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SirT1 inhibition reduces IGF-I/IRS-2/Ras/ERK1/2 signaling and protects neurons

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Abstract

Sirtuins have been shown to protect cells and delay aging, but our laboratory showed that *S. cerevisiae* Sir2 can also increase stress sensitivity and limit life span extension. Here we provide evidence for a role of the mammalian Sir2 ortholog SirT1 in the activation of a pathway that sensitizes neurons to oxidative damage. SirT1-inhibition increased acetylation and decreased phosphorylation of IRS-2, reduced Ras activation and reduced ERK1/2 phosphorylation suggesting that SirT1 may enhance IGF-I signaling in part by deacetylating IRS-2. Either of the inhibition of SirT1 or of Ras/ERK1/2 were associated with resistance to oxidative damage. Although both markers of oxidized proteins and lipids were reduced in the brain of old SirT1 deficient mice, the life span of the homozygote knock out mice was shorter under both normal and calorie restricted conditions. These results are consistent with findings in *S. cerevisiae* and other model organisms suggesting that mammalian sirtuins can play both protective and pro-aging roles.

Introduction

Sirtuins, or Sir2 family proteins, are NAD^+-dependent class III histone deacetylases conserved from bacteria to humans (Frye, 2000). Sir2 modulates aging and life span in yeast, *C. elegans and Drosophila* (Fabrizio et al., 2005; Kaeberlein et al., 1999; Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). Though earlier studies showed the anti-aging effect of Sir2 and its function in calorie restriction (CR) in lower eukaryotes, later studies found that Sir2 is not required for CR-induced life span extension in yeast (Kaeberlein et al., 2004) or worms (Hansen et al., 2007; Kaeberlein et al., 2006; Lee et al., 2006). Notably, our lab showed that lack of Sir2 in yeast further extended the lifespan of calorie restricted cells, or long-lived Sch9 (Fabrizio et al., 2005) or of mutants with deficiencies in the Ras/cAMP pathway suggesting that Sir2 can also promote aging. SirT1, the mammalian ortholog of yeast Sir2, has been shown to regulate numerous physiological processes including glucose metabolism, DNA repair and apoptosis (Bordone et al., 2006; Cohen et al., 2004; Luo et al., 2001; Moynihan et al., 2005; Rodgers et al., 2005; Sun et al., 2007). In mammalian cells, SirT1 regulates several stress-

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response factors, such as p53 tumor suppressor (Langley et al., 2002; Vaziri et al., 2001), forkhead transcription factors (Brunet et al., 2004; Motta et al., 2004), and NF-κB (Yeung et al., 2004) yet it remains unclear if/how SirT1 regulates resistance to oxidative stress. Notably, SirT1 is expressed at a high level in the brain compared to other organs (Michishita et al., 2005).

Here we studied primary rat cortical neurons and other mammalian cells to test the role of SirT1 in stress resistance. Because we have shown that the down-regulation of either the Ras or Sch9 pathway extends longevity and increases stress resistance in yeast, and because homologs of these signal transduction proteins are major components of the pro-aging insulin/IGF-I-like pathways, we focused on the potential role of SirT1 in regulating mammalian Ras and Akt/S6k signaling. In agreement with our results in *S. cerevisiae*, we found that inhibition of SirT1 increased resistance to oxidative stress in neurons. Our data indicate that this effect is mediated in part by the deacetylation of IRS-2 and up-regulation of insulin/IGF-1R/IRS-2/Ras/ERK1/2 signaling. Although we also show that mice lacking SirT1 have reduced levels of markers of oxidative damage in the brain, these cellular effects did not translate into a longer life span and in fact the mice lacking SirT1 displayed severe developmental defects and were short lived under both ad lib and calorie restricted conditions. These results indicate that the role of SirT1 deficiency in protecting aginst oxidative damage is eclipsed by the important role of this deacetylase in many normal functions.

Results

Inhibition of SirT1 increases oxidative stress resistance in neurons

Previous studies in our lab showed that deletion of *SIR2* increases stress resistance in *S. cerevisiae* (Fabrizio et al., 2005). Here we investigated whether SirT1, the ortholog of yeast Sir2, plays a similar role in mammalian cells. Oxidative stress was induced in cultured neurons by hydrogen peroxide (H_2O_2) or menadione (which generates both superoxide and H_2O_2) and cell viability was measured by MTT assay. 10 day old (DIV) cortical neuron cultures were incubated with 400 μM H_2O_2 or 7.5μM menadione for 24 hr followed by MTT assay. After H_2O_2 treatment about 26% neurons survived, whereas 50% survived menadione treatment (Fig. 1 A, B, C, D). We also pretreated neurons with SirT1 inhibitors, nicotinamide or sirtinol for 48 hrs and then subjected them to the above oxidative stress protocol. Nicotinamide (Nico) dose-dependently increased neuronal survival after H_2O_2 treatment. 500 μM and 5 mM of Nico pretreatment increased the survival of H_2O_2 treated cells to 52.3± 4.7% (P<0.01, H_2O_2 vs. H_2O_2+500 μM Nico, n=3, one-way ANOVA, Turkey's test) and 58.7±2.3% (P<0.001, H_2O_2 vs. H_2O_2+5 mM Nico, n= 3, one-way ANOVA, Turkey's test), respectively (Fig. 1 A); while 10 μM (P>0.05, H_2O_2 vs. H_2O_2+ 10 μM Nico, n=3, one-way ANOVA, Turkey's test) and 100 μM (P>0.05, H_2O_2 vs. H_2O_2+ 100 μM Nico, n=3, one-way ANOVA, Turkey's test) of Nico showed no improvement. Nico also dose-dependently rescued neurons in menadione-induced oxidative stress (Fig. 1 B). 500 μM and 5 mM Nico increased the survival of menadione-treated cells to 77.0 ± 2.2 % (P<0.01, menadione *vs.* menadione + 500 μM Nico, n=3, one-way ANOVA, Turkey's test) and 86.1±2.4% (P<0.001, menadione vs. menadione + 5 mM Nico, n=3, one-way ANOVA, Turkey's test), respectively; while 10 μM (P>0.05, menadione vs. menadione + 10 μM Nico, n=3, one-way ANOVA, Turkey's test) and 100 μM (P>0.05, menadione vs. menadione + 100 μM nicotinamide, n=3, one-way ANOVA, Turkey's test) of Nico showed no significant effect. These values were adjusted to the effect of Nico alone (Nico alone was not significantly different from control; **Fig. S1**).

Similar effects were achieved with another SirT1 inhibitor sirtinol. 25 μM and 50μM of sirtinol pretreatment increased the survival of H_2O_2 -treated neurons to 49.4 ± 3.6% (P<0.01, H_2O_2 vs. H_2O_2+25 μM sirtinol, n=3, one-way ANOVA, Turkey's test) and 70.4 ±1.2% (P<0.001, H_2O_2 vs. H_2O_2+ 50 μM sirtinol, n=3, one-way ANOVA, Turkey's test), respectively (Fig. 1

C, D); while 5 μM sirtinol (P>0.05, H_2O_2 vs. H_2O_2+5 μM sirtinol, n=3, one-way ANOVA, Turkey's test) showed no benefit. Sirtinol also dose-dependently increased neuronal survival in menadione-induced oxidative stress. 25 μM and 50μM of sirtinol pretreatment increased the viability of menadione-treated cells to 86.7±5.7% (P<0.01, H_2O_2 vs. H_2O_2+ 25 μM sirtinol, n=3, one-way ANOVA, Turkey's test) and 93.4±1.9% (P<0.01, H_2O_2 vs. H_2O_2+ 50 μM sirtinol, n=3, one-way ANOVA, Turkey's test), respectively, whereas 5 μM of sirtinol (P>0.05, H_2O_2 vs. H_2O_2+ 5 μM sirtinol, n=3, one-way ANOVA, Turkey's test) showed no effect. The ODs are adjusted to the effects of sirtinol alone (sirtinol decreased MTT reduction by 13–21% at 25–50 μM compared to control (**Fig. S1**).

To confirm that the stress resistance is caused by reduced SirT1 activity we transfected either control or SirT1 siRNA into 5 DIV neurons and assessed survival after H_2O_2 or menadione induced oxidative stress (Fig. 1 E). The sequence specificity of siRNA (aagtgcctcaaatattaataa) against rat SirT1 was verified by genome-wide search against the rat build 4 genome database (8014 sequences; 5,387,086,425 total letters, NCBI). At least 2 nt difference was found in the nearest match and no predicted rat sirtuins (SIRT2-7) were in the top 20 hits.

Knockdown of SirT1 increased the viability of H_2O_2 treated cells from 26.2±2.7 % to 57.5 ±3.7% (P<0.001, H_2O_2 vs. H_2O_2 + SirT1 siRNA, n=4, t-test). Inhibition of SirT1 also boosted the survival of menadione-treated cells from 54.2±2.7% to 78.5±3.7% (P<0.01, menadione vs. menadione + SirT1 siRNA, n=4, t-test). Viability of SirT1 siRNA treated cells did not differ from that of control. To confirm that SirT1 was indeed knocked down by siRNA we initially attempted to examine protein expression, however, none of the commercial SirT1 antibodies we have tried (05-707 Anti-Sirt1; 07-131 Anti-Sir2; Millipore, Billerica, MA) appear to bind specifically to SirT1 in rat neurons; so we carried out SirT1 deacetylase activity using a fluorometric kit assay 2 days after transfection (Fig. 1 F). SirT1 siRNA decreased SirT1 deacetylase activity to 53.2 ± 4.2% of control (P<0.01, n=4, control vs. SirT1 siRNA, t-test; due to possible detection of other deacetylases as well the down regulation effect of SirT1 siRNA could be even higher). Consistent with our research in yeast, these results in neurons suggest that inhibiting SirT1 can protect against oxidative stress.

