

# SirT1 Inhibition Reduces IGF-I/IRS-2/Ras/ERK1/2 Signaling and Protects Neurons

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## SUMMARY

Sirtuins are known to protect cells and extend life span, but our previous studies indicated 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 sensitization of neurons to oxidative damage. SirT1 inhibition increased acetylation and decreased phosphorylation of IRS-2; it also reduced activation of the Ras/ERK1/2 pathway, suggesting that SirT1 may enhance IGF-I signaling in part by deacetylating IRS-2. Either the inhibition of SirT1 or of Ras/ERK1/2 was associated with resistance to oxidative damage. Markers of oxidized proteins and lipids were reduced in the brain of old SirT1-deficient mice, but the life span of the homozygote knockout mice was reduced under both normal and calorie-restricted conditions. These results are consistent with findings in *S. cerevisiae* and other model systems, suggesting that mammalian sirtuins can play both protective and proaging roles.

## INTRODUCTION

Sirtuins, or Sir2 family proteins, are NAD<sup>+</sup>-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 antiaging 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 life span of calorie-restricted cells, 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 apopto-

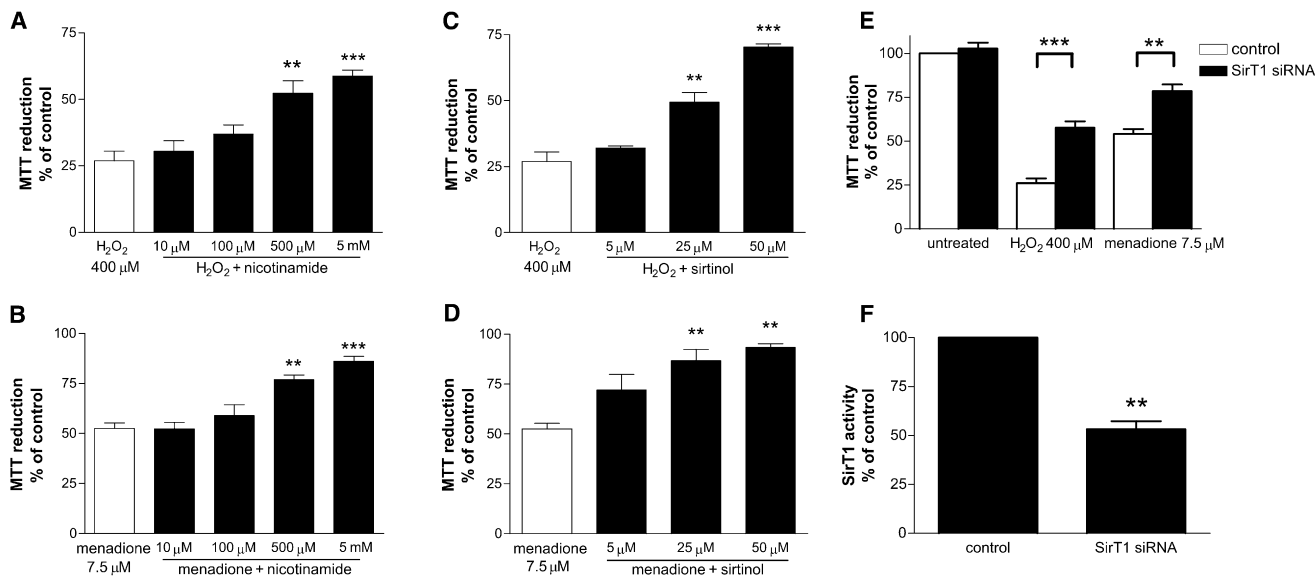
sis (Bordone et al., 2006; Cohen et al., 2004; Luo et al., 2001; Moynihan et al., 2005; Rodgers et al., 2005; Sun et al., 2007). In mammalian cells, SirT1 regulates several stress-response factors, such as p53 tumor suppressor (Langley et al., 2002; Vaziri et al., 2001), forkhead transcription factors (Brunet et al., 2004; Motta et al., 2004), and NF- $\kappa$ B (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. Our results indicate that the role of SirT1 deficiency in protecting against 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 rat neurons by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or menadione, which generates both superoxide and H<sub>2</sub>O<sub>2</sub>. Cell viability was measured by MTT assay. Ten days in vitro (DIV) cortical neuron cultures were incubated with 400  $\mu$ M H<sub>2</sub>O<sub>2</sub> or 7.5  $\mu$ M menadione for 24 hr followed by MTT assay. After H<sub>2</sub>O<sub>2</sub> treatment, about 26% of the neurons survived, whereas 50% survived menadione treatment (Figures 1A–1D). We also pretreated neurons with SirT1 inhibitors, nicotinamide (Nico), or sirtinol for 48 hr and then subjected them to the above oxidative-stress protocol. Nicotinamide dose dependently increased neuronal survival after H<sub>2</sub>O<sub>2</sub> treatment. Five-hundred  $\mu$ M and five mM of Nico pretreatment increased the survival of H<sub>2</sub>O<sub>2</sub>-treated cells to 52.3  $\pm$  4.7% ( $p$  < 0.01, H<sub>2</sub>O<sub>2</sub> versus H<sub>2</sub>O<sub>2</sub> + 500  $\mu$ M Nico,  $n$  = 3, one-way ANOVA, Turkey's test) and 58.7  $\pm$  2.3% ( $p$  < 0.001, H<sub>2</sub>O<sub>2</sub> versus H<sub>2</sub>O<sub>2</sub> + 5 mM Nico,  $n$  = 3, one-way ANOVA, Turkey's test), respectively (Figure 1A), while 10  $\mu$ M ( $p$  > 0.05, H<sub>2</sub>O<sub>2</sub> versus H<sub>2</sub>O<sub>2</sub> + 10  $\mu$ M Nico,  $n$  = 3, one-way ANOVA, Turkey's test) and 100  $\mu$ M ( $p$  > 0.05, H<sub>2</sub>O<sub>2</sub> versus H<sub>2</sub>O<sub>2</sub> + 100  $\mu$ M Nico,  $n$  = 3, one-way ANOVA, Turkey's test) of Nico showed no improvement. Nico also dose dependently



