-Meier survival curve shows significantly increased survival, $p < 0.01$, of *AC5* KO mice (n = 14) compared to their WT littermates (n = 17) studied anterospectively from birth to death. The dotted line indicates the time of 50% survival. Roughly 50% of WT mice died by 25 months. In striking contrast, 50% of *AC5* KO mice died by 33 months. These differences are significant, $p < 0.01$. The maximum survival was also significantly different, $p < 0.02$, by the chi-square test. Data for males and females were pooled since there were no gender differences for survival.

Bone Quality Is Preserved in Old *AC5* KO Mice

Bone integrity was only studied in female mice. Necropsies revealed weakened bone structure with susceptibility to fractures in old WT mice, but not in old *AC5* KO mice. Radiographs indicated reduced bone density, healing stress fractures, reduced calcification, and absence of the fibula in old WT mice (Figure 2A). In contrast, bones appeared normal in radiographs of old *AC5* KO mice (Figure 2A). The differences in bone structure were quantified by torsional mechanical testing (Manigrasso and O'Connor, 2004). Figure 2B shows three indices of (femur) bone strength in WT and *AC5* KO mice. The bone peak torque, shear modulus, and shear stress were greater in *AC5* KO mice, demonstrating that the bone in aging *AC5* KO mice is stronger and more resistant to stress.

AC5 KO Mice Have Lower Body Weights and Lower GH Levels

Reduced body weight (BW) and GH signaling have been observed in CR mice and several other genetic mutations (Al-Regaiey et al., 2005; Bartke and Brown-Borg, 2004; Coschigano et al., 2000; Miller, 1999; Miller et al., 2002) that lead to increased lifespan. In order to determine whether knocking out *AC5* affects mouse BW and GH signaling, we measured BW and GH levels in *AC5* KO mice. Body weights were found to be similar in young *AC5* KO and WT mice, but significantly decreased in old *AC5* KO compared to old WT mice ($p < 0.05$). A significant reduction ($p < 0.05$) in the GH level was also observed in *AC5* KO mice (Figures S1A and S1B). In view of the lower body weights in *AC5* KO mice, we examined whether CR contributes to the extended lifespan in *AC5* KO mice. We measured food intake in young *AC5* KO mice and WT controls over a 3 week period. The data demonstrated that *AC5* KO mice tend to eat more than WT. Thus, lifespan extension in *AC5* KO is not caused by reduced caloric intake in young animals. It is still possible that older *AC5* KO eat less (Figure S1C).

AC5 KO Mice Are Protected from Aging-Induced Cardiomyopathy

We measured the key features of aging cardiomyopathy including left ventricular (LV) weight/BW (LVW/BW) ratio, myocyte size, LV ejection fraction (LVEF), apoptosis, and fibrosis. LVW/BW ratio (Figure 3A), an index of LV hypertrophy (LVH), was not different between young WT and *AC5* KO mice but was significantly elevated in old WT ($p < 0.05$) and not in old *AC5* KO mice. Myocyte cross-sectional area was significantly less, $p < 0.02$, in old *AC5* KO ($259.5 \pm 4.9\ \mu m^2$) than in old WT mice ($282.9 \pm 5.2\ \mu m^2$), confirming that less LVH developed in old *AC5* KO mice. LVEF (Figure 3B), an index of LV systolic function, was significantly higher in old *AC5* KO mice compared to old WT mice ($p < 0.05$), indicating better LV function. Myocardial fibrosis was increased in old versus young WT, $p < 0.05$, but was not increased in old *AC5* KO mice (Figure 3D).

Aging cardiomyopathy is also characterized by increased apoptosis, which was also observed in the present study in the old WT mice, but not in the old *AC5* KO mice (Figure 3C). One important signaling pathway mediating cell survival involves ERK signaling (see below). Several downstream apoptosis-related elements of this pathway, e.g., RSK, p-Bad, and Bcl-xl, were significantly increased in heart, kidney, and brain of *AC5* KO mice (Figure S2A). Other antiapoptotic markers such as XIAP and Hsp70 were also increased in *AC5* KO mice (Figure S2B), consistent with protection from apoptosis in *AC5* KO mice.

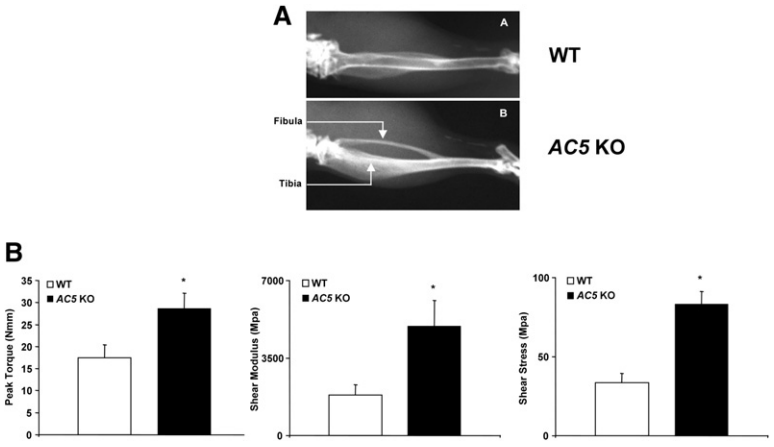

Figure 2. Bone Integrity Is Maintained in Aging *AC5* KO Mice

The data obtained in (A) and (B) were from six female *AC5* KO and five female WT mice at 20–25 months old. (A) Radiographs taken of the tibia of WT and *AC5* KO mice. The WT mice exhibit reduced bone density, apparent healing stress fractures, reduced calcification, and absence of the fibula. This was not observed in *AC5* KO mice of the same age. In this example, both the WT and *AC5* KO mice were 23 months old.

(B) Three indices of bone strength and integrity comparing old WT (open bars) and old *AC5* KO (solid bars) mice. All three indices—peak torque (left), shear modulus (middle), and shear stress (right)—were measured by torsional mechanical testing and were significantly greater (*$p < 0.05$) in *AC5* KO, demonstrating that the bone in *AC5* KO is stronger and more resistant to stress, which supports the example shown in Figure 2A. Data are expressed as mean ± SEM.