Since IGF-I/Akt pathway has been shown to both protect and sensitize cells against oxidative damage (Kops et al., 2002; Song et al., 2005) we tested whether Akt phosphorylation was affected by SirT1 inhibitors but did not find significant change (**Fig. S5 A**), suggesting that the effect of SirT1 inhibition is unlikely to be mediated by Akt pathway.

SirT1 inhibition decreases Ras/ERK activation in culture and *in vivo*

Neuronal Ras/ERK (extracellular signal-regulated kinase) is a major pathway regulating oxidative stress. In our yeast studies we described the Ras pathway as a second pro-aging pathway in parallel to the Sch9 pathway (Fabrizio et al., 2003; Fabrizio et al., 2004). Deletion of *SIR2* in yeast further increased stress resistance in mutants lacking *SCH9* but not in those with reduced Ras/cAMP signaling (Fabrizio et al., 2005) raising the possibility that Sir2 may regulate components of the Ras/cAMP pathway. Based on our yeast results and because mammalian Ras functions downstream of the pro-aging IGF-I receptor we studied the role of SirT1 inhibitors on Ras/ERK signaling. Neurons cultured in neurobasal media supplemented with B27 (containing ~4 μg/ml insulin) showed a high basal activation of Ras (GTP-bound Ras), which was reduced by a 4 hr treatment of 60 μM of sirtinol (Fig. 2 A), indicating that SirT1 modulates Ras activity.

We also checked if ERK1/2, a major effector of Ras, is altered by SirT1 inhibition. 10–14 DIV rat cortical neurons were treated with vehicle, SirT1 inhibitors Nico for 48 hr, or sirtinol for 4 hr and cell lysates were collected for SDS-PAGE and blotted with anti-phospho-$ERK_{1/2}$ (P-ERK1/2) and anti-$ERK_{1/2}$, respectively. 10 mM and 25 mM of Nico decreased phosphorylated

ERK1/2 (Fig. 2 B). The effect of sirtinol was dose-dependent as 30 μM of sirtinol reduced the ratio of P-ERK/ERK by 45% and 60 μM of sirtinol reduced the ratio by 68% (Fig. 2 C, D) ($P<0.05$, control vs. sirtinol 30 μM; $P<0.01$, control vs. sirtinol 60 μM; n=4, One-way ANOVA, Turkey's test).

To confirm that the above effects are due to specific inhibition of SirT1, a plasmid encoding SirT1 siRNA was co-transfected with GFP to neurons. This plasmid has been shown to reduce SirT1 expression (Cohen HY et al., 2004). 48 hr after transfection the cells were immunostained with anti-P-ERK1/2 and anti-GFP (Fig. 2 E). The immunodensity of P-ERK1/2 in transfected cells was significantly lower than that of surrounding non-transfected cells ($P<0.05$, n=12, Student's t-test). As a control a plasmid encoding a dominant-negative SirT2, GFP-SirT2, was transfected into neurons. P-ERK1/2 staining remained the same in SirT2DN transfected cells compared to non-transfected ones ($P>0.05$, n=12, Student's t-test) (Fig. 2 F). These data confirm that SirT1 inhibition attenuates the activation of ERK1/2. We also checked whether the same occurs *in vivo* and indeed a decrease in ERK1/2 activation was observed in the hippocampus of 17-day-old SirT1 knockout (KO) mice ($P<0.05$, SirT1+/+ vs. SirT1−/−, n=6, t-test) (Fig. 2 G, H). Together, these data suggest that inhibition of SirT1 can down-regulate ERK1/2 activity.

We also tested whether the effect of SirT1 on ERK1/2 is observed in a human cell line and whether it inhibits IGF-I signaling. HEK293 cells were serum starved for 15 hrs and treated with IGF-I (500 ng/ml) for 5 min. Cell lysates were immediately collected for SDS-PAGE and immunoblotted for P-ERK or ERK (Fig. 2 I, J). Starved cells showed low levels of P-ERK1/2, whereas IGF-I induced a 12-fold increase in P-ERK1/2 ($P<0.01$, control vs. IGF-I, n=3, one-way ANOVA, Turkey's test). 4 hr pretreatment of 60 μM sirtinol markedly reduced this effect of IGF-I on ERK1/2 phosphorylation (60% decrease, $P<0.05$, IGF-I vs. IGF-I+sirtinol, n=3, one-way ANOVA, Turkey's test). To begin to determine how SirT1 inhibition affects ERK activation, we applied PMA (phorbol 12-myristate 13-acetate, a phorbol ester, 1 μg/ml), which is known to activate ERK1/2 through the PKC/Raf-1/MEK1 pathway (Liebmann, 2001), to starved cells. As expected, it induced a 24-fold increase in P-ERK1/2 ($P<0.001$, control vs. PMA, n=3, one-way ANOVA, Turkey's test) (Fig. 2 K, L). However, pretreatment with sirtinol did not block this effect ($P>0.05$, PMA vs. PMA + sirtinol, n=3, one-way ANOVA, Turkey's test) indicating that the target molecule(s) of SirT1 lies between insulin/IGF-IR and Ras.

Subcellular localization of SirT1 in the brain

For SirT1 to regulate the activation of Ras/ERK, SirT1 may either regulate the gene expression of upstream molecules, or act directly upon those molecules through post-translational modification. SirT1 had long been considered a nuclear protein until recently (Tanno et al., 2007). The subcellular localization of SirT1 in neuronal cells is poorly understood. We investigated the possibility that SirT1 acts on non-nuclear substrates by examining its localization in mouse brains (Fig. 3). Brains from postnatal day 3 (P3, Fig. 3 A) and adult (Fig. 3 B) mice were collected, homogenized and separated into nuclear, cytosol and membrane fractions through sucrose-gradient centrifugation. These fractions were then immunoblotted for SirT1, NeuN (as a nucleus marker), GAPDH (as a cytosol marker), and $GluR_{2/3}$ (a membrane associated protein). SirT1 antibody recognizes one band at 110 kDa, which is absent from the brain of SirT1 KO mice (Fig. 3 C), confirming the specificity of the signal. In P3 brains SirT1 is localized to both nuclear and cytosolic fraction with the majority residing in the cytosol; whereas in the adult brain it is found predominantly in the cytosolic fraction (Fig. 3 A, B).

Increased acetylation and decreased phosphorylation of IRS-2 by SirT1 inhibition

The localization of SirT1 to the cytosol could allow direct deacetylation of substrate protein (s) in the cytosol. The signaling pathway upstream of Ras is composed of sequential activation of receptors, insulin receptor substrates (IRS) or Shc, Grb2 and SOS. Among the signaling molecules between insulin/IGF-I receptors and Ras, insulin receptor substrate-1 (IRS-1) has been shown to be an acetylated protein (Kaiser and James, 2004). Therefore, we hypothesized that SirT1 may regulate insulin/IGF-I signal transduction by deacetylating IRS. To test this hypothesis we first conducted co-immunoprecipitation to see whether SirT1 physically interacts with IRS-1 or IRS-2. HEK293 cells were transfected with a plasmid encoding V5-tagged SirT1 and cell lysates were immunoprecipitated with anti-V5 or control IgG and probed with for IRS1 or IRS2, respectively (Fig 4. A). IRS-2, but not IRS-1, co-immunoprecipitated with V5-SirT1, suggesting that IRS-2 might be a substrate of SirT1. To determine whether SirT1 can directly deacetylate IRS-2, we set up an *in vitro* deacetylation experiment. We purified IRS-2 protein from HEK293 cells by immunoprecipitation and incubated equal amounts of purified IRS-2 with different doses of SirT1 and 1 mM of NAD for 1.5 hr and then blotted for acetylated-lysine (AcK) or IRS-2 (Fig 4. B). This experiment confirmed that IRS-2 is acetylated and that SirT1 can directly deacetylate IRS-2 in a cell-free system in agreement with a recent finding (Zhang, 2007).

Next we examined if SirT1 could deacetylate IRS-1 or IRS-2 in live cells and if this deacetylation affects IGF-I signaling. HEK cells were serum starved (0.5% FBS) for 15 hr and treated with 500 ng/ml of IGF-I for 5 min. IRS-1 or IRS-2 were immunoprecipitated from the cells and probed with AcK (Fig. 4 C, D, **Fig. S7**). IGF-I did not significantly alter the acetylation level of IRS-1 or IRS-2 (Fig. 4 D). However, pretreatment with 5 mM of Nico for 24 hr or 60 μM of sirtinol for 4 hr significantly increased the acetylation level of IRS-2 by 93.5% or 79.7%, respectively ($P<0.01$, IGF-I vs. IGF-I+Nico, n=4; $P<0.05$, IGF-I vs. IGF-I+sirtinol, n=4, one-way ANOVA, Turkey's test) but had no effect on IRS-1. To study whether the alteration of the acetylation level of IRS-2 affects its signaling we measured its phosphorylation level. Serum-starved HEK cells were treated with IGF-I with or without SirT1 inhibitors and IRS-2 was immunoprecipitated from the cells and probed for phosphotyrosine (PY). IGF-I induced a 14-fold increase in phosphorylation of IRS-2, which was significantly diminished by pre-treatment with Nico (46.7% decrease, $P<0.05$, IGF-I vs. IGF-I+Nico, n=4, one-way ANOVA, Turkey's test) or sirtinol (55.3% decrease, $P<0.05$, IGF-I vs. IGF-I+sirtinol, n=4, one-way ANOVA, Turkey's test).