**Figure 1. Inhibiting SirT1 Deacetylase Increases Oxidative Stress Resistance in Neurons**

(A–D) Ten to fourteen DIV neurons were treated with nicotinamide (A and B; Nico) or sirtinol (C and D) at indicated concentrations for 48 hr. Oxidative stress was then induced by 400  $\mu$ M of hydrogen peroxide (A and C) or 7.5  $\mu$ M of menadione (B and D), respectively. Twenty-four hr later, cell viability was measured by MTT assay. The absorbance values are normalized to respective controls (H<sub>2</sub>O<sub>2</sub>/menadione relative to vehicle-treated control; H<sub>2</sub>O<sub>2</sub> + Nico/sirtinol relative to Nico/sirtinol alone; menadione + Nico/sirtinol relative to Nico/sirtinol alone).

(E) Five DIV neurons were transfected with control or SirT1 siRNA. Two days later, neurons were subjected to H<sub>2</sub>O<sub>2</sub> or menadione, followed by MTT assay 24 hr later.

(F) Five DIV neurons were transfected with control or SirT1 siRNA, and 2 days later, SirT1 deacetylase activity was measured. Data represent mean  $\pm$  SEM.

Cortical neurons were cultured in 96-well plates (A–E) or 10 cm dishes (F).

rescued neurons in menadione-induced oxidative stress (Figure 1B). Five-hundred  $\mu$ M and five mM Nico increased the survival of menadione-treated cells to  $77.0 \pm 2.2\%$  ( $p < 0.01$ , menadione versus menadione + 500  $\mu$ M Nico,  $n = 3$ , one-way ANOVA, Turkey's test) and  $86.1 \pm 2.4\%$  ( $p < 0.001$ , menadione versus menadione + 5 mM Nico,  $n = 3$ , one-way ANOVA, Turkey's test), respectively, while 10  $\mu$ M ( $p > 0.05$ , menadione versus menadione + 10  $\mu$ M Nico,  $n = 3$ , one-way ANOVA, Turkey's test) and 100  $\mu$ M ( $p > 0.05$ , menadione versus menadione + 100  $\mu$ M 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; Figure S1).

