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Calorie Restriction Promotes Mammalian Cell Survival by Inducing the SIRT1 Deacetylase

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A major cause of aging is thought to result from the cumulative effects of cell loss over time. In yeast, caloric restriction (CR) delays aging by activating the Sir2 deacetylase. Here we show that expression of mammalian Sir2 (SIRT1) is induced in CR rats as well as in human cells that are treated with serum from these animals. Insulin and insulin-like growth factor 1 (IGF-1) attenuated this response. SIRT1 deacetylates the DNA repair factor Ku70, causing it to sequester the proapoptotic factor Bax away from mitochondria, thereby inhibiting stress-induced apoptotic cell death. Thus, CR could extend life-span by inducing SIRT1 expression and promoting the long-term survival of irreplaceable cells.

In mammals, caloric restriction (CR) delays the onset of numerous age-associated diseases including cancer, atherosclerosis, and diabetes, and can greatly increase life-span (1). The molecular mechanisms underlying this effect are not known. In the budding yeast *Saccharomyces cerevisiae*, CR extends life-span by increasing the activity of the Sir2 protein (2–5), a member of the conserved sirtuin family of nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylases (6). In yeast and *Caenorhabditis elegans*, life-span is extended by the presence of extra copies of the *SIR2/Sir-2.1* gene (7, 8), or by small-molecule sirtuin agonists called STACs (9). In mammals, it is becoming increasingly apparent that SIRT1 is a key regulator of cell defenses and survival in response to stress (10–14).

In response to damage or stress, cells attempt to repair and defend themselves, but if unsuccessful, they often undergo programmed cell death, or apoptosis. Numerous studies show that aging is associated with increased rates of stress-induced apoptosis (15), and the cumulative effects of cell loss have been implicated in various diseases including neurodegeneration, retinal degeneration, cardiovascular disease, and frailty (15–17). Consistent with this, rodents subjected to

CR and long-lived genetic mutants such as the p66^{sch} knockout mouse are typically less prone to stress-induced apoptosis (18, 19).

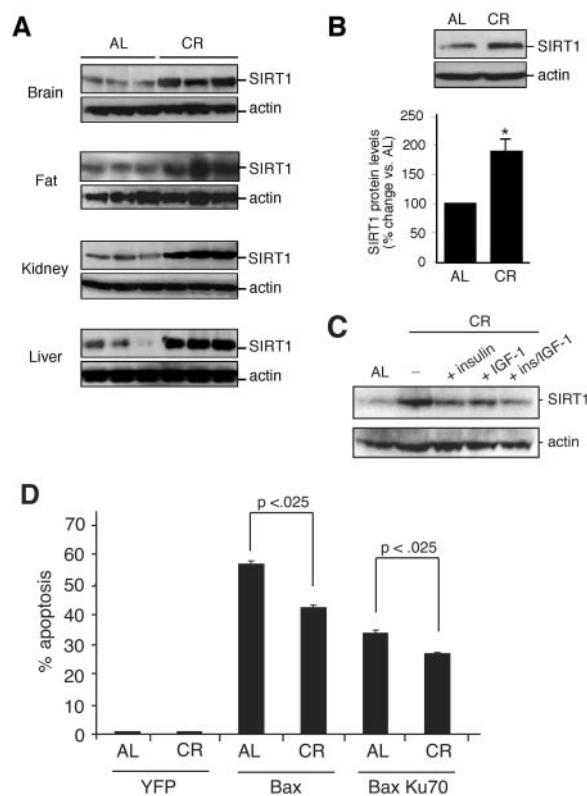
A critical step in initiating stress-induced apoptosis is the relocalization of the Bax protein from the cytoplasm to the outer mitochondrial membrane and the subsequent

release of cytochrome c. Downstream events include caspase activation and cleavage of poly(ADP-ribose) polymerase (PARP). Under normal conditions, Bax is rendered inactive in the cytoplasm by its tight association with Ku70, a DNA repair factor (20, 21). In response to acute cell damage or stress, two critical lysines in Ku70 (K539, K542) become acetylated by the acetyltransferases CBP and PCAF, and the Ku70-Bax interaction is disrupted, allowing Bax to localize to mitochondria and initiate apoptosis (21).