To corroborate the results in HEK293 cells we also examined rat cortical neurons transected with control or SirT1 siRNA (Fig 4. E). Inhibition of SirT1 increased acetylation level of IRS-2 and decreased phosphorylation of IRS-2. Concurrently, activation of $ERK_{1/2}$ was reduced by SirT1 inhibition.

MEK/ERK1/2 inhibition protects neurons against oxidative stress

To test if down-regulation of ERK1/2 can account for the effect of SirT1 inhibition on stress resistance we applied MEK1/2 (immediately upstream of ERK1/2) inhibitors to cultured neurons. Oxidative stress was induced by H_2O_2 or 7.5 μM of menadione for 24 hr. The indicated groups of cells were pretreated with MEK1/2 inhibitor 10 μM U0126 or 10 μM SL327 for 4hr. MTT assay suggests that 400 μM H_2O_2 killed 71.4±5.4% of neurons (Fig. 5 A). Pretreatment with U0126 or SL327 increased the survival to 45.9±4.5% ($P>0.05$, H_2O_2 vs. H_2O_2+U, n=3, one-way ANOVA, Turkey's test) or 49.2±5.5% ($P<0.05$, H_2O_2 vs. H_2O_2+SL, n=3, one-way ANOVA, Turkey's test), respectively. Menadione (Md) reduced the viability to 49.9±5.3% compared to control. Pretreatment of U0126 or SL327 increased the survival to 73.9±3.7% ($P<0.05$, Md vs. Md+U, n=3, one-way ANOVA, Turkey's test) or 68.3±1.5% ($P>0.05$, Md vs. Md+SL, n=3, one-way ANOVA, Turkey's test), respectively. Similar results were obtained

with Live/Dead assay (Fig. 5 B, C). 100 μM of H_2O_2 led to 57.5±3.3% survival compared to control. Pretreatment of U0126 increased viability to 74.4±3.5% (P<0.05, H_2O_2 vs. H_2O_2+U, n=4, one-way ANOVA, Turkey's test), and SL327 increased survival to 74.1±3.6% (P<0.05, H_2O_2 vs. H_2O_2+SL, n=4, one-way ANOVA, Turkey's test). Similarly, menadione reduced viability to 52.1±2.6% compared to control. Pretreatment of U0126 increased viability to 65.8 ±3.8% (P>0.05, Md vs. Md+U, n=4, one-way ANOVA, Turkey's test), while SL327 increased survival to 69.4±2.6% (P<0.05, Md vs. Md+SL, n=4, one-way ANOVA, Turkey's test). Thus, inhibition of MEK1/ERK1/2 is sufficient to increase the stress resistance of neurons, suggesting that SirT1 can sensitize neurons to oxidative stress at least in part by up-regulating ERK1/2 activity.

As both SirT1 and ERK1/2 are involved in the regulation of apoptosis we studied how inhibition of SirT1 or ERK1/2 affects apoptosis in our system. We transfected neurons with SirT1 siRNA or treated them with MEK1/2 inhibitor SL327 and then exposed the cells to 400 μM H_2O_2 or 7.5 μM menadione for 24 hrs. We estimated necrosis by measuring LDH release in the media and quantified apoptosis by measuring cytoplasmic histone-associated mono- and oligonucleosomes with Cell Death Detection ELISA^{PLUS} kit (Fig. 5 D, E). H_2O_2 and menadione increased LDH release to 5.5±0.7 and 4.4±0.4 fold of control, respectively. Inhibition of SirT1 or ERK1/2 significantly alleviated the increase in LDH release (Fig. 5 D; P<0.01 compared to H_2O_2, P<0.05 compared to menadione; n=3, t-test). Meanwhile H_2O_2 and menadione induced apoptosis to 1.6±0.2 and 2.0±0.2 fold of control, respectively. Neither SirT1 nor ERK1/2 inhibition effectively reduced H_2O_2 induced apoptosis but SirT1 inhibition had a small but significant effect in reducing apoptosis induced by menadione (Fig. 5 E; 1.6 ±0.2 fold of control, P<0.05, n=10, t-test).

Reduced oxidative stress in SirT1−/− mouse brain

We have shown in neurons SirT1 inhibition protects against exogenous oxidative insult of H_2O_2 or menadione. To determine whether SirT1 can have similar effects *in vivo* we measured markers of oxidative damage in the brain of 18 month old mice. We removed brains (minus cerebellum) from SirT1 +/+ and −/− mice and measured protein carbonyl content with an ELISA kit (Fig 6. A) and lipid peroxidation with TBARS method (Fig 6. B). Compared to wild type (WT) mice (2.3±0.14 nmol/mg), KO brains showed 17% decrease in protein carbonyl content (1.9±0.1 nmol/mg; P<0.05, n=7, t-test). Lipid peroxidation was also reduced in KO brain (3.7±0.2 nmol malondialdehyde/mg), 20% less than that in WT (4.5±0.2 nmol/mg; P<0.05, n=7, t-test). The reduction of markers of oxidative stress in SirT1 KO brain is consistent with our findings in culture. Although a recent report discovered that SirT1 protects the heart against oxidative stress (Alcendor et al., 2007) these results suggest that SirT1 may play a different role in the brain and other cells.

We previously showed that lack of Sir2 in yeast extended further the lifespan of CR cells, or long-lived Sch9 but not under standard nutrient conditions (Fabrizio et al., 2005). Our current results suggest that SirT1 can also sensitize mammalian neurons to oxidative damage. To determine the role of SirT1 *in vivo* we monitored the life span of WT mice and those homozygous or heterozygous (HET) for disruption mutation in SirT1 gene (McBurney et al., 2003). The mice were fed ad-lib (Fig. 6 C) or on a 40% reduced calorie diet for 2 years (Fig. 6 D). Compared to WT and HET mice, SirT1 KO mice survived shorter on both normal and CR diet. This is in agreement with recent findings by others (Boily et al., 2008).

Discussion

Sirtuins including Sir2 and SirT1 have been described as mediators of the effect of calorie restriction on longevity and are widely believed to protect against aging (Cohen et al., 2004; Guarente, 2005). However, others have proposed that sirtuins are not required for the effects

of CR on life span (Hansen et al., 2007; Kaeberlein et al., 2004; Kaeberlein et al., 2006; Lee et al., 2006) and our previous results in yeast suggested that the lack of Sir2 can also increase resistance to stress and extend further the chronological life span of calorie restricted cells or yeast also lacking components of the Ras/cAMP/PKA or Sch9 pathway (Fabrizio et al., 2003; Fabrizio et al., 2001)(Fabrizio et al., 2005; Longo and Kennedy, 2006). In agreement with our results in *S. cerevisiae*, here we found that inhibition of SirT1 reduces IGF-I signaling and increases the resistance of mammalian cells to oxidative stress. Our data indicates that SirT1 increases insulin/IGF-I signaling in part by acetylating IRS-2, which promotes its phosphorylation. These post-translation modifications in IRS-2 are followed by Ras/ERK activation and sensitization of primary rat neurons to oxidative stress. Inhibition of Ras/Mek1/ERK1/2 activity was sufficient to protect the neurons against oxidative stress.

Whereas the level of markers of oxidative damage was reduced in the brain of SirT1−/− mice compared to controls, these apparently protective effects were not sufficient to counterbalance the positive effects of SirT1 as evident from the reduced mean life span of the homozygote knock out mice under both ad lib or calorie restricted diets. These results are consistent with the existence of a pro-oxidative stress role for mammalian SirT1 and Ras similar to that described for Sir2 and Ras in *S. cerevisiae* but confirm that sirtuins can play both positive and negative roles.

SirT1 was originally identified as a histone deacetylase. Consistent with this function, it was found to be localized in the nucleus in numerous studies (Michishita et al., 2005). Recently, however, Tanno *et al* examined different tissues and found SirT1 predominantly localized to the cytosol in mouse brain tissue (Tanno et al., 2007). We checked SirT1's localization in the brain. We performed sucrose-gradient centrifugation to separate the brain tissue into nuclear, cytosolic and membrane fractions. Immunoblots with these fractions indicate that SirT1 lies in both the nucleus and the cytosol with the preference of cytosol in postnatal day 3 brain; while SirT1 is almost exclusively retained in the cytosol in the adult brain. This is in line with increasing evidence pinpointing SirT1 as exhibiting cytosolic localization (Jin et al., 2007; Kim et al., 2007; Tanno et al., 2007; Zhang, 2007). Besides histones, various transcription factors have been identified as substrates for SirT1. SirT1-mediated deacetylase activity on these substrates seems to occur in the nucleus.