Similar effects were achieved with another SirT1 inhibitor sirtinol. Twenty-five  $\mu$ M and fifty  $\mu$ M of sirtinol pretreatment increased the survival of H<sub>2</sub>O<sub>2</sub>-treated neurons to  $49.4 \pm 3.6\%$  ( $p < 0.01$ , H<sub>2</sub>O<sub>2</sub> versus H<sub>2</sub>O<sub>2</sub> + 25  $\mu$ M sirtinol,  $n = 3$ , one-way ANOVA, Turkey's test) and  $70.4 \pm 1.2\%$  ( $p < 0.001$ , H<sub>2</sub>O<sub>2</sub> versus H<sub>2</sub>O<sub>2</sub> + 50  $\mu$ M sirtinol,  $n = 3$ , one-way ANOVA, Turkey's test), respectively (Figures 1C and 1D), while 5  $\mu$ M sirtinol ( $p > 0.05$ , H<sub>2</sub>O<sub>2</sub> versus H<sub>2</sub>O<sub>2</sub> + 5  $\mu$ M sirtinol,  $n = 3$ , one-way ANOVA, Turkey's test) showed no benefit. Sirtinol also dose dependently increased neuronal survival in menadione-induced oxidative stress. Twenty-five  $\mu$ M and fifty  $\mu$ M of sirtinol pretreatment increased the viability of menadione-treated cells to  $86.7 \pm 5.7\%$  ( $p < 0.01$ , H<sub>2</sub>O<sub>2</sub> versus H<sub>2</sub>O<sub>2</sub> + 25  $\mu$ M sirtinol,  $n = 3$ , one-way ANOVA, Turkey's test) and  $93.4 \pm 1.9\%$  ( $p < 0.01$ , H<sub>2</sub>O<sub>2</sub> versus H<sub>2</sub>O<sub>2</sub> + 50  $\mu$ M sirtinol,  $n = 3$ , one-way ANOVA, Turkey's test), respectively, whereas 5  $\mu$ M of sirtinol ( $p > 0.05$ , H<sub>2</sub>O<sub>2</sub>

versus H<sub>2</sub>O<sub>2</sub> + 5  $\mu$ M sirtinol,  $n = 3$ , one-way ANOVA, Turkey's test) showed no effect. The absorbance values are adjusted to the effects of sirtinol alone (sirtinol decreased MTT reduction by 13%–21% at 25–50  $\mu$ M compared to control) (Figure S1).

To confirm that the stress resistance is caused by reduced SirT1 activity, we transfected either control or SirT1 siRNA into five DIV neurons and assessed survival after H<sub>2</sub>O<sub>2</sub>- or menadione-induced oxidative stress (Figure 1E).

Knockdown of SirT1 increased the viability of H<sub>2</sub>O<sub>2</sub>-treated cells from  $26.2 \pm 2.7\%$  to  $57.5 \pm 3.7\%$  ( $p < 0.001$ , H<sub>2</sub>O<sub>2</sub> versus H<sub>2</sub>O<sub>2</sub> + SirT1 siRNA,  $n = 4$ ,  $t$  test). Inhibition of SirT1 also boosted the survival of menadione-treated cells from  $54.2 \pm 2.7\%$  to  $78.5 \pm 3.7\%$  ( $p < 0.01$ , menadione versus 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-Sirt2; Millipore, Billerica, MA) appear to bind specifically to SirT1 in rat neurons. Hence, we carried out SirT1 deacetylase activity assay using a fluorometric kit 2 days after transfection (Figure 1F). SirT1 siRNA decreased SirT1 deacetylase activity to  $53.2 \pm 4.2\%$  of control ( $p < 0.01$ ,  $n = 4$ , control versus SirT1 siRNA,  $t$  test). 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 (Figure S5A), which suggests that the effect of SirT1 inhibition is unlikely to be mediated by the 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 proaging pathway in parallel to the Sch9 pathway (Fabrizio et al., 2003; 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 proaging IGF-I receptor, we studied the role of SirT1 inhibitors on Ras/ERK signaling. Neurons cultured in neurobasal media supplemented with B27 (containing ~4  $\mu$ g/ml insulin) showed a high-basal activation of Ras (GTP-bound Ras), which was reduced by a 4 hr treatment of 60  $\mu$ M of sirtinol (Figure 2A), indicating that SirT1 modulates Ras activity.

We also checked if ERK1/2, a major effector of Ras, is altered by SirT1 inhibition. Ten to fourteen 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-ERK1/2 (P-ERK1/2) and anti-ERK1/2, respectively. Ten mM and twenty-five mM of Nico decreased phosphorylated ERK1/2 (Figure 2B). The effect of sirtinol was dose dependent as 30  $\mu$ M of sirtinol reduced the ratio of P-ERK/ERK by 45% and 60  $\mu$ M of sirtinol reduced the ratio by 68% (Figures 2C and 2D) ( $p < 0.05$ , control versus sirtinol 30  $\mu$ M;  $p < 0.01$ , control versus sirtinol 60  $\mu$ M;  $n = 4$ , 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 cotransfected with GFP in neurons. This plasmid has been shown to reduce SirT1 expression (Cohen et al., 2004). Forty-eight hours after transfection, the cells were immunostained with anti-P-ERK1/2 and anti-GFP (Figure 2E). The immunodensity of P-ERK1/2 in transfected cells was significantly lower than that of surrounding nontransfected 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 nontransfected ones ( $p > 0.05$ ,  $n = 12$ , Student's *t* test) (Figure 2F). 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 hippocampi of 17-day-old SirT1 knockout (KO) mice ( $p < 0.05$ , SirT1<sup>+/+</sup> versus SirT1<sup>-/-</sup>,  $n = 6$ , *t* test) (Figures 2G and 2H). Together, these data suggest that inhibition of SirT1 can downregulate 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 hr 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 (Figures 2I and 2J). Starved cells showed low levels of P-ERK1/2, and IGF-I induced a 12-fold increase in P-ERK1/2 ( $p < 0.01$ , control