Given the role of yeast Sir2 in life-span extension by CR (2), we investigated whether mammalian SIRT1 might also be involved in the CR response. Tissues were extracted from 12-month-old rats that had either been fed ad libitum (AL) or a CR diet (60% of AL) since weaning. Compared to expression in AL animals, SIRT1 expression was higher in many tissues of the CR animals, including brain, visceral fat pads, kidney, and liver (Fig. 1A).

To investigate whether SIRT1 might be responsible for the ability of CR to protect the cells of animals from stress-induced apoptosis, we used an in vitro cell culture model that recapitulates key in vivo proliferative and phenotypic features of CR (22). In this system, cells are cultured in the presence of serum from calorically restricted rats, resulting in the induction of characteristic stress-

Fig. 1. Caloric restriction increases SIRT1 expression in a variety of rat tissues and inhibits Bax-mediated apoptosis. **(A)** Twelve-month-old male Fisher 344 rats were fed NIH-31 standard feed ad libitum (AL) or a daily food allotment of 60% of that eaten by the AL animals (CR) immediately after weaning. Extracts of tissues (~25 µg) from AL and CR animals were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and probed with a rabbit polyclonal antibody against SIRT1, or monoclonal antibody against β-actin, which served as a loading control **(B)** HEK 293 cells were grown for 48 hours in Dulbecco's minimum essential medium (DMEM) supplemented with serum from AL or CR animals. Total protein extracts (50 µg) were separated by SDS-PAGE and probed for SIRT1 and β-actin. **(C)** FaO cells were grown in serum from CR animals supplemented with insulin (ins, 2.2 ng/dl) or IGF-1 (429 ng/dl), or both (22). **(D)** 293T cells were grown in DMEM containing 10% serum from either AL rats or CR rats. After 24 hours, cells were transfected with YFP (1 µg), YFP-Bax (1 µg), or YFP-Bax (1 µg) and Ku70 (2 µg). The percentage of YFP-positive cells with apoptotic nuclei was scored 24 hours after transfection. Values are the average of three experiments, and >200 cells were scored. Error bars represent the SEM, and statistical significance was assessed using logistic regression.



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response genes and the attenuation of stress-induced apoptosis (22). SIRT1 expression was ~twofold higher in human embryonic kidney (HEK) 293T cells grown in the presence of CR serum as compared to cells grown in AL serum (Fig. 1B).

In worms, flies, and mice, mutations in the insulin/insulin-like growth factor 1 (IGF-1) pathway can extend life-span (6, 23). The levels of insulin and IGF-1 are 8- and 1.4-fold lower, respectively, in CR rodents as compared to AL rodents (22). To determine whether the induction of SIRT1 expression by CR serum was due, in part, to reduced levels of these factors, FaO rat hepatoma and HEK 293 cells were treated with CR serum supplemented with insulin or IGF-1, or both. Addition of either factor reduced SIRT1 expression, and together there was a slight additive effect (Fig. 1C) (24).

To test the effect of the CR serum on rates of stress-induced apoptosis, we used a well-characterized assay whereby 293T cells were transfected with Bax tagged with yellow fluorescent protein (Bax-YFP), in the presence or absence of a Ku70 expression construct

(20, 21). YFP-positive cells were scored 24 hours later for a fragmented nucleus, a common marker of apoptosis (20, 21). Because Ku70 sequesters Bax from mitochondria, cells overexpressing Bax alone are more susceptible to apoptosis than are cells overexpressing both Bax and Ku70. In the presence or absence of Ku70 overexpression, 293T cells grown in serum from CR animals were less susceptible to Bax-mediated apoptosis as compared to cells grown in media with AL serum (Fig. 1D).

Based on these results and the role of Ku70 acetylation in the regulation of Bax-mediated apoptosis (21), we speculated that SIRT1 and Ku70 might work together to modulate the susceptibility of cells to stress-induced apoptosis. When 293T cells were either treated with resveratrol, a small-molecule agonist of SIRT1 (9), or transfected with a SIRT1 expression vector, a dose-dependent suppression of Bax-mediated apoptosis was observed within 24 hours (Fig. 2). Conversely, expression of a dominant-negative SIRT1 allele (H363Y) increased the susceptibility of cells to Bax-mediated apoptosis (Fig. 2D). The H363Y allele also increased the amount

of cleaved PARP seen after 12 hours (Fig. 2E) (24). Small interfering RNAs (siRNAs) against SIRT1 had a similar effect (Fig. 2F and fig. S1).