AC5 KO Mice Are Resistant to Oxidative Stress

To test the hypothesis that knocking out *AC5* renders cells resistant to environmental stresses, we investigated whether neonatal *AC5* KO cardiac myocytes and fibroblasts from *AC5* KO embryos and adult *AC5* KO mice are resistant to oxidative (H_2O_2) and DNA (UV light) damage. Cardiac myocytes were isolated from 1-day-old neonates of homozygous *AC5* KO mice or WT mice. After culture for 48 hr, cells were treated by H_2O_2 (25, 50, and 100 μM) or UV light (10 and 25 mJ/cm^2) for 24 hr. Cell viability was higher in *AC5* KO than in WT myocytes (Figure 4A, left upper and lower panels). Mouse embryonic fibroblasts (MEFs) were isolated from 13 day embryos of homozygous *AC5* KO mice or WT mice. The third passage cells were treated by H_2O_2 (50, 100, 200, and 400 μM) or UV light (25 and 100 mJ/cm^2) for 24 hr. The proportion of surviving cells was higher in *AC5* KO than in WT MEFs (Figure 4A, right upper and lower panels). Furthermore, fibroblasts were grown from tail skin of adult *AC5* KO and WT mice. The third passage cells were exposed to 25 μM H_2O_2 or 25 mJ/cm^2 UV for 24 hr. Similar to MEFs, WT fibroblasts were more sensitive to H_2O_2 and UV light, whereas *AC5* KO fibroblasts displayed increased resistance to both H_2O_2 and UV light (data not shown). H_2O_2- or UV-light-treated MEFs and adult fibroblasts were also subjected to apoptosis analysis. Apoptosis was lower in *AC5* KO than in WT MEFs (Figure 4B, left) and adult fibroblasts (Figure 4B, right). These results indicate that disruption of *AC5* enhances resistance to oxidative stress in vitro. In order to examine the mechanisms which confer resistance to oxidative stress, we examined the levels of the antioxidant MnSOD in heart, kidney, brain, and bone by western blotting. The protein level of MnSOD was increased in the heart, kidney, and brain of aging *AC5* KO mice, providing further evidence that the *AC5* KO mice are resistant to oxidative damage (Figure 4C).

The Raf/MEK/ERK Signaling Is Stimulated in *AC5* KO Mice

To investigate the molecular mechanisms mediating longevity in *AC5* KO mice, we utilized an unbiased proteomic analysis to search for novel proteins that are altered in *AC5* KO mice. We initially separated proteins from brain tissue using 2D gel electrophoresis and compared the gel patterns of WT and *AC5* KO mice (20 months old). We found that nine proteins were upregulated, five downregulated, and three altered by posttranslational modifications in *AC5* KO mice (Table S1). Among these changes, we are interested in the alteration which is shown in Figure 5A; the pattern of spots in the circles was different between WT and *AC5* KO. The two spots in WT and one in *AC5* KO were observed, and all of them were identified as mitogen-activated protein kinase kinase 1 (MEK1; Figure S3) using peptide mapping by mass spectrometry and sequence database searches, which suggests the alteration of MEK1 posttranslational modification (most likely phosphorylation) in *AC5* KO mice.

Since we observed alterations of MEK1 in *AC5* KO mice from 2D gel electrophoresis, we examined the levels of p-MEK by western blotting in the heart. p-MEK was confirmed to be elevated in the heart of *AC5* KO mice (Figure 5B). Furthermore, we examined its upstream molecule, Raf-1, and its downstream molecule, ERK, by western blotting. We found that phosphorylation of ERK was significantly increased in the heart and kidney (Figure 5C). Phosphorylation of Raf-1 was also elevated (data not shown). The phosphorylation of ERK was also elevated in MEFs isolated from *AC5* KO mice (Figure 5C, right). Therefore, Raf/MEK/ERK signaling is upregulated in *AC5* KO mice.

ERK Mediates Resistance to Oxidative Stress and Upregulation of SOD in *AC5* KO Mice

We hypothesized that knocking out *AC5* enhances cellular resistance to oxidative stress through activation of ERK.

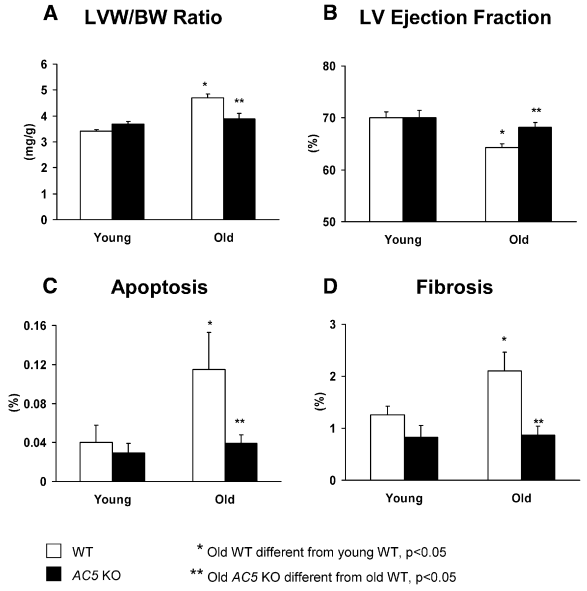

Figure 3. *AC5* KO Mice Are Protected from Aging Cardiomyopathy

Comparison of LVW/BW (A), LV ejection fraction (B), LV apoptosis (C), and LV fibrosis (D) in *AC5* WT and KO, young (3–6 months, n = 4–9) and old (20–30 months, n = 4–9). There were no significant differences between young WT and young *AC5* KO mice. However, all of the four parameters were significantly different in old WT compared with young WT (*p < 0.05), characteristic of aging cardiomyopathy. In contrast, none of the four parameters were different in old versus young *AC5* KO, but all four parameters were significantly different in old *AC5* KO versus old WT (**p < 0.05). For panels (A)–(D), all data are expressed as mean ± SEM.

To test this hypothesis, we inhibited ERK with PD98059 (the levels of p-ERK were significantly reduced after treatment; data not shown), a specific inhibitor, to examine whether inhibition of ERK reduces oxidative stress tolerance in *AC5* KO mice. *AC5* KO MEFs were pretreated with PD98059 (30 μM in 0.04% DMSO and 60 μM in 0.08% DMSO) for 30 min before 24 hr incubation with 50 μM or 100 μM H_2O_2. The proportion of surviving cells pretreated with PD98059 was lower than the cells with vehicle treatment alone (Figure 5D, left and middle), indicating a possible role of ERK activation in mediating resistance to oxidative stress. To further confirm whether ERK is involved in stress resistance downstream of *AC5*, we used RNAi to knock down ERK1 and ERK2 in *AC5* KO MEFs (the levels of p-ERK were significantly reduced after treatment; data not shown), and then cells were treated with 400 μM H_2O_2. In an ERK knockdown experiment, cell viability was reduced (Figure 5D, right). As shown in Figure 5D, the reduction of tolerance to oxidative stress was more in *AC5* KO than in WT. To further test whether MnSOD could be a downstream enzyme of the ERK signaling pathway, we determined the level of MnSOD in MEFs from WT and *AC5* KO mice treated with PD98059 and ERK siRNA. As shown in Figure 5E, the level of MnSOD was reduced in MEFs pretreated with PD98059 (left) and in MEFs with ERK siRNA (right), suggesting that ERK plays an essential role in mediating upregulation of MnSOD.