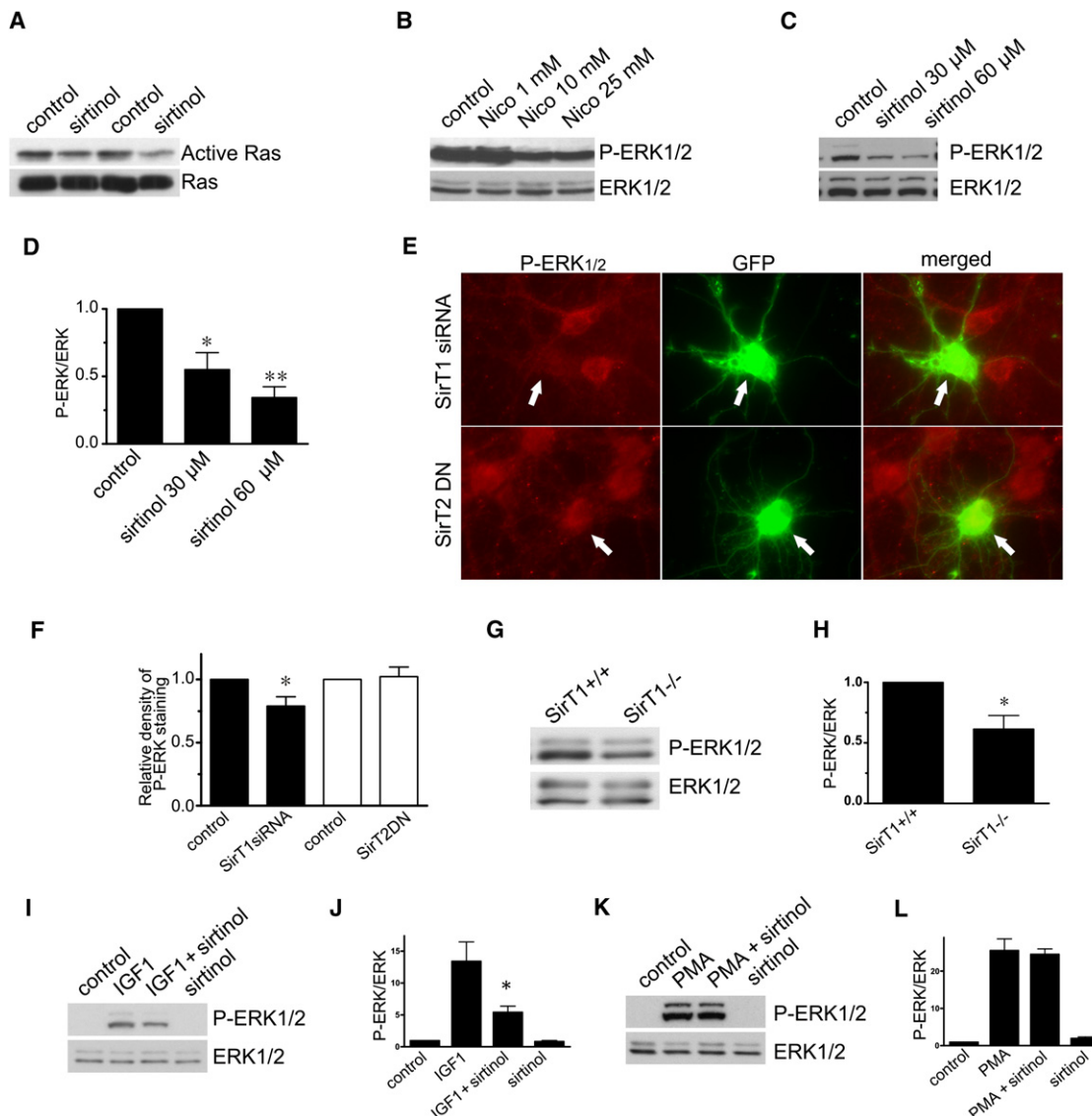
versus IGF-I,  $n = 3$ , one-way ANOVA, Turkey's test). Four hr pretreatment of 60  $\mu$ M sirtinol markedly reduced this effect of IGF-I on ERK1/2 phosphorylation (60% decrease,  $p < 0.05$ , IGF-I versus IGF-I + sirtinol,  $n = 3$ , one-way ANOVA, Turkey's test). To begin to determine how SirT1 inhibition affects ERK activation, we applied PMA (phorbol 12-myristate 13-acetate, a phorbol ester, 1  $\mu$ g/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 versus PMA,  $n = 3$ , one-way ANOVA, Turkey's test) (Figures 2K and 2L). However, pretreatment with sirtinol did not block this effect ( $p > 0.05$ , PMA versus PMA + sirtinol,  $n = 3$ , one-way ANOVA, Turkey's test), indicating that the target molecule(s) of SirT1 lie(s) 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 posttranslational 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 nonnuclear substrates by examining its localization in mouse brains (Figure 3). Brains from postnatal day 3 (P3, Figure 3A) and adult (Figure 3B) 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<sub>2/3</sub> (a membrane-associated protein). SirT1 antibody recognizes one band at 110 kDa, which is absent from the brain of SirT1 KO mice (Figure 3C), 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 (Figures 3A and 3B).