Coimmunoprecipitation experiments indicated that SIRT1 physically associates with Ku70 in vivo (Fig. 3A). Two specific lysines in Ku70 (K539 and K542) promote the release of Bax when acetylated (Fig. 3B). Overexpression of wild-type SIRT1 reduced the overall acetylation level of Ku70 in vivo, whereas overexpression of the SIRT1-H363Y allele had the opposite effect (Fig. 3C). To identify which lysines on Ku70 are targeted for deacetylation by SIRT1, we performed two assays. Recombinant SIRT1 was incubated with an acetylated Ku70 peptide, and after a set time the remaining level of acetylation was ascertained by probing a blot of the reaction with a pan-acetyl-lysine-specific antibody (Fig. 3D). In a more quantitative assay, SIRT1 was incubated with an acetylated Ku70 fluorogenic peptide and assayed as previously described (9) (Fig. 3E). A p53 peptide, acetylated on lysine 320, served as a positive control. Both assays indicated that SIRT1 efficiently deacetylated the two lysines in the C-terminus of Ku70 that are critical for regulating Bax (Fig. 3, D and E).

To test whether the regulation of Bax by SIRT1 involves these two Ku70 residues in vivo, we replaced each residue with arginine to mimic a constitutively deacetylated state (21) and assessed the mutant alleles for suppressing apoptosis in the absence of SIRT1 function. Residue K331 of Ku70 served as a negative control because this residue is acetylated in vivo (21), but is both a poor substrate of SIRT1 (Fig. 3D) and plays no apparent role in Bax-mediated apoptosis in vivo (21). Human 293 cells stably expressing the SIRT1-H239Y allele were transfected with each of the mutant Ku70 alleles, and the percentage of Bax-mediated apoptosis was determined. The H363Y allele of SIRT1 promoted Bax-mediated apoptosis in the K331R-transfected cells, but not in the K539R- or K542R-transfected cells, indicating that SIRT1 targets K539 and K542 in vivo and that deacetylation of either K539 or K542 is sufficient to suppress Bax-mediated apoptosis (Fig. 3F).

To determine whether interfering with SIRT1 function would block CR-mediated attenuation of apoptosis, we expressed SIRT1-H363Y, or siRNA against SIRT1, in the presence of AL or CR serum, and assessed apoptosis. Overexpression of H363Y abrogated the ability of CR to attenuate Bax-mediated apoptosis (Fig. 4A), and siRNA against SIRT1 completely abolished the effect of CR serum (Fig. 4B). Consistent with this, both the dominant-negative SIRT1 and SIRT1 siRNA reduced the association be-

Fig. 2. SIRT1 promotes the ability of Ku70 to suppress Bax-mediated apoptosis. (A) 293T cells were transfected with YFP (1 μ g) or YFP-Bax (1 μ g) and Ku70 (2 μ g). Twelve hours after transfection, the medium was supplemented with resveratrol (0, 50, or 100 nM), and the percentage of YFP-positive cells with apoptotic nuclei was scored 24 hours after transfection. Values are the average of three experiments, and >200 cells were scored. Error bars represent the SEM. (B) Protein extracts (50 μ g) from 293 cells stably expressing SIRT1 or a dominant-negative version of SIRT1 (H363Y) were separated by SDS-PAGE and probed for SIRT1 and then β -actin. (C and D) Cells were transfected with YFP (1 μ g), YFP-Bax (1 μ g), or YFP-Bax (1 μ g) and Ku70 (2 μ g), and the percentage of apoptosis was scored. Statistical significance was assessed using logistic regression. (E) 293 cells or 293 cells expressing SIRT1-H363Y were transfected with YFP (1 μ g) or YFP-Bax (1 μ g). Twelve hours after transfection, protein extracts were separated by SDS-PAGE and probed for PARP and then β -actin. (F) 293 cells were transfected with either siRNA empty vector or siRNA-SIRT1 vector (1 μ g). Twenty-four hours after transfection, cells were transfected with siRNA vector or siRNA-SIRT1 together with either YFP, YFP-Bax, or YFP-Bax and Ku70, and the percentage of apoptosis was scored 24 hours later. The sequence of siRNA-SIRT1 is GAA GTT GAC CTC CTC ATT GT.