It has been proposed that SirT1 plays an anti-aging role in mammals by deacetylating and activating FOXO (Brunet et al., 2004; Kobayashi et al., 2005). Similarly, SIRT1 exhibits antagonistic effects of insulin on PGC-1α in gluconeogenesis (Rodgers et al., 2005). On the other hand, evidence is also accumulating in support of a synergic relationship between Sir2 and insulin/IGF-I. SirT1 was shown to increase the release of insulin or improve insulin sensitivity (Bordone et al., 2006; Moynihan et al., 2005; Sun et al., 2007). Moreover, SirT1 lowered the expression of IGF-binding proteins (IGFBP), a secreted inhibitory modulator of IGF function (Yang et al., 2005) and SirT1 knockout mice have increased expression of IGFBP1 (Lemieux et al., 2005). In agreement with these results, knockdown of liver SirT1 in mice reduced blood glucose concentration (Rodgers and Puigserver, 2007).

In our previous study in *S. cerevisiae* the deletion of *SIR2* increased further the resistance of mutants lacking *sch9* (homologous to S6kinase and Akt) to heat shock and oxidative stress but did not increase the resistance of mutants with defects in the Ras/cAMP pathway raising the possibility that Sir2 and Ras function in the same pathway (Fabrizio et al., 2005; Longo and Kennedy, 2006). Although a Ras/cAMP/PKA pathway has not been described in mammalian cells, we provide a mechanism linking SirT1 activity, the IGF-I/IRS-2/Ras/ERK pathway and stress resistance in mammalian cells. Our results suggest that inhibition of SirT1 downregulates insulin/IGF-I-dependent activation of ERK1/2 in part through decreased IRS-2 phosphorylation and decreased Ras activation. These results are consistent with findings that

Ras induces premature replicative senescence in primary mammalian cells and that SirT1-deficient mouse embryonic fibroblasts (MEF) have a dramatically extended replicative life span (Chua et al., 2005; Serrano et al., 1997). As expected if SirT1 acts upstream of Ras, SirT1 was not required for Ras-dependent accelerated replicative senescence (Chua et al., 2005). Thus, it is possible that inhibition of IRS-2 upstream of Ras is responsible for this effect of SirT1 deficiency on replicative senescence. Interestingly, the PI3K/Akt pathway, another major effector downstream of IRS-2 was unaffected by SirT1 inhibition, although over-expression of SirT1 was reported to increase Akt activation under insulin-resistant conditions but not under normal conditions (Sun et al., 2007). By contrast, only the ERK1/2 pathway but not the PI3K/Akt pathway appears to be affected by SirT1 inhibition in our system. These differences may be explained by the observation that in some organs, IRS proteins mediate the effects of insulin through PI3K whereas in other tissues they activate ERK signaling, (Byron et al., 2006; Huang et al., 2005). These imply that the coupling of IRS to the downstream signaling may depend on the cell type or other environmental factors. The effect of SirT1 on IRS-2 may have important implications considering that brain irs2−/− mice live longer (Taguchi et al., 2007).

ERK is crucial for oxidative stress. ERK signaling cascade is known to be activated by oxidative stress (Gaitanaki et al., 2003; Guyton et al., 1996; Zhang et al., 1998) and to exhibit dual effects on cell death depending on its kinetics, duration, intensity and context of its activation (Chu et al., 2004). It has been shown to promote cell survival under some conditions (Cheung and Slack, 2004), but to increase the sensitivity to oxidative stress under other conditions. Inhibition of ERK abrogates cell death induced by H_2O_2 in pancreatic cancer cells (Osada et al., 2007) and protects against glutamate-induced neuronal death (Satoh et al., 2000). Furthermore, certain drugs protect neurons partly by inhibiting ERK activation (Xu et al., 2006). Our results suggest that SirT1 inhibition protects neurons by decreasing Ras/ERK signaling and provide evidence for the pro-aging role of the Ras/ERK pathway downstream of IGF-I, analogously to that of the Ras pathways in *S. cerevisiae*. In agreement with our present results, a reduction in brain IRS-2 increased the life span of mice and stabilized MnSOD activity after fasting (Taguchi et al., 2007). These results in brain Irs2+/− mice are also consistent with our results in *S. cerevisiae* showing that MnSOD is required for life span extension in both mutants with defects in *SCH9* or RAS/cAMP signaling (Fabrizio et al., 2003).

Numerous studies point to SirT1 as a key regulator of cell survival in response to stress. It exhibits both pro- and anti- survival functions depending on the conditions. For instance, SirT1 countered p53-dependent apoptosis caused by etoposide in mouse embryonic fibroblasts (MEF) (Luo et al., 2001). In an ALS mouse model SirT1 rescued neurons (Kim et al., 2007). But SirT1 can also exacerbate cell death. As described earlier SirT1 KO MEFs showed higher replicative life span under chronic sublethal oxidative stress (Chua et al., 2005). SirT1 also sensitized HEK293 cells to TNFα-induced apoptosis (Yeung et al., 2004). Many factors may contribute to the seemingly contradictory effects of SirT1. First different nutrient, growth or stress signals may be sensed by SirT1 and integrated into divergent outputs. Secondly, SirT1's subcellular localization may also play a role in its regulation of cell death. Some studies suggest that cytoplasm-localized SirT1 may promote apoptosis (Jin et al., 2007; Zhang, 2007) while the anti-apoptotic effect may come only from the nuclear-localized SirT1 (Tanno et al., 2007). Thirdly, SirT1 has a wide array of targets which may become preferentially (de) activated in different contexts. Deacetylation of p53 and FOXO contributes to the pro-survival effect of SirT1 (Brunet et al., 2004; Langley et al., 2002; Motta et al., 2004), while NFkB and $p19^{ARF}$ mediate the pro-death effect (Chua et al., 2005; Yeung et al., 2004). It is possible that these transcription factors are also implicated in the increased protection in neurons with reduced SirT1 and ERK activity.

The detection of lower oxidative stress in the brain of SirT1 knockout mice is consistent with our cell culture data. The reduced production of hydrogen peroxide by mitochondria and major changes in electron transport and leakage in SirT1 KO mice recently shown by McBurney and colleagues (Boily et al., 2008) may explain part of the protective effect observed after SirT1 inhibition. Yet we cannot conclude that SirT1 promotes oxidative damage in all organs *in vivo* as SirT1 may play vastly different roles in various organs. For example, Alcendor et al reported the beneficial effect of SirT1 overexpression in the heart against oxidative stress, although they showed that this anti-oxidant effect becomes a pro-oxidant effect at a higher overexpression level (Alcendor RR et al., 2007). In agreement with the very different roles of SirT1, here we show that the pro-oxidative stress role of SirT1 in neurons and in the mouse brain is not translated into a longer life span. In fact, SirT1 KO mice live shorter than wild type controls under both normal and calorie restricted diets (Fig. 6). Thus, differently from our studies in yeast, we did not find that CR extends further the life span of SirT1 KO mice, which is consistent with a recent report (Boily et al., 2008). Considering that SirT1 +/− mice display a normal mean life span and that SirT1−/− mice have severe developmental defects including a dwarf phenotype (McBurney et al., 2003), it is likely that these defects contribute to shortening the life span independently of the rate of aging. A brain specific SirT1 KO mouse model may provide further clues.

In summary, this study suggests that SirT1 contributes to oxidative damage in mammals by activating IRS-2/Ras/ERK signaling downstream of insulin/IGF-I receptors but also plays a number of roles important for normal growth and life span (McBurney et al., 2003; Moynihan et al., 2005; Picard et al., 2004). There has been a keen interest in developing SirT1 activators for human consumption. Our studies implicating SirT1 in both pro-aging and protective functions in yeast and mammalian cells suggest that additional studies should be carried out before SirT1 activators are considered for chronic use.

Experimental Procedures

Materials

Antibodies for SirT1 (07-131), IRS2 (06-506) and phosphotyrosine 4G10 (05-321), NeuN (MAB377), GluR2/3 (AB1506), Ras activation assay kit (17-218) and SirT1 deacetylase (17-370) were from Millipore. Antibodies for phospho-p44/42 MAP Kinase (Thr202/Tyr204;9101), p44/42 MAP Kinase (9102), IRS-1 (2382), IRS-2 (4502) and acetylated-lysine (AcK103;9681) were from Cell Signaling (Danvers, MA). Anti-GAPDH (ab9484) was from Abcam (Cambridge, MA). Anti-V5 (R960-25) was from Invitrogen (Carlsbad, CA). Other chemicals were from Sigma-Aldrich (St. Louis, MO). U6Pro-SirT1-siRNA was from Dr David Sinclair encoding siRNA targeting GAAGTTGACCTCCTCATTGT. SirT2DN (pEGFP-C1-hSIRT2 N168A) was from Dr Eric Verdin. V5-SIRT1 was from Dr Marty Mayo.

Cell culture

Neurons from E18 rat cortex were dissociated in neurobasal medium supplemented with 0.5 mM glutamine, 25 μM glutamate and B-27 and plated at 3×10^4/well in 96-well plates for viability assays, at 10^5/well in 6-well plates for immunostaining or 5×10^5 for immunoblotting, at 3×10^6 onto 10 cm dishes for activity assay or immunoprecipitation and maintained in neurobasal medium supplemented with B-27 and glutamine until 5–7 DIV for transfection or 10–14 DIV for treatments.