### 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, 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 coimmunoprecipitation 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 for IRS-1 or IRS-2, respectively (Figure 4A). IRS-2, but not IRS-1, coimmunoprecipitated 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 immunoprecipitated IRS-2 from HEK293 cells and incubated equal amounts of purified IRS-2 with different doses of SirT1 and 1 mM of NAD for 1.5 hr, then, we blotted for acetylated-lysine (AcK) or IRS-2 (Figure 4B). This experiment



**Figure 2. Inhibition of SirT1 Deacetylase Decreases Ras/ERK1/2 Activation in Cultured Neurons and In Vivo**

(A) Representative blots showing the effect of SirT1 inhibitor sirtinol on Ras activation. Ten to fourteen DIV neurons were treated with vehicle or 60  $\mu$ M sirtinol for 4 hr and subjected to Ras activation assay or immunoblotted with anti-Ras.

(B and C) Representative blots showing the effect of SirT1 inhibitors nicotinamide (B) and sirtinol (C) on ERK1/2 signaling. Ten to fourteen DIV neurons were treated with Nico for 48 hr or sirtinol for 4 hr. Total cell lysates were collected for SDS-PAGE and blotted with anti-phospho-ERK1/2 and anti-ERK1/2, respectively.

(D) Quantification of immunoblots showing the effect of sirtinol on ERK1/2 activation.

(E) Seven DIV cortical neurons were transfected with U6Pro-SirT1-siRNA<sup>+</sup>GFP or dominant-negative GFP-SirT2 (SirT2DN); 48 hr later, they were immunostained with anti-phospho-ERK1/2.

(F) Quantification of immunodensity from 12 transfected and 12 nearby nontransfected cells for SirT1 siRNA and SirT2DN, respectively.

(G and H) Representative blots (G) and quantification (H) showing the effect of SirT1 deacetylase on ERK1/2 signaling in mice hippocampi.

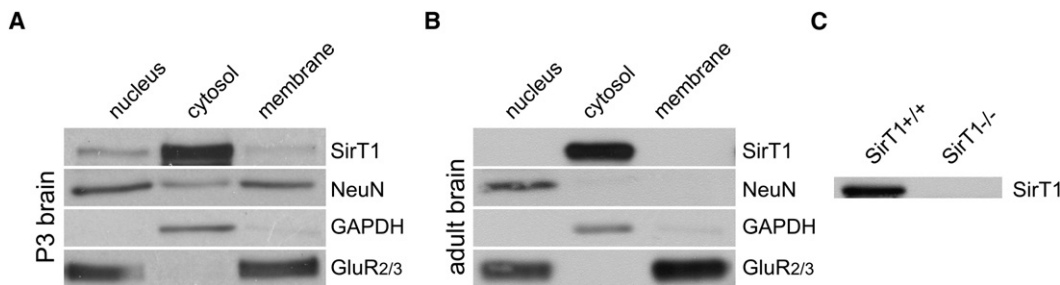
(I–L) Representative blots (I and K) and quantification (J and L) showing the effect of sirtinol on ERK1/2 signaling in HEK cells. HEK cells were starved for 15 hr in media containing 0.5% FBS and incubated with 60  $\mu$ M sirtinol for 4 hr and then treated with IGF-1 or PMA for 5 min. Cell lysates were subjected to SDS-PAGE and blotted with anti-phospho-ERK1/2 or anti-ERK1/2. Quantifications are shown as mean  $\pm$  SEM.