Overexpression of Mammalian ERK2 in Budding Yeast Results in Extended Lifespan and Resistance to Heat Shock and Oxidative Stress

To study the effects of the Raf/MEK/ERK pathway on longevity, we overexpressed human ERK2 in yeast (Figure 6A) since expression of human ERK in yeast can partially substitute for the absence of *KSS1*, the ortholog of ERK (Atienza et al., 2000). We found that ERK2 overexpressing yeast strains were resistant to heat shock and oxidative stress (Figure 6B), and chronological lifespan was longer compared to a WT strain that does not express ERK2, e.g., the survival at 5 days in the strain with ERK2 overexpression was 62 ± 3.2%, significantly greater, $p < 0.05$, than WT (51 ± 4.4%; Figure 6C). We expressed ERK2 also in strains that have a mutation in the gene encoding adenylyl cyclase, *CYR1* (mutant: *cyr1-1*; Fabrizio et al., 2001). The *cyr1-1* mutant strain expressing ERK2 was not more resistant to heat shock and oxidative stress compared to the nonERK2-expressing *cyr1-1* mutant strain (Figure 6B), and also the chronological lifespan was not increased significantly (Figure 6C). Furthermore, when we deleted *KSS1* (ERK homolog gene) from the yeast, the yeast strains lacking Kss1p showed increased sensitivity to heat shock and oxidative stress (Figure 6D) and a shorter lifespan, e.g., the survival at 5 days in the strain lacking Kss1p was 36 ± 1.5%, significantly less, $p < 0.05$, than WT (53 ± 2.1%; Figure 6E). Lack of Kss1p also increases sensitivity to heat shock and oxidative stress in a mutant *cyr1-1* strain (Figure 6D) with a concomitant decrease in chronological lifespan, e.g., the survival at 5 days in a mutant *cyr1-1* strain lacking Kss1p was 69 ± 2.5%, significantly less, $p < 0.05$, than the *cyr1-1* strain (90 ± 5.2%; Figure 6E). The data for all survival results at 5 days and statistics are included in Table S2. These results are consistent with the notion that the corresponding Raf/MEK/ERK signaling pathway in yeast (Ste11/Ste7/Kss1) interacts genetically with the Cyr1/cAMP/PKA pathway in mediating regulation of lifespan and stress resistance. It should be noted that our results also suggest that the effect of Cry1-1 mutation could be mediated by Kss1-independent mechanisms as well.

DISCUSSION

The central finding in this work is that *AC5* KO mice exhibit extended lifespan. Our observation for the first time indicates that AC is not only a fundamentally important mechanism regulating lifespan in a wide variety of organisms but also provides a novel molecular mechanism by which genetic deletion of *AC5* leads to this lifespan extension.

AC is the keystone of sympathetic transmission in β-AR signaling in myocardium. The sympathetic nervous system is designed to respond to stress. The activity of the sympathetic nervous system and β-AR stimulation