Negative control and SirT1 siRNA (SirT1 target sequence: AAGTGCCTCAAATATTAATAA) were from Qiagen. On 5 DIV rat cortical neurons were transfected with 25 nM control or SirT1 siRNA using HiPerFect Transfection Reagent. 2 days later cells were treated with chemicals or collected for assays. Neurons were transfected with

Clontech CalPhos™ Mammalian Transfection Kit. HEK293 cells were transfected with Lipofectamine reagent (Invitrogen).

SirT1 deacetylase activity was measured using fluorometric SIRT1 Assay Kit (Sigma CS1040) per manufacturer instructions except that 1 μM trichostatin A was also added to the reaction. Fluorescence intensity at 444 nm (exc. 355 nm) was recorded and normalized to μg of protein and values represented as % of control.

Viability

Cell survival was measured by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Absorptions at 570nm were normalized to control and 3–4 wells were used for each condition per experiment. Data from 3 independent experiments were averaged and shown as mean±SEM. LDH assay was performed with CytoTox 96® Non-Radioactive Cytotoxicity kit (Promega, Madison, WI). Data from 3 independent experiments are shown as mean±SEM and normalized to control. Apoptosis was measured with Cell Death Detection ELISA^{PLUS} kit (Roche, Indianapolis, IN). Data from 10 wells from 3 independent experiments are shown as mean±SEM and normalized to control. In Live/Dead Assay (Invitrogen L3224) cells were stained with 2 μM calcein AM (green fluorescent due to esterase in living cells) and 2 μM ethidium homodimer 1 (EthD-1, red fluorescent once binding to DNA) for 10 min at 37° C followed by washing with HBSS and observation under the microscope. In each experiment 2 coverslips were used for each condition and 4 random fields captured for each coverslip. Live (green) and dead (red) cells were counted from 3 independent experiments. A portion of a representative image is shown for each condition.

Immunocytochemistry was performed with standard protocol as previously described (Xu et al., 2007).

Western blotting and immunoprecipitation (IP)

Cells or tissue were homogenized in 1% SDS, 10 mM Tris, pH 7.4, protease inhibitor cocktail, 1 mM Na_3VO_4, 1 mM NaF, followed by boiling at 95°C for 5 min and centrifugation at 14,000 rpm at 4°C for 30 min. Equal amounts of supernatant protein were resolved on SDS-PAGE and immunoblotted following standard procedures. Blots were quantified with NIH image. For coIP cells were lysed in modified radioimmunoprecipitation buffer and lysates incubated with 5 μg of anti-V5 or control IgG and protein was pulled down by protein A agarose. For IP cells were lysed in modified RIPA containing 1 μM TSA and 5 mM nicotinamide and incubated with IRS1 or IRS2 antibodies and protein was pulled down by protein A agarose. Ras activation assay (Upstate 17-218) was performed per manufacturer's instructions.

In vitro deacetylation

IRS-2 was immunoprecipitated from HEK293 cells and equal amount of IRS-2 was incubated with different amounts of SirT1 deacetylase (0, 1, 2 μg) in 1 mM NAD, 25 mM Tris pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.2 mM DTT for 1.5 hr at RT. The reaction was stopped by addition of denaturing buffer and subjected to western blotting.

Animals

Wild-type C57BL/6J male (3 day and 2 month old) mice were used for brain fractionation. SirT1+/+, +/− and −/− genotypes have been described previously (McBurney et al., 2003). Mice were deeply anesthetized with isoflurane followed immediately by decapitation and brains (and hippocampus) were collected right away. In life span studies ad libitum (AL) mice were fed NIH-31 standard feed while CR mice were fed daily with NIH31/NIA fortified food containing 60% of calories consumed by AL mice. Water was available ad libitum for all mice.

Brain Fractionation

Forebrain homogenates were separated into nuclear, cytosol and membrane fractions through sucrose-gradient centrifugation. Briefly, the forebrain was homogenized in chilled buffer containing 0.32 M sucrose, 10 mM HEPES pH 7.4, 2 mM EDTA, protease inhibitors, phosphatase inhibitors and centrifuged at 1000 × g for 15 min to yield the nuclear fraction pellet. The supernatant was then centrifuged at 200,000 × g for 15 min to yield cytosol in the supernatant. The resulting pellet was resuspended and centrifuged 4 more times to obtain the membrane fraction.

Determination of Oxidative Stress

Half brain minus cerebellum (HFB) was homogenized in 50 mM phosphate buffer pH7.4 and centrifuged to yield supernatant for assaying for protein carbonyl content with OxiSelect™ Protein Carbonyl ELISA Kit (CellBiolabs Inc, San Diego, CA). For lipid peroxidation HFB was homogenized in TBARS homogenization buffer and subjected to TBARS assay as previously described (Liu et al., 2003).

Statistical analysis

Student's t-test was used to compare 2 groups. One-way ANOVA was used to compare multiple groups with Turkey's test as post-hoc test. Groups were determined significantly different when $P<0.05$.

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References

Alcendor RR, Gao S, Zhai P, Zablocki D, Holle E, Yu X, Tian B, Wagner T, Vatner SF, Sadoshima J. Sirt1 regulates aging and resistance to oxidative stress in the heart. Circ Res 2007;100:1512–1521. [PubMed: 17446436]

Berdichevsky A, Viswanathan M, Horvitz HR, Guarente L. C. elegans SIR-2.1 interacts with 14-3-3 proteins to activate DAF-16 and extend life span. Cell 2006;125:1165–1177. [PubMed: 16777605]

Bluher M, Kahn BB, Kahn CR. Extended longevity in mice lacking the insulin receptor in adipose tissue. Science 2003;299:572–574. [PubMed: 12543978]

Boily G, Seifert EL, Bevilacqua L, He XH, Sabourin G, Estey C, Moffat C, Crawford S, Saliba S, Jardine K, Xuan J, Evans M, Harper ME, McBurney MW. SirT1 regulates energy metabolism and response to caloric restriction in mice. PLoS ONE 2008;3:e1759. [PubMed: 18335035]

Bordone L, Motta MC, Picard F, Robinson A, Jhala US, Apfeld J, McDonagh T, Lemieux M, McBurney M, Szilvasi A, Easlon EJ, Lin SJ, Guarente L. Sirt1 regulates insulin secretion by repressing UCP2 in pancreatic beta cells. PLoS Biol 2006;4:e31. [PubMed: 16366736]

Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW, Greenberg ME. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science 2004;303:2011–2015. [PubMed: 14976264]

Byron SA, Horwitz KB, Richer JK, Lange CA, Zhang X, Yee D. Insulin receptor substrates mediate distinct biological responses to insulin-like growth factor receptor activation in breast cancer cells. Br J Cancer 2006;95:1220–1228. [PubMed: 17043687]

Cheng C, Fabrizio P, Ge H, Longo VD, Li LM. Inference of transcription modification in long-live yeast strains from their expression profiles. BMC Genomics 2007;8:219. [PubMed: 17617911]

Cheung EC, Slack RS. Emerging role for ERK as a key regulator of neuronal apoptosis. Sci STKE 2004 2004:PE45.

Chu CT, Levinthal DJ, Kulich SM, Chalovich EM, DeFranco DB. Oxidative neuronal injury. The dark side of ERK1/2. Eur J Biochem 2004;271:2060–2066. [PubMed: 15153095]

Chua KF, Mostoslavsky R, Lombard DB, Pang WW, Saito S, Franco S, Kaushal D, Cheng HL, Fischer MR, Stokes N, Murphy MM, Appella E, Alt FW. Mammalian SIRT1 limits replicative life span in response to chronic genotoxic stress. Cell Metab 2005;2:67–76. [PubMed: 16054100]

Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science 2004;305:390–392. [PubMed: 15205477]

Fabrizio P, Gattazzo C, Battistella L, Wei M, Cheng C, McGrew K, Longo VD. Sir2 blocks extreme life-span extension. Cell 2005;123:655–667. [PubMed: 16286010]

Fabrizio P, Liou LL, Moy VN, Diaspro A, Valentine JS, Gralla EB, Longo VD. SOD2 functions downstream of Sch9 to extend longevity in yeast. Genetics 2003;163:35–46. [PubMed: 12586694]

Fabrizio P, Pletcher SD, Minois N, Vaupel JW, Longo VD. Chronological aging-independent replicative life span regulation by Msn2/Msn4 and Sod2 in Saccharomyces cerevisiae. FEBS Lett 2004;557:136–142. [PubMed: 14741356]

Fabrizio P, Pozza F, Pletcher SD, Gendron CM, Longo VD. Regulation of longevity and stress resistance by Sch9 in yeast. Science 2001;292:288–290. [PubMed: 11292860]

Frye RA. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochem Biophys Res Commun 2000;273:793–798. [PubMed: 10873683]

Gaitanaki C, Konstantina S, Chrysa S, Beis I. Oxidative stress stimulates multiple MAPK signalling pathways and phosphorylation of the small HSP27 in the perfused amphibian heart. J Exp Biol 2003;206:2759–2769. [PubMed: 12847121]

Guarente L. Calorie restriction and SIR2 genes--towards a mechanism. Mech Ageing Dev 2005;126:923–928. [PubMed: 15941577]