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 anti-AcK (Figures 4C, 4D, and S7). IGF-I did not significantly alter the acetylation level of IRS-1 or IRS-2 (Figure 4D). However, pretreatment with 5 mM of Nico for 24 hr or 60  $\mu$ M of sirtinol for 4 hr significantly increased the acetylation level of IRS-2 by 93.5% or 79.7%, respectively





**Figure 3. Subcellular Localization of SirT1 in the Brain**

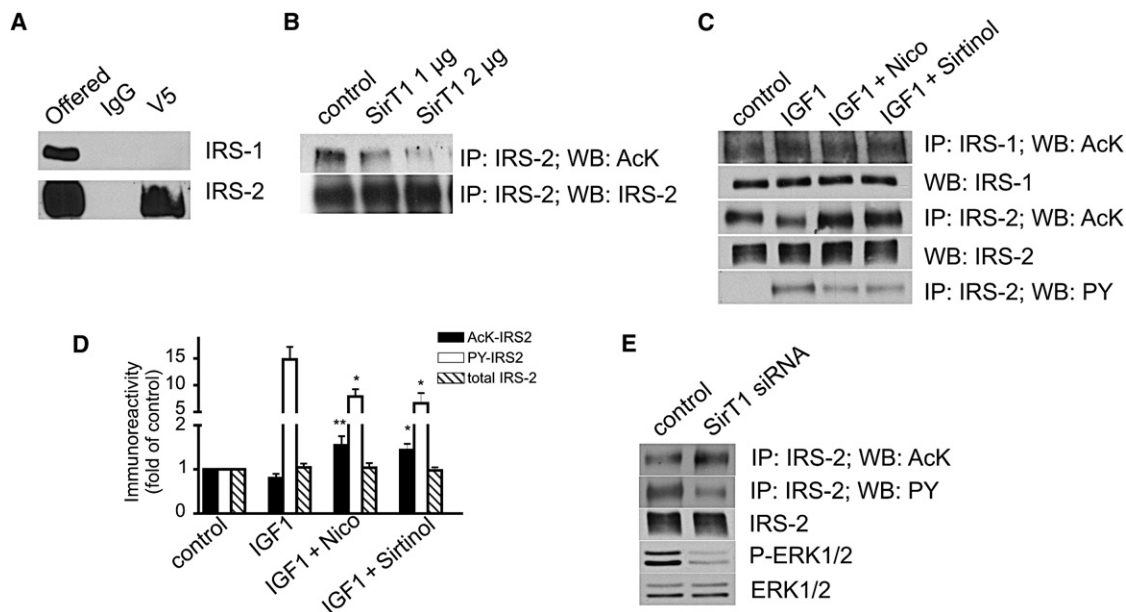
(A and B) Forebrains from postnatal 3 days (P3, A; adult mice, B) were homogenized and separated into nuclear, cytosol, and membrane fractions through sucrose-gradient centrifugation and immunoblotted with SirT1, NeuN, GAPDH, and GluR2/3, respectively. In P3, brain SirT1 is localized in both nucleus and cytosol, while it is found predominantly in cytosol in the adult brain.

(C) The specificity of SirT1 immunoblot was confirmed with SirT1<sup>-/-</sup> mice.

( $p < 0.01$ , IGF-I versus IGF-I + Nico,  $n = 4$ ;  $p < 0.05$ , IGF-I versus 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 pretreatment with Nico

(46.7% decrease,  $p < 0.05$ , IGF-I versus IGF-I + Nico,  $n = 4$ , one-way ANOVA, Turkey's test) or sirtinol (55.3% decrease,  $p < 0.05$ , IGF-I versus IGF-I + sirtinol,  $n = 4$ , one-way ANOVA, Turkey's test).

To corroborate the results in HEK293 cells, we also examined rat cortical neurons transfected with control or SirT1 siRNA (Figure 4E). Inhibition of SirT1 increased acetylation level of IRS-2 and decreased phosphorylation of IRS-2. Concurrently, activation of ERK1/2 was reduced by SirT1 inhibition.