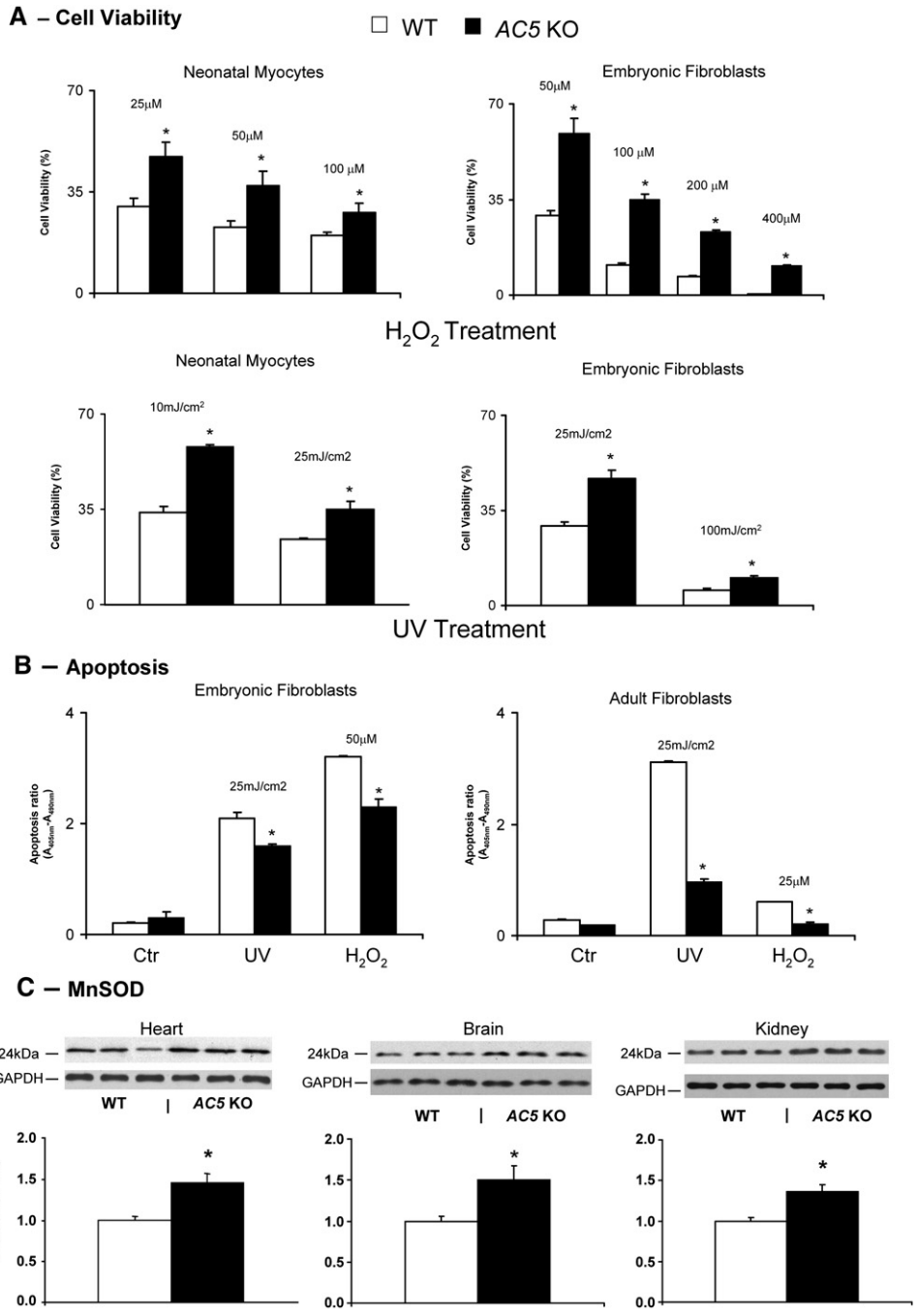

Figure 4. *AC5* KO Mice Are Resistant to Oxidative Stress

(A) Cell viability was evaluated in response to oxidative stress in neonatal cardiac myocytes and MEFs isolated from *AC5* KO and WT mice. Myocytes were treated with H_2O_2 (25, 50, and 100 μM) (upper left) or UV (10 and 25 mJ/cm^2; lower left) and evaluated for cell viability using CellTiter-Blue Cell Viability Assay. *AC5* KO neonatal myocytes showed more tolerance to oxidative and DNA damage (*$p < 0.05$ versus WT). MEFs were treated with H_2O_2 (50, 100, 200, and 400 μM; upper right) or UV (25 and 100 mJ/cm^2; lower right) and evaluated for survival rate using trypan blue exclusion. *AC5* KO MEFs showed more tolerance to oxidative and DNA damage (*$p < 0.05$ versus WT). Data are expressed as mean ± SEM.

(B) Apoptosis was analyzed in response to UV or H_2O_2 in MEFs and adult fibroblasts isolated from *AC5* KO and WT mice by histone-associated DNA fragmentation using Cell Death Detection ELISA. Apoptosis was lower in *AC5* KO than in WT MEFs and adult fibroblasts (*$p < 0.05$), indicating more tolerance to oxidative and DNA damage in *AC5* KO than in WT mice. Data are expressed as mean ± SEM.

(C) Western blotting of MnSOD from the heart, brain, and kidney of *AC5* WT and KO mice (20 months, n = 4). The level of MnSOD is significantly greater in the heart, brain, and kidney of *AC5* KO mice compared to WT mice (*$p < 0.05$). Data are expressed as mean ± SEM.

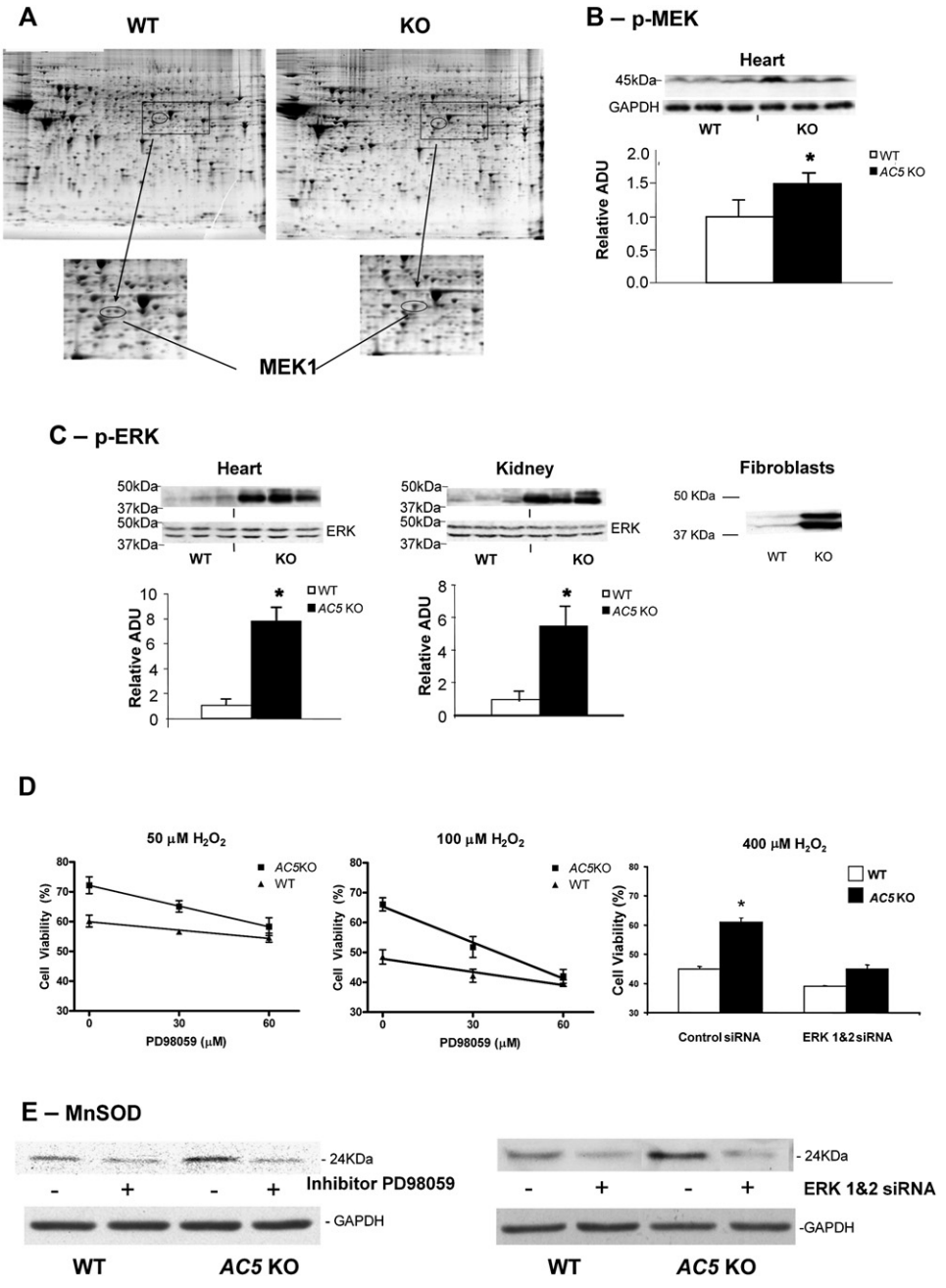

Figure 5. The Raf/MEK/ERK Pathway Plays an Essential Role in Mediating Stress Resistance in *AC5* KO Mice

(A) 2D gel images from brain protein extracts of *AC5* WT (top left panel) and KO mice (top right panel). The lower panel shows a magnified gel region from WT (left) and *AC5* KO (right). Gels were stained by SYPRO Ruby and quantified by Compugen Z3 software. The circled spots show an alteration in the pattern of protein migration between WT and *AC5* KO mice, which were later identified as MEK1 by peptide mass mapping using MALDI TOF mass spectrometry and protein database search (Figure S3).