Guyton KZ, Liu Y, Gorospe M, Xu Q, Holbrook NJ. Activation of mitogen-activated protein kinase by H2O2. Role in cell survival following oxidant injury. J Biol Chem 1996;271:4138–4142. [PubMed: 8626753]

Hansen M, Taubert S, Crawford D, Libina N, Lee SJ, Kenyon C. Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans. Aging Cell 2007;6:95–110. [PubMed: 17266679]

Holzenberger M, Dupont J, Ducos B, Leneuve P, Geloen A, Even PC, Cervera P, Le Bouc Y. IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature 2003;421:182–187. [PubMed: 12483226]

Huang C, Thirone AC, Huang X, Klip A. Differential contribution of insulin receptor substrates 1 versus 2 to insulin signaling and glucose uptake in l6 myotubes. J Biol Chem 2005;280:19426–19435. [PubMed: 15764603]

Hwangbo DS, Gershman B, Tu MP, Palmer M, Tatar M. Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature 2004;429:562–566. [PubMed: 15175753]

Jin Q, Yan T, Ge X, Sun C, Shi X, Zhai Q. Cytoplasm-localized SIRT1 enhances apoptosis. J Cell Physiol 2007;213:88–97. [PubMed: 17516504]

Kaeberlein M, Kirkland KT, Fields S, Kennedy BK. Sir2-independent life span extension by calorie restriction in yeast. PLoS Biol 2004;2:E296. [PubMed: 15328540]

Kaeberlein M, McVey M, Guarente L. The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. Genes Dev 1999;13:2570–2580. [PubMed: 10521401]

Kaeberlein M, Powers RW 3rd, Steffen KK, Westman EA, Hu D, Dang N, Kerr EO, Kirkland KT, Fields S, Kennedy BK. Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. Science 2005;310:1193–1196. [PubMed: 16293764]

Kaeberlein TL, Smith ED, Tsuchiya M, Welton KL, Thomas JH, Fields S, Kennedy BK, Kaeberlein M. Lifespan extension in Caenorhabditis elegans by complete removal of food. Aging Cell 2006;5:487–494. [PubMed: 17081160]

Kaiser C, James SR. Acetylation of insulin receptor substrate-1 is permissive for tyrosine phosphorylation. BMC Biol 2004;2:23. [PubMed: 15522123]

Kenyon C. A conserved regulatory system for aging. Cell 2001;105:165–168. [PubMed: 11336665]

Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R. A C. elegans mutant that lives twice as long as wild type. Nature 1993;366:461–464. [PubMed: 8247153]

Kim D, Nguyen MD, Dobbin MM, Fischer A, Sananbenesi F, Rodgers JT, Delalle I, Baur JA, Sui G, Armour SM, Puigserver P, Sinclair DA, Tsai LH. SIRT1 deacetylase protects against neurodegeneration in models for Alzheimer's disease and amyotrophic lateral sclerosis. EMBO J 2007;26:3169–3179. [PubMed: 17581637]

Kobayashi Y, Furukawa-Hibi Y, Chen C, Horio Y, Isobe K, Ikeda K, Motoyama N. SIRT1 is critical regulator of FOXO-mediated transcription in response to oxidative stress. Int J Mol Med 2005;16:237–243. [PubMed: 16012755]

Kops GJ, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffer PJ, Huang TT, Bos JL, Medema RH, Burgering BM. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature 2002;419:316–321. [PubMed: 12239572]

Langley E, Pearson M, Faretta M, Bauer UM, Frye RA, Minucci S, Pelicci PG, Kouzarides T. Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence. EMBO J 2002;21:2383–2396. [PubMed: 12006491]

Lee GD, Wilson MA, Zhu M, Wolkow CA, de Cabo R, Ingram DK, Zou S. Dietary deprivation extends lifespan in Caenorhabditis elegans. Aging Cell 2006;5:515–524. [PubMed: 17096674]

Lemieux ME, Yang X, Jardine K, He X, Jacobsen KX, Staines WA, Harper ME, McBurney MW. The Sirt1 deacetylase modulates the insulin-like growth factor signaling pathway in mammals. Mech Ageing Dev 2005;126:1097–1105. [PubMed: 15964060]

Liebmann C. Regulation of MAP kinase activity by peptide receptor signalling pathway: paradigms of multiplicity. Cell Signal 2001;13:777–785. [PubMed: 11583913]

Liu R, Liu IY, Bi X, Thompson RF, Doctrow SR, Malfroy B, Baudry M. Reversal of age-related learning deficits and brain oxidative stress in mice with superoxide dismutase/catalase mimetics. Proc Natl Acad Sci U S A 2003;100:8526–8531. [PubMed: 12815103]

Longo VD, Finch CE. Evolutionary medicine: from dwarf model systems to healthy centenarians? Science 2003;299:1342–1346. [PubMed: 12610293]

Longo VD, Kennedy BK. Sirtuins in aging and age-related disease. Cell 2006;126:257–268. [PubMed: 16873059]

Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A, Guarente L, Gu W. Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell 2001;107:137–148. [PubMed: 11672522]

McBurney MW, Yang X, Jardine K, Hixon M, Boekelheide K, Webb JR, Lansdorp PM, Lemieux M. The mammalian SIR2alpha protein has a role in embryogenesis and gametogenesis. Mol Cell Biol 2003;23:38–54. [PubMed: 12482959]

Medvedik O, Lamming DW, Kim KD, Sinclair DA. MSN2 and MSN4 link calorie restriction and TOR to sirtuin-mediated lifespan extension in Saccharomyces cerevisiae. PLoS Biol 2007;5:e261. [PubMed: 17914901]

Michishita E, Park JY, Burneskis JM, Barrett JC, Horikawa I. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. Mol Biol Cell 2005;16:4623–4635. [PubMed: 16079181]

Motta MC, Divecha N, Lemieux M, Kamel C, Chen D, Gu W, Bultsma Y, McBurney M, Guarente L. Mammalian SIRT1 represses forkhead transcription factors. Cell 2004;116:551–563. [PubMed: 14980222]

Moynihan KA, Grimm AA, Plueger MM, Bernal-Mizrachi E, Ford E, Cras-Meneur C, Permutt MA, Imai S. Increased dosage of mammalian Sir2 in pancreatic beta cells enhances glucose-stimulated insulin secretion in mice. Cell Metab 2005;2:105–117. [PubMed: 16098828]

Osada S, Sakashita F, Hosono Y, Nonaka K, Tokuyama Y, Tanaka H, Sasaki Y, Tomita H, Komori S, Matsui S, Takahashi T. Extracellular signal-regulated kinase phosphorylation due to menadione-induced arylation mediates growth inhibition of pancreas cancer cells. Cancer Chemother Pharmacol. 2007

Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, Machado De Oliveira R, Leid M, McBurney MW, Guarente L. Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. Nature 2004;429:771–776. [PubMed: 15175761]

Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. Nature 2005;434:113–118. [PubMed: 15744310]

Rodgers JT, Puigserver P. Fasting-dependent glucose and lipid metabolic response through hepatic sirtuin 1. Proc Natl Acad Sci U S A 2007;104:12861–12866. [PubMed: 17646659]

Rogina B, Helfand SL. Sir2 mediates longevity in the fly through a pathway related to calorie restriction. Proc Natl Acad Sci U S A 2004;101:15998–16003. [PubMed: 15520384]

Satoh T, Nakatsuka D, Watanabe Y, Nagata I, Kikuchi H, Namura S. Neuroprotection by MAPK/ERK kinase inhibition with U0126 against oxidative stress in a mouse neuronal cell line and rat primary cultured cortical neurons. Neurosci Lett 2000;288:163–166. [PubMed: 10876086]

Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 1997;88:593–602. [PubMed: 9054499]

Song G, Ouyang G, Bao S. The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med 2005;9:59–71. [PubMed: 15784165]

Sun C, Zhang F, Ge X, Yan T, Chen X, Shi X, Zhai Q. SIRT1 Improves Insulin Sensitivity under Insulin-Resistant Conditions by Repressing PTP1B. Cell Metab 2007;6:307–319. [PubMed: 17908559]

Taguchi A, Wartschow LM, White MF. Brain IRS2 signaling coordinates life span and nutrient homeostasis. Science 2007;317:369–372. [PubMed: 17641201]

Tanno M, Sakamoto J, Miura T, Shimamoto K, Horio Y. Nucleocytoplasmic shuttling of the NAD+-dependent histone deacetylase SIRT1. J Biol Chem 2007;282:6823–6832. [PubMed: 17197703]

Tissenbaum HA, Guarente L. Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. Nature 2001;410:227–230. [PubMed: 11242085]

Urban J, Soulard A, Huber A, Lippman S, Mukhopadhyay D, Deloche O, Wanke V, Anrather D, Ammerer G, Riezman H, Broach JR, De Virgilio C, Hall MN, Loewith R. Sch9 is a major target of TORC1 in Saccharomyces cerevisiae. Mol Cell 2007;26:663–674. [PubMed: 17560372]

Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L, Weinberg RA. hSIR2 (SIRT1) functions as an NAD-dependent p53 deacetylase. Cell 2001;107:149–159. [PubMed: 11672523]

Wang Y, Tissenbaum HA. Overlapping and distinct functions for a Caenorhabditis elegans SIR2 and DAF-16/FOXO. Mech Ageing Dev 2006;127:48–56. [PubMed: 16280150]