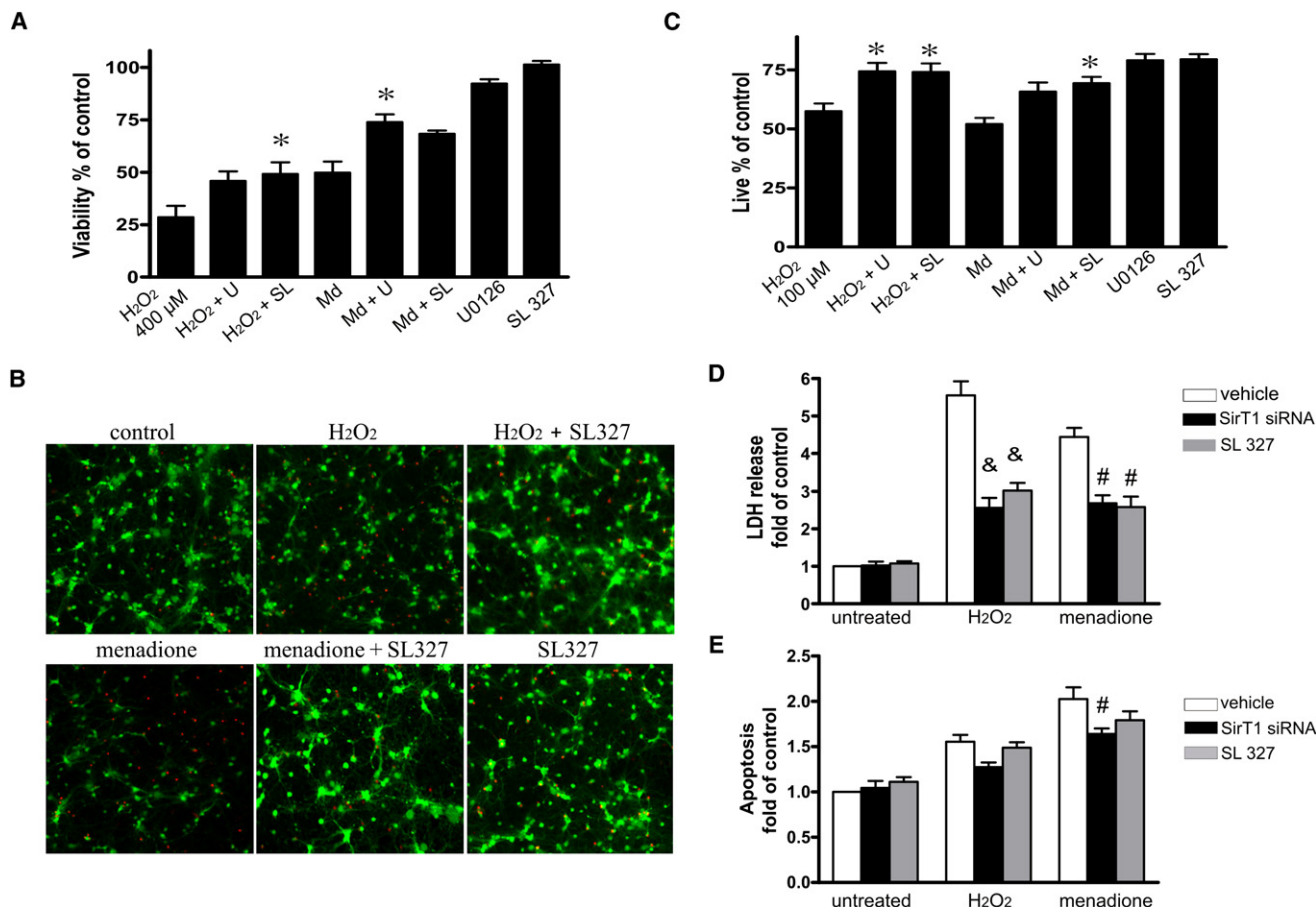
**Figure 4. SirT1 Regulates Ras/ERK1/2 Signaling via Deacetylation of IRS-2**

(A) Physical interaction between SirT1 and IRS-2. HEK293 cells were transfected with V5-SirT1, and 48 hr later cell lysates were immunoprecipitated with V5 antibody or control IgG and probed with anti-IRS-1 or anti-IRS-2, respectively.

(B) SirT1 deacetylates IRS-2 in vitro. HEK cell lysates were precipitated with anti-IRS-2, divided into three equal parts, and incubated with 0, 1, or 2  $\mu$ g SirT1 deacetylase, respectively, for 1.5 hr. They were then subjected to SDS-PAGE and blotted with antiacetylated lysine (AcK).

(C and 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. In (C), HEK cells were treated as indicated and cell lysates were precipitated with anti-IRS-1 or anti-IRS-2 and blotted with anti-AcK or anti-phosphotyrosine (PY). Cell lysates were also blotted for IRS-1 or IRS-2. In (D), quantification is shown from four to five blots (mean  $\pm$  SEM).

(E) Representative blots showing the effect of SirT1 on IRS-2 deacetylation in neurons. Five DIV neurons were transfected with either control or SirT1 siRNA. Two days later, cell lysates were immunoprecipitated with anti-IRS-2 and blotted for AcK or PY. Cell lysates were also blotted for IRS-2, P-ERK1/2, and ERK1/2, respectively.



**Figure 5. Inhibiting MEK/ERK1/2 Protects Neurons against Oxidative Stress**

Neurons were cultured in 96-well plates (A, D, and E) or glass coverslips in 6-well plates (B).