(B) Western blotting of p-MEK in the heart of *AC5* WT and KO mice (20 months, n = 4 in each group). The level of p-MEK is significantly increased in *AC5* KO mice, *$p < 0.05$. Data are expressed as mean ± SEM.

(C) Western blotting of p-ERK in the heart and kidney of *AC5* WT and KO mice (20 months, n = 4 in each group). The levels of p-ERK are significantly increased in old *AC5* KO mice for both kidney and heart, *$p < 0.05$. The levels of p-ERK are also increased in MEFs. Data are expressed as mean ± SEM.

(D) The cell viability of *AC5* WT and KO MEFs pretreated with PD98059 (30 μM and 60 μM) for 30 min before 24 hr incubation with 50 μM (left) or 100 μM H_2O_2 (middle). MEFs were isolated from three KO and two WT mice. Two cell viability data points from each animal in each group at different concentrations of PD98059 were used to create graph and regression lines. The regression lines were compared statistically for differences in the slope using ANCOVA. Clearly, the cell viability in the presence of PD98059 was lower than that without PD98059. The differences between the slopes are

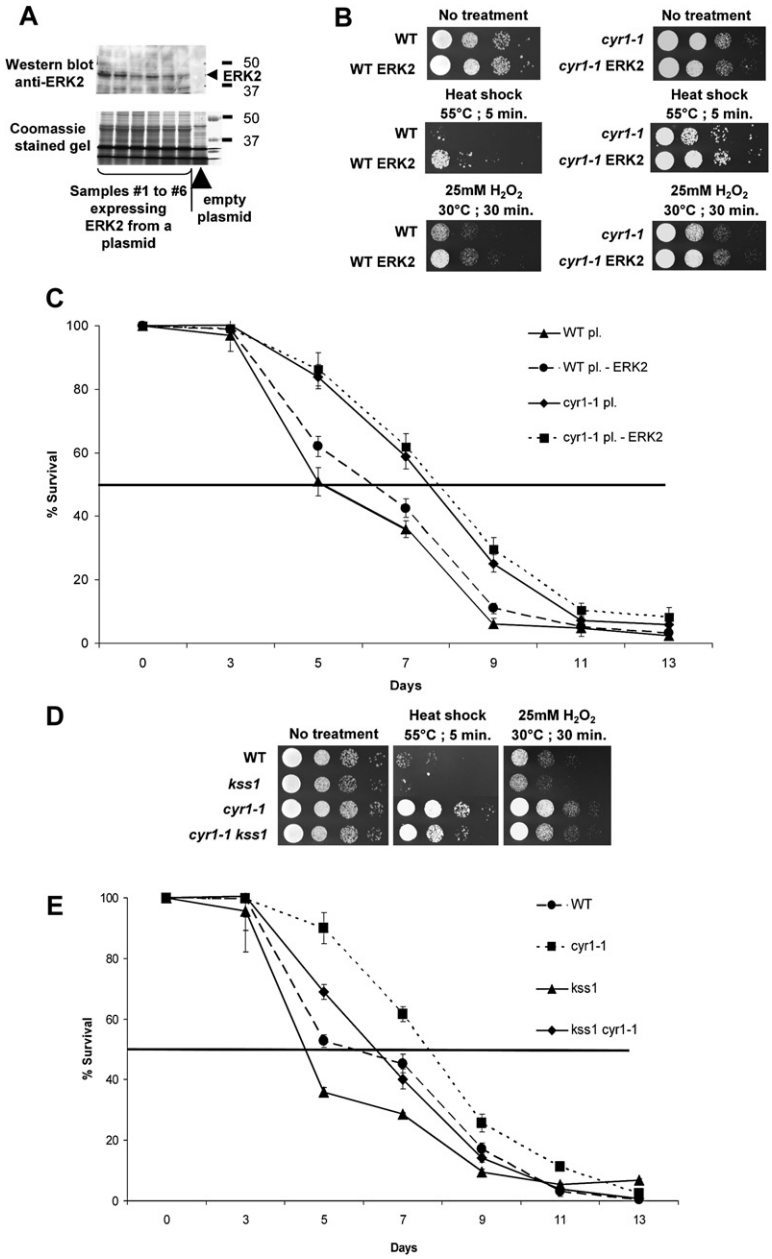

Figure 6. The Cyr1/cAMP/PKA Pathway and the Raf/MEK/ERK Pathway Are Interacting Genetically in the Regulation of Resistance to Heat Shock and Oxidative Stress and in Controlling Chronological Lifespan

(A) ERK2 is expressed from a yeast plasmid and regulated by the ADH1 promoter. WT cells were transformed with an empty plasmid and ERK2-expressing plasmid. Expression of ERK2 was examined by western blotting (upper panel). The lower left panel is a Coomassie-stained gel.

(B) Yeast strains (WT, WT expressing ERK2 in a plasmid, *cyr1-1* mutant cells, and *cyr1-1* mutant cells expressing ERK2) were incubated at 55°C for five minutes or incubated in the presence of 25 mM hydrogen peroxide (H_2O_2) at 30°C for 30 min.

(C) Chronological lifespan was determined from a WT strain, a WT strain expressing ERK2 on a plasmid (pl), a *cyr1-1* mutant strain, and a *cyr1-1* mutant strain expressing ERK2. Standard errors are shown. The median lifespans are: WT (5.1 days), WT ERK2 (6.3 days), *cyr1-1* (7.5 days), and *cyr1-1* ERK2 (7.8 days). Data are expressed as mean ± SEM.

(D) Yeast strains (WT, *cyr1-1, kss1*, and *kss1 cyr1-1* mutant cells) were incubated at 55°C for five minutes or incubated in the presence of 25 mM hydrogen peroxide (H_2O_2) at 30°C for 30 min.