Xu W, Wong TP, Chery N, Gaertner T, Wang YT, Baudry M. Calpain-mediated mGluR1alpha truncation: a key step in excitotoxicity. Neuron 2007;53:399–412. [PubMed: 17270736]

Xu X, Chua CC, Gao J, Hamdy RC, Chua BH. Humanin is a novel neuroprotective agent against stroke. Stroke 2006;37:2613–2619. [PubMed: 16960089]

Yan L, Vatner DE, O'Connor JP, Ivessa A, Ge H, Chen W, Hirotani S, Ishikawa Y, Sadoshima J, Vatner SF. Type 5 adenylyl cyclase disruption increases longevity and protects against stress. Cell 2007;130:247–258. [PubMed: 17662940]

Yang Y, Hou H, Haller EM, Nicosia SV, Bai W. Suppression of FOXO1 activity by FHL2 through SIRT1-mediated deacetylation. EMBO J 2005;24:1021–1032. [PubMed: 15692560]

Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, Mayo MW. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J 2004;23:2369–2380. [PubMed: 15152190]

Zhang J. The direct involvement of SirT1 in insulin-induced IRS-2 tyrosine phosphorylation. J Biol Chem. 2007

Zhang J, Jin N, Liu Y, Rhoades RA. Hydrogen peroxide stimulates extracellular signal-regulated protein kinases in pulmonary arterial smooth muscle cells. Am J Respir Cell Mol Biol 1998;19:324–332. [PubMed: 9698606]

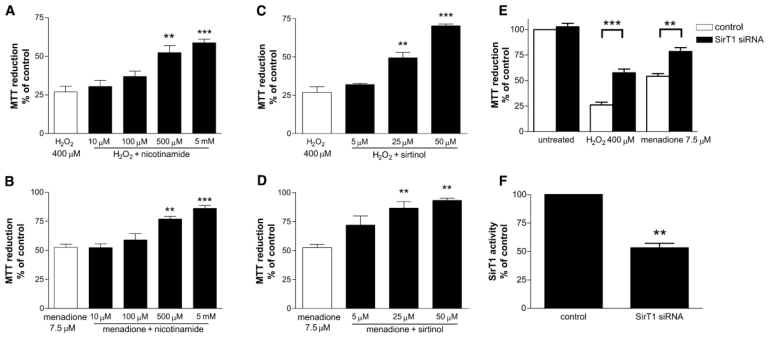

Figure 1.

Inhibiting SirT1 deacetylase increases oxidative stress resistance in neurons. Cortical neurons were cultured in 96-well plates (**A**–**E**) or 10 cm dishes (**F**). (**A**, **B**, **C**, **D**) 10–14 DIV neurons were treated with nicotinamide (**A, B**; Nico) or sirtinol (**C, D**) at indicated concentrations for 48 hrs. Oxidative stress was then induced by 400 μM of hydrogen peroxide (**A, C**) or 7.5 μM of menadione (**B, D**), respectively. 24 hrs later cell viability was measured by MTT assay. The ODs are normalized to respective controls (H_2O_2/menadione relative to vehicle-treated control; H_2O_2+Nico/sirtinol relative to Nico/sirtinol alone; menadione+Nico/sirtinol relative to Nico/sirtinol alone). (**E**) 5 DIV neurons were transfected with control or SirT1 siRNA. 2 days later some neurons were subjected to H_2O_2 or menadione, followed by MTT assay 24 hrs later. (**F**) 5 DIV neurons were transfected with control or SirT1 siRNA and 2 days later SirT1 deacetylase activity was measured. Data represent mean±SEM.

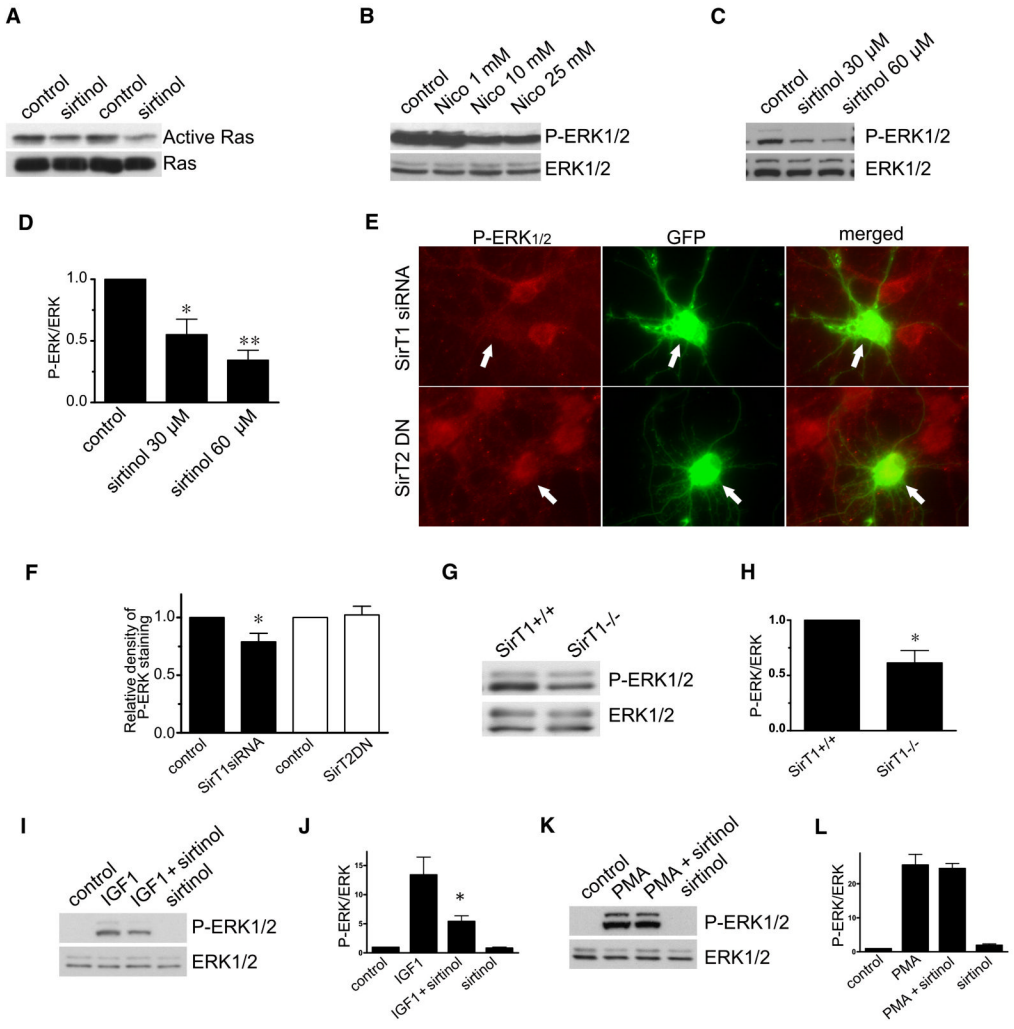

Figure 2.

Inhibition of SirT1 deacetylase decreases Ras/ERK$_{1/2}$ activation in cultured neurons and *in vivo*. (**A**) Representative blots showing the effect of SirT1 inhibitor sirtinol on Ras activation. 10–14 DIV neurons were treated with vehicle or 60 μM sirtinol for 4 hrs and subjected to Ras activation assay or immunoblotted with anti-Ras. (**B, C**) Representative blots showing the effect of SirT1 inhibitors nicotinamide (**B**) and sirtinol (**C**) on ERK$_{1/2}$ signaling. 10–14 DIV neurons were treated with Nico for 48 hrs or sirtinol for 4 hrs. Total cell lysates were collected for SDS-PAGE and blotted with anti-phospho-ERK$_{1/2}$ and anti-ERK$_{1/2}$, respectively. (**D**) Quantification of immunoblots showing the effect of sirtinol on ERK$_{1/2}$ activation. (**E**) 7 DIV cortical neurons were transfected with U6Pro-SirT1-siRNA+GFP or dominant-negative GFP-SirT2 (SirT2DN) and 48 hrs later were immunostained with anti-phospho-ERK$_{1/2}$. (**F**) Quantification of immunodensity from 12 transfected and 12 nearby non-transfected cells for SirT1 siRNA and SirT2DN, respectively. (**G, H**) Representative blots (**G**) and quantification (**H**) showing the effect of SirT1 deacetylase on ERK$_{1/2}$ signaling in mice hippocampus. (**I, J, K, L**) Representative blots (**I, K**) and quantification (**J, L**) showing the effect of sirtinol on ERK$_{1/2}$ signaling in HEK cells. HEK cells were starved for 15 hrs in media containing 0.5% FBS and incubated with 60 μM sirtinol for 4 hrs and then treated with IGF-1 or PMA for 5 min. Cell lysates were subjected to SDS-PAGE and blotted with anti-phospho-ERK$_{1/2}$ or anti-ERK$_{1/2}$. Quantifications are shown as mean±SEM.