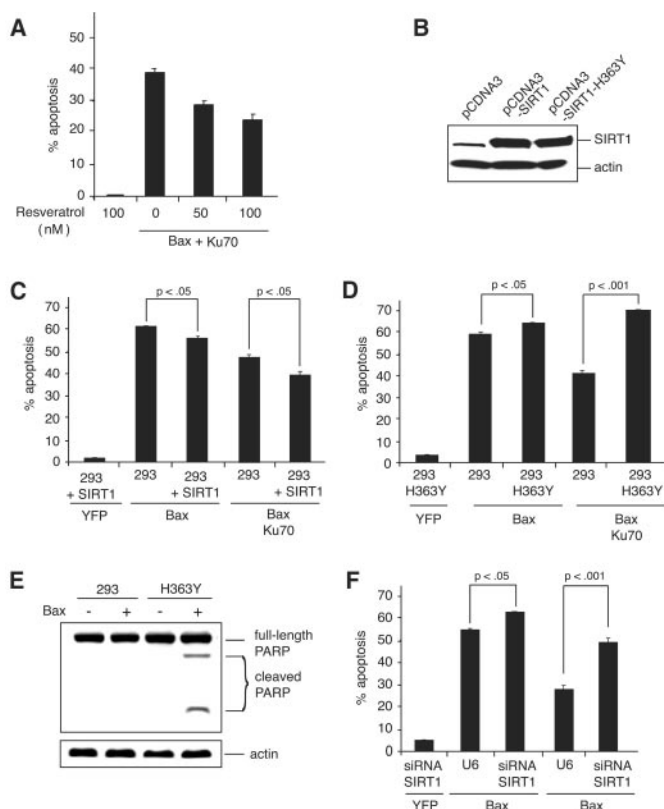


Fig. 3. SIRT1 attenuates Bax-mediated apoptosis by deacetylating two critical lysines in the C-terminus of Ku70. (A) SIRT1-Ku70 coimmunoprecipitation experiments were performed as previously described (21) (B) Ku70 Bax-binding domain and acetylated lysines K331, K539, and K542. (C) Protein extracts (1 mg) from 293 cells stably transfected with pCDNA, pCDNA-SIRT1-H363Y, and pCDNA-SIRT1 were incubated with protein A/G Sepharose beads, and then precipitated with a goat polyclonal antibody to Ku70. Acetylation levels were determined as previously described (21), and the membrane was re-probed for Ku70. (D) Two peptides—DYN-PEGK-AcVTKRKC and PEGKVTK-AcRKHDNC, corresponding to acetylated K539 and K542 of Ku70—were incubated in deacetylase buffer with or without recombinant SIRT1 (0.5 μg of protein, 37°C, 60 min). Reactions were run on a 10-kD size exclusion column, and the flow-through was slot blotted and probed for acetylation. (E) SIRT1 fluorogenic deacetylation assays using acetylated Ku70-K331, K539, and K542 (9). (F) 293 cells or 293 cells expressing SIRT1-H363Y were transfected with YFP-Bax (1 μg) and pCDNA-Ku70 (2 μg) or Ku70 mutants bearing K → R substitutions for K331, K539, and K542 (2 μg).