(E) Chronological lifespan determination of WT, *cyr1-1, kss1*, and *kss1 cyr1-1* mutant cells. Standard errors are shown. The median lifespans are: WT (6 days), *cyr1-1* (7.7 days), *kss1* (4.5 days), and *kss1 cyr1-1* (6.4 days). Data are expressed as mean ± SEM.

increases with aging and may play a role in the development of aging cardiomyopathy (Lakatta, 1993; Swynghedauw et al., 1995). For these reasons, it has been thought that desensitization in the β-AR signaling pathway is protective in aging cardiomyopathy (Asai et al., 1999). β-AR blockers improve survival after myocardial infarction, have salutary effects in patients with heart failure, and may have antiatherosclerosis effects. On the other hand, chronically enhanced β-AR stimulation with age is deleterious to the heart; indeed a number of mouse models of increased β-AR stimulation at the level of the receptor (Du et al., 2000; Engelhardt et al., 1999; Liggett et al., 2000), G protein (Iwase et al., 1996), or PKA (Antos et al., 2001) have been shown to have increased mortality and decreased resistance to stress. The effect of chronic inhibition of β-AR signaling upon aging of the heart has not

significant, $p < 0.05$, as denoted by the asterisks, indicating that the reduction of tolerance to oxidative stress was greater in *AC5* KO than in WT. The cell viability of *AC5* WT and KO MEFs with ERK1 and ERK2 knockdown using ERK siRNA and then incubation with 400 μM H_2O_2 are shown (right).The cell viability with ERK1 and ERK2 knockdown was less than that without ERK1 and ERK2 knockdown. These observations demonstrate that inhibition of ERK activation reduces the tolerance to different concentrations of oxidative stress. Data are expressed as mean ± SEM.

(E) Western blotting of MnSOD from MEFs of WT and *AC5* KO mice with and without PD98059 (left) and with and without ERK siRNA (right). The level of MnSOD was reduced in MEFs pretreated with PD98059 and ERK siRNA.

been examined using specific loss-of-function models. The protection of *AC5* KO mice from both aging cardiomyopathy (LVH, fibrosis, and apoptosis; this study) and stresses (pressure overload and chronic catecholamine stimulation; our previous studies in Okumura et al., 2003a, 2003c) may result from a desensitization of β-AR signaling in the heart since *AC5* is a major isoform of AC in cardiac myocardium.

It is generally believed that lifespan of the organism is regulated by key, evolutionarily conserved, molecular mechanisms, such as sirtuin family proteins and FOXO family transcription factors. Although it has been shown that the genetic mutations in the cAMP-PKA pathway extend both replicative and chronological lifespan in yeast (Fabrizio et al., 2001; Lin et al., 2000), whether or not similar mechanisms regulate lifespan of mammals is a critically important issue, which remains to be demonstrated. Our observation clearly indicates that genetic deletion of *AC5*, a mammalian homolog of yeast *Cyr1*, extends the lifespan of mice.

To investigate the molecular mechanisms in the regulation of longevity in *AC5* KO mice, an unbiased proteomic analysis followed by confirmation by western analysis demonstrated activation in the Raf/MEK/ERK signaling pathway. Previous findings suggested that activation of the Raf/MEK/ERK pathway declines with age (Ajuh et al., 2000; Hutter et al., 2000; Lorenzini et al., 2002; Meloche et al., 2000; Miller et al., 1997; Torres et al., 2003; Zhen et al., 1999). Increased levels of p-ERK were found in long-lived CR mice (Ikeyama et al., 2002) and Snell dwarf mice (Madsen et al., 2004). ERK has also been considered as a cardioprotective agent in aged myocardium (Taylor and Starnes, 2003). All of these results are consistent with a notion that the Raf/MEK/ERK pathway is critical in the aging process. However, a beneficial effect of ERK in longevity has not been investigated previously. We used yeast as a model to investigate the role of Raf/MEK/ERK activation in longevity. Overexpression of human ERK2 in a yeast strain caused extended chronological lifespan and stress resistance, strongly suggesting that the mammalian ERK functions in controlling lifespan. Furthermore, lack of Kss1p, an ERK2 homolog in yeast, significantly reduced the lifespan and resistance to heat and oxidative stress in the *cyr1-1* mutant, consistent with the notion that ERK2 homologs play an essential role in mediating lifespan extension in the *cyr1-1* mutant. It should be noted, however, that a parallel pathway, presumably another yeast homolog of mammalian ERKs or even ERK-independent mechanisms, may also regulate lifespan and resistance to oxidative stress and heat because the *KSS1* deletion generated upon the *cyr1-1* mutant showed significantly longer lifespan than the *KSS1* deletion alone.

Another important finding is that *AC5* KO mice are resistant to oxidative stress. Apoptosis, which may result from oxidative damage, was reduced in the myocardium of *AC5* KO mice compared to WT mice, and molecular mechanisms to be involved in protection against apoptosis, p-Bad, Bcl-xl, Hsp70, and XIAP, were increased not only in heart but also in kidney and brain. Resistance to oxidative stress has been identified in some longevity mouse models; e.g., IGF-1-receptor-deficient mice (Holzenberger et al., 2003), $p66^{Shc-/-}$ mice (Migliaccio et al., 1999), mice with overexpression of mitochondria-specific catalase (Schriner et al., 2005), Snell and Ames dwarf mice (Murakami et al., 2003; Romanick et al., 2004), and CR mice (Sohal and Weindruch, 1996). In addition, increased lifespan in nematodes (Feng et al., 2001; Ishii, 2000; Lithgow, 1996) and *Drosophila* (Morrow et al., 2004) also has been attributed at least in part to increased resistance to oxidative stress. The Raf/MEK/ERK signaling pathway is one of the central mediators in response to oxidative damage (Finkel and Holbrook, 2000; Yoon et al., 2002). Activation of the ERK pathway in response to oxidative stress is reduced with age, and the loss of oxidative stress tolerance with age is linked to reduced ERK activity (Ikeyama et al., 2002). These findings suggest that the increased ERK activation in *AC5* KO mice may result in an increased tolerance to oxidative stress. Our data indicate that MnSOD expression is reduced when ERK activation is inhibited, suggesting that MnSOD could be a downstream effector of the ERK signaling pathway. We suggest that knocking out *AC5* activates ERK signaling, which, in turn, promotes the upregulation of MnSOD, leading to resistance to oxidative stress (Figure 7).