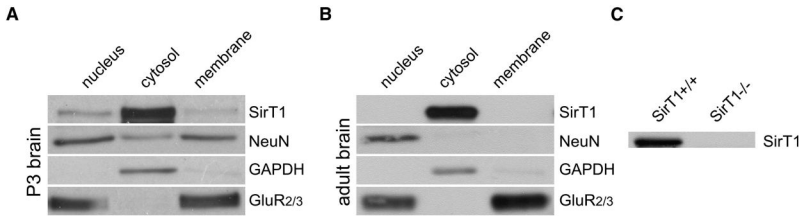

Figure 3.

Subcellular localization of SirT1 in the brain. (**A, B**) Forebrains from postnatal 3 days (P3, **A**) and adult (**B**) mice were homogenized and separated into nuclear, cytosol and membrane fractions through sucrose-gradient centrifugation and immunoblotted with SirT1, NeuN, GAPDH, $GluR_{2/3}$, respectively. In P3 brain SirT1 is localized in both nucleus and cytosol, while it is found predominantly in cytosol in the adult brain. (**C**) The specificity of SirT1 immunoblot was confirmed with SirT1−/− mice.

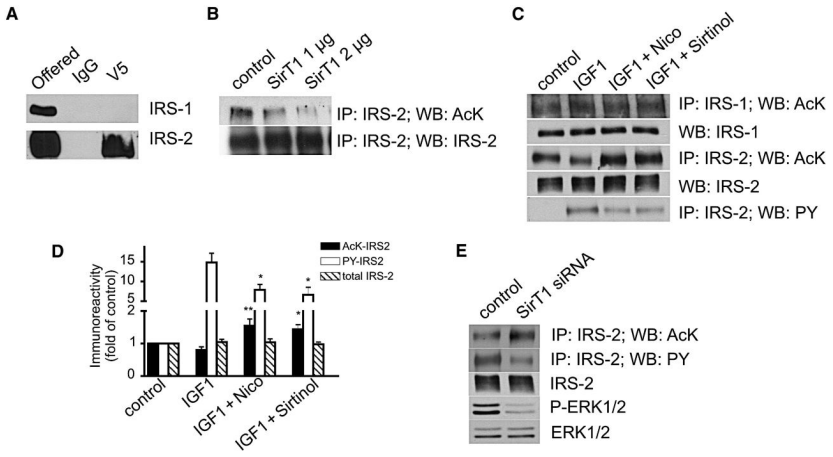

Figure 4.

SirT1 regulates Ras/ERK$_{1/2}$ signaling via deacetylation of IRS-2. (**A**) Physical interaction between SirT1 and IRS-2. HEK293 cells were transfected with V5-SirT1 and 48 hrs later cell lysates were immunoprecipitated with V5 antibody or control IgG and probed with anti-IRS1 or anti-IRS2, respectively. (**B**) SirT1 deacetylates IRS-2 *in vitro*. HEK cell lysates were precipitated with anti-IRS2, divided to 3 equal parts and incubated with 0, 1, or 2 μg SirT1 deacetylase respectively for 1.5 hr. They were then subjected to SDS-PAGE and blotted with anti-acetylated-lysine (AcK). (**C, D**) Representative blots (**C**) and quantification (**D**) showing that inhibition of SirT1 deacetylase in HEK cells increased acetylation of IRS-2 and decreased phosphorylation of IRS-2 while having no effect on IRS-1. (**C**) HEK cells were treated as indicated and cell lysates were precipitated with anti-IRS1 or anti-IRS2 and blotted with anti-AcK or anti-phosphotyrosine (PY). Cell lysates were also blotted for IRS1 or IRS2. (**D**) Quantification from 4–5 blots (mean±SEM). (**E**) Representative blots showing the effect of SirT1 on IRS-2 deacetylation in neurons. 5 DIV neurons were transfected with either control or SirT1 siRNA. 2 days later cell lysates were immunoprecipitated with anti-IRS2 and blotted for AcK or PY. Cell lysates were also blotted for IRS2, P-ERK$_{1/2}$ and ERK$_{1/2}$, respectively.

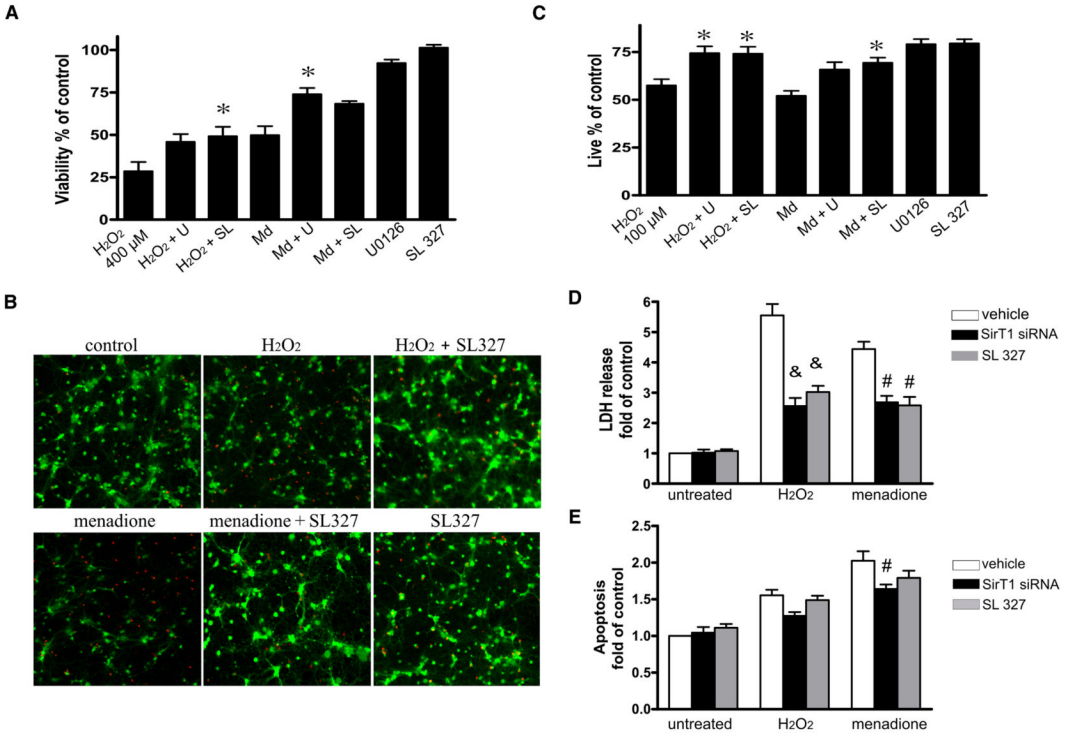

Figure 5.

Inhibiting $ERK_{1/2}$ protects neurons against oxidative stress. Neurons were cultured in 96-well plates (**A, D, E**) or glass coverslips in 6-well plates (**B**). 10–14 DIV neurons were pretreated with $ERK_{1/2}$ inhibitors U0126 or SL327 for 4 hr. Oxidative stress was induced by H_2O_2 or menadione. Viability was measured by MTT (**A**) or live/dead assay (**B**). (**C**) % live was calculated from the number of live (green) and dead (red) cells. (**D, E**) $ERK_{1/2}$ inhibition primarily attenuates necrosis. Neurons were transfected with control or SirT1 siRNA, or incubated with SL327 before exposed to 400 μM H_2O_2 or 7.5 μM menadione. 24 hrs later culture media were collected for LDH assay and cells were lysed for apoptosis assay. Data represent mean±SEM.

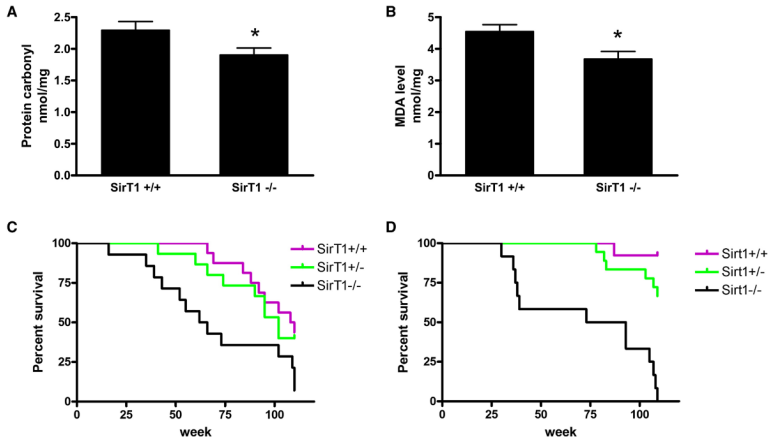

Figure 6.

Reduced oxidative stress in SirT1−/− mice brain and the reduced life span of SirT1−/− mice. (**A**, **B**) Reduced oxidative stress in SirT1 knockout mice brain. Protein carbonyl content (**A**; nmol protein carbonyl per mg protein, mean±SEM) and lipid peroxidation (**B,** TBARS assay; nmol malondialdehyde [MDA] equivalent per mg protein) were measured in 18 month old SirT1 +/+ and −/− mice brains. (**C**, **D**) Calorie restriction does not extend life span of SirT1 −/− mice. (**C**) Survival curves for SirT1 +/+ (n=16), +/−(n=15), −/− mice (n=14) on ad-lib diet (P<0.05, log-rank test). (**D**) Survival curves for SirT1 +/+ (n= 13), +/− (n=18), −/− (n=12) mice on 40% reduced calorie diet (P<0.001, log-rank test). Mice were 2–5 months old at the onset of CR.