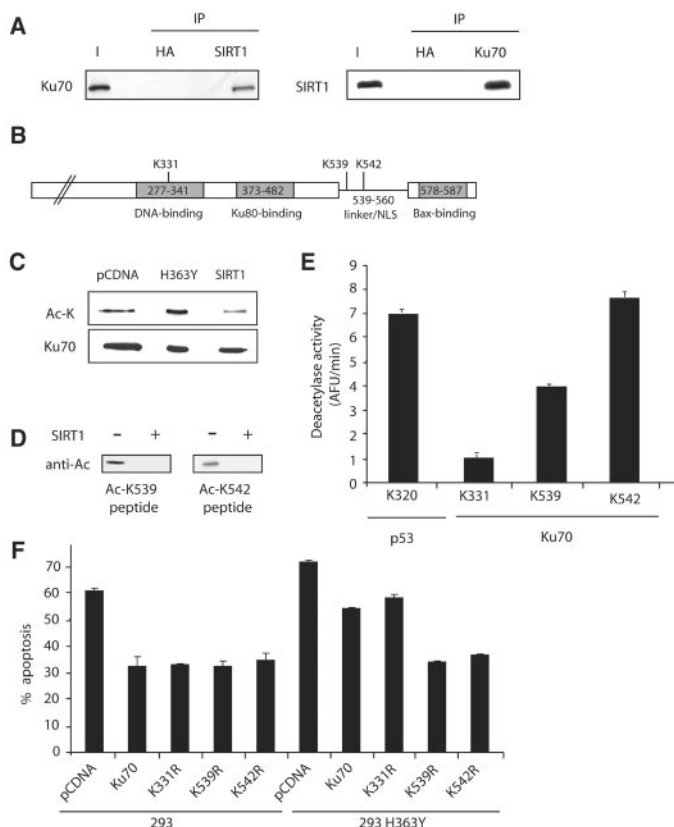
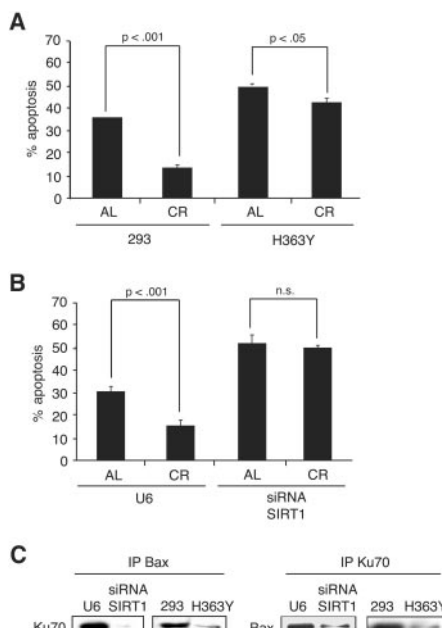


Fig. 4. Attenuation of apoptosis by CR serum and the interaction between Ku70 and Bax is SIRT1 dependent. (A) 293 cells and 293 cells stably expressing the dominant-negative SIRT1 (H363Y) were grown in DMEM containing 10% serum from AL or CR rats. After 24 hours, cells were transfected with YFP-Bax (1 μg) and Ku70 (2 μg); the percentage apoptosis was determined 24 hours after transfection. (B) 293 cells were grown in AL or CR serum-supplemented media then transfected with either siRNA vector or siRNA-SIRT1 vector (1 μg). Twenty-four hours after transfection, the cells were again transfected with siRNA empty vector or siRNA-SIRT1 (1 μg) together with YFP, YFP-Bax, or YFP-Bax and Ku70, as in (A). The percentage of YFP-positive cells with apoptotic nuclei was scored 24 hours after the second transfection. Statistical significance was assessed using logistic regression analysis. (C) Endogenous Ku70 and Bax from 293 cells or 293 cells stably expressing H363Y were coimmunoprecipitated in CHAPS buffer and analyzed by Western blotting.



tween Ku70 and Bax (Fig. 4C). We conclude that SIRT1 maintains residues K539 and K542 of Ku70 in a deacetylated state to keep Bax sequestered from mitochondria.

Thus, CR induces SIRT1 expression in a wide array of tissues, and this shifts the balance away from cell death toward cell survival. In addition to the Ku70-Bax pathway,

numerous other SIRT1 targets are presumably affected by CR, including p53 and FOXO3 (10–14). These data also indicate that the systemic regulation of mammalian SIRT1 is mediated, in part, by insulin and IGF-1, two serum factors that are involved in life-span regulation in a variety of species.

We postulate that the induction of SIRT1 by CR is an evolutionarily ancient biological stress response that slows aging by increasing the long-term function and survival of critical cell types. Calorically restricted rodents and long-lived mutant mice are protected from cancer despite attenuating apoptosis possibly because their cells possess heightened defenses and repair mechanisms, and they retain the ability to undergo apoptosis if the damage is beyond repair. The induction of SIRT1 in visceral fat pads is particularly interesting in light of the recent demonstration that this tissue is involved in the regulation of rodent life-span (23).

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