Development of osteoporotic and osteosclerotic bone, demonstrated by loss of the nonweight-bearing fibula, is commonly observed in inbred and outbred mice as they approach two years of age (Globocnik and Rajtova, 1978; Loutit et al., 1976) and is similar to our observations made in the old WT mice. In contrast, bone quality was preserved in the old female *AC5* KO mice. In osteoblasts, ERK activation has been associated with proliferation, mechanical responsiveness, and differentiation (Kapur et al., 2004; Lai et al., 2001). Furthermore, ERK signaling is necessary for bone morphogenetic protein-induced osteoblast differentiation (Gallea et al., 2001). Thus, loss of *AC5* function leads to enhanced osteoblast function through ERK activation. In the current investigation, we observed protection against adverse effects of aging on bone and on the heart, and we also observed similar cellular mechanisms in kidney and brain. However, it is conceivable that the protective mechanisms observed in *AC5* KO mice are not expressed in all tissues.

The mechanism for longevity has been studied most extensively in CR (Sinclair, 2005). Like mice with CR, old *AC5* KO mice exhibit lower body weights. Interestingly, the food intake in young *AC5* KO mice was enhanced, suggesting that lifespan extension in *AC5* KO mice may not be mediated by reduced caloric intake in young animals, although we cannot exclude the possibility that *AC5* KO may cause a reduction in food intake in later stages of life, thereby mimicking CR. Alternatively, *AC5* KO may induce metabolic effects similar to CR, which stimulates the eating behavior of young *AC5* KO mice. In fact, inhibition of the cAMP-PKA pathway and CR function

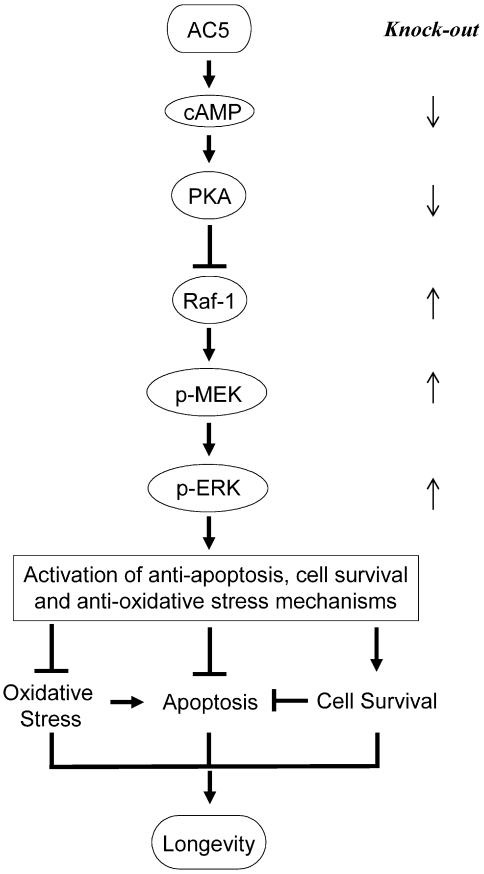

Figure 7. The Proposed Mechanism Mediating Longevity and Stress Resistance in *AC5* KO Mice

Knocking out *AC5* activates the Raf/MEK/ERK signaling pathway. The activation of ERK activates antioxidative stress, antiapoptosis, and cell survival mechanisms, which lead to longevity in *AC5* KO mice. The arrows indicate the direction of signaling (↑ indicates increase, and ↓ indicates decrease).

in the same pathway to extend replicative lifespan in yeast, and mutations that decrease PKA have been suggested as genetic models of CR (Lin et al., 2000). Thus, further investigation will be required to determine whether *AC5* KO and CR utilize a common mechanism for lifespan extension in mice.

Review of the literature and our own preliminary studies indicate that with the exception of particular neoplasms that vary from mouse strain to strain, the pathology at the time of death is quite diverse (Chrisp et al., 1996; Storer, 1966; Wolf et al., 1988). Malignant lymphoma and alveologenic neoplasms are the principle causes of death in the C57BL/6J strain (Wolf et al., 1988). One of few studies of 129/SvJ mice (Storer, 1966) summarized the major pathology of the mice as "nonspecific" with tumors in 20% of females and 10% of males. The summary of tumor incidence in mouse strains from Jackson Lab (Naf et al., 2002) indicates five organs with moderate tumor incidence in 129/SvJ mice: mammary gland, lymphohematopoietic, lung, liver, and vulva. By autopsy of mice with 129/SvJ-C57BL/6J strain, we observed that lymphoma was a common tumor in WT, and this type of tumor was ameliorated in AC KO mice (unpublished data). Cancer protection has also been observed in other longevity models, e.g., CR and GH/IGF-I deficiency (Yang et al., 2005; Zhu et al., 2005). The mechanism of lifespan extension in *AC5* KO mice is still not established, but delayed tumor incidence compared to WT mice could be one mechanism, whereas protection against the cardiomyopathy of aging could be another.

In summary, the data from the current investigation suggests that disruption of *AC5* mediates activation of the Raf/MEK/ERK signaling pathway and results in protection from oxidative stress, apoptosis, and osteoporosis, leading to longevity in *AC5* KO mice (Figure 7). Aging increases the susceptibility of organs to various age-related diseases. Retarding the aging of the individual organ could be a fundamental therapy to prevent age-related diseases. *AC5* could be a novel target to prevent age-related heart disease and possibly prolong the lifespan of humans.

EXPERIMENTAL PROCEDURES

Generation of Knockout Mice

The *AC5* gene was disrupted by deleting the exon with the first translation initiation site using homologous recombination as described previously (Okumura et al., 2003c). All mice were 129/SvJ-C57BL/6 mixed-background littermates from F1 heterozygote crosses. Equal numbers of male and female mice were studied, except for the bone integrity experiments, where only female mice were studied. This study was approved by the Institutional Animal Care and Use Committee at New Jersey Medical School.

Evaluation of Bone Integrity

The X-ray radiography was taken for the comparison of bone density, healing stress fracture, and calcification between WT and *AC5* KO mice. Femur structural properties (peak torque) and material properties (shear stress and shear modulus) were determined from the force-displacement curves and bone dimensions as previously described (Manigrasso and O'Connor, 2004).

Measurement of GH Level

GH level was measured using a Mouse/Rat GH ELISA Kit from Diagnostic Systems Laboratories (Webster, TX).

Evaluation of Apoptosis

DNA fragmentation was detected in situ using terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining, as published previously (Geng et al., 1999).

2D Gel Electrophoresis and Protein Identification

Brain tissues from 20-month-old *AC5* KO and WT mice were homogenized in a 2D compatible buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Biolytes, 1% Triton, 1% DTT, and phosphatase and protease inhibitors) and centrifuged at 12,000 × g for 30 min at 4°C, and the protein concentration was determined using the Bradford method. Proteins were separated in the first dimension according to their isoelectric point by isoelectric focusing (IEF) and then resolved in the second dimension, according to the molecular weight by SDS-PAGE. IEF was performed using Immobilized pH Gradient (IPG) strips with a pH range of 5–8 on a BioRad IEF cell with a programmed voltage gradient. SDS-PAGE was performed on a 12.5% polyacrylamide gel. Gels were

stained with SYPRO Ruby (Bio-Rad) and scanned with a Typhon 9400 imager (Amersham Biosciences). 2D gel spots of interest were excised, destained, and digested with modified trypsin. In gel tryptic digests were extracted and analyzed by ABI 4700 MALDI-TOF/TOF mass spectrometers. Spectra were acquired, and measured peptide masses were searched in the NCBInr protein sequence database using the MS-Fit and ProFound search engines with a mass tolerance of 50 ppm and a required four peptide minimum match.

Western Blotting

The blots were probed with primary antibodies incubated overnight at 4°C. The immunopositive bands were visualized by using Western Lightning chemiluminescence reagent (Perkin Elmer Life Sciences, MA). All western blot exposures were in the linear range of detection, and the intensities of the resulting bands were quantified by Quantity One software on GS-800 densitometer (Bio-Rad).

Primary Culture of Neonatal Mouse Ventricular Myocytes and Oxidative Stress Treatment

Primary cultures of ventricular cardiac myocytes were prepared from 1-day-old *AC5* KO and WT mice. Briefly, ventricular myocytes were enzymatically dissociated and preplated for 1 hr to enrich for myocytes. Cells were plated onto either gelatin-coated 60 mm culture dishes or coverslips and cultured in cardiac myocyte culture media containing DMEM/F-12 supplemented with 5% horse serum, 4 μg/ml transferrin, 0.7 ng/mL sodium selenite (GIBCO BRL), 2 g/L bovine serum albumin (fraction V), 3 mM pyruvic acid, 15 mM HEPES (pH 7.6), 100 μM ascorbic acid, 100 μg/ml ampicillin, 5 μg/ml linoleic acid, and 100 μM 5-bromo-2′-deoxyuridine (Sigma Chemical Co.). Culture media was changed to serum-free media after 24 hr. Myocytes were further cultured under serum-free conditions for 48 hr before experiments. We obtained myocyte cultures in which more than 95% are myocytes, as assessed by immunofluorescence staining with a mAb against sarcomeric myosin (MF20). After culturing for 48 hr, myocytes were treated by UV (10 and 25 mJ/cm^2) and H_2O_2 (25–100 μM) and evaluated for cell viability using CellTiter-Blue Cell Viability Assay (Promega Co., Madison, WI).

Primary Culture of Fibroblasts and Oxidative Stress Treatment

MEFs were isolated from embryos of homozygous *AC5* KO mice or *AC5* WT mice. Mouse adult skin-derived fibroblasts were isolated from tails of homozygous *AC5* KO mice or *AC5* WT mice. The genotypes of MEFs were identified by PCR analysis using genomic DNA. The fibroblasts were expanded for another two passages before use. All fibroblasts were cultured in complete DMEM (Dulbecco's modified Eagle's medium) with high glucose (Hyclone) containing 10% FBS, 200 μM L-glutamine, and 1% penicillin-streptomycin. The third passage cells were treated by H_2O_2 (25–400 μM) or UV light (25–500 mJ/cm^2). Cell survival rate was determined after 1–2 days using trypan blue exclusion method. Histone-associated DNA fragmentation was analyzed for apoptotic cell death by ELISA (Cell Death Detection ELISA Plus, Roche).

ERK1 and ERK2 siRNA Transfection

ERK1 (sc-29308) and ERK2 (sc-35336) siRNA from Santa Cruz Biotechnology were used to knock down gene expression of ERK1 and ERK2 in *AC5* MEFs. Briefly, in a 96-well tissue culture plate, 1.3 × 10^4 cells were seeded per well in antibiotic-free normal growth medium supplemented with FBS. The cells were incubated at 37°C until 60%–80% confluent. Cells were then incubated at room temperature for 5 min with Lipofectamine 2000 (0.94 μl) and OPTI medium (9.4 μl). siRNA control (sc-37007; 40 μl) or of ERK1 (20 μl) and of ERK2 (20 μl) siRNA was then added, and cells were incubated for an additional 20 min. The cells were washed with 200 μl OPTI medium. 94 μl of OPTI medium was added to the well, followed by siRNA transfection mixture, and incubated for 4 hr at 37°C. This mixture was then replaced by normal growth medium and incubated for 50 hr. The cells were treated overnight with 100 μl 2% FBS growth medium containing different concentrations of H_2O_2 and were then analyzed for viability.

Yeast Methods

The *cyr1-1* mutant strain and its corresponding WT strain were used (Fabrizio et al., 2001). The cDNA encoding ERK2 was inserted into the YEplac195 plasmid (containing a *LEU2* gene); ERK2 is under the control of the ADH1 promoter (YEplac195-ADH-ERK2). Expression levels were tested by western blotting analysis using polyclonal antibodies against ERK2 protein. The YEplac195-ADH (empty plasmid) and YEplac195-ADH-ERK2 plasmids were transformed into both a WT and a *cyr1-1* mutant yeast strain. The plasmids were maintained in a complete synthetic medium lacking leucine (YC-Leu). The ORF of the *KSS1* gene was knocked out precisely by gene disruption using a *HIS3* marker gene. Yeast strains were tested for responses to different stresses: cells were incubated for 5 min at 55°C or in the presence of 25 mM hydrogen peroxide at 30°C for 30 min. Chronological lifespan was determined as previously described (Fabrizio et al., 2003; Sun et al., 2002). Experiments were repeated four times.

Statistical Analysis

Data are expressed as mean ± SEM. For comparison of two groups, Student's t test was used. Survival curves were compared using Chi Square, Kaplan-Meier survival analysis or ANOVA with Fisher's PLSD test. Regression lines were compared for differences in slope using the Analysis of Covariance (ANCOVA). Significance was accepted at $p < 0.05$.

Supplemental Data

Supplemental Data include two tables and four figures and can be found with this article online at http://www.cell.com/cgi/content/full/130/2/247/DC1/.

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