Ten to fourteen DIV neurons were pretreated with ERK1/2 inhibitors U0126 or SL327 for 4 hr. Oxidative stress was induced by H<sub>2</sub>O<sub>2</sub> or menadione. Viability was measured by MTT (A) or live/dead assay (B).

(C) Percent live was calculated from the number of live (green) and dead (red) cells.

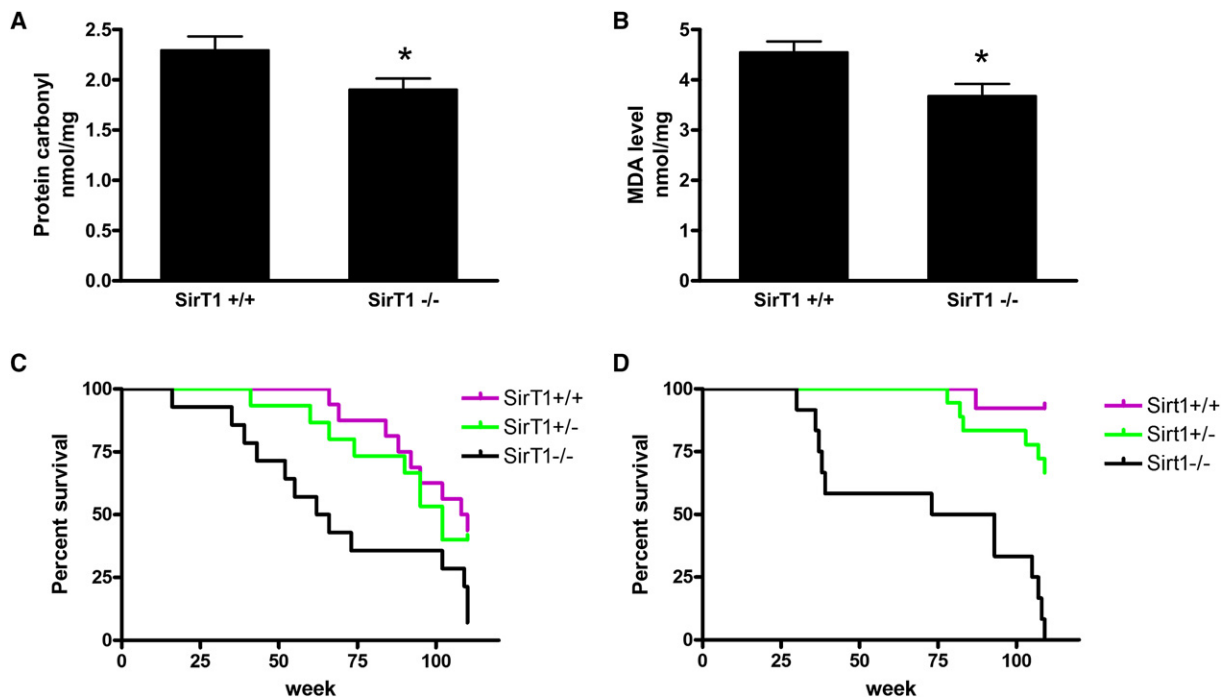
(D and E) ERK1/2 inhibition primarily attenuates necrosis. Neurons were transfected with control or SirT1 siRNA, or incubated with SL327 before exposed to 400  $\mu$ M H<sub>2</sub>O<sub>2</sub> or 7.5  $\mu$ M menadione. Twenty-four hr later, culture media were collected for LDH assay, and cells were lysed for apoptosis assay (&  $p < 0.01$  compared with H<sub>2</sub>O<sub>2</sub>; #  $p < 0.05$  compared to Md, t test). Data represent mean  $\pm$  SEM.

### MEK/ERK1/2 Inhibition Protects Neurons Against Oxidative Stress

To test if downregulation 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<sub>2</sub>O<sub>2</sub> or 7.5  $\mu$ M of menadione for 24 hr. The indicated groups of cells were pretreated with MEK1/2 inhibitor—10  $\mu$ M U0126 or 10  $\mu$ M SL327 for 4 hr. MTT assay suggests that 400  $\mu$ M H<sub>2</sub>O<sub>2</sub> killed 71.4%  $\pm$  5.4% of neurons (Figure 5A). Pretreatment with U0126 or SL327 increased the survival to 45.9%  $\pm$  4.5% ( $p > 0.05$ , H<sub>2</sub>O<sub>2</sub> versus H<sub>2</sub>O<sub>2</sub> + U,  $n = 3$ , one-way ANOVA, Turkey's test) or 49.2%  $\pm$  5.5% ( $p < 0.05$ , H<sub>2</sub>O<sub>2</sub> versus H<sub>2</sub>O<sub>2</sub> + SL,  $n = 3$ , one-way ANOVA, Turkey's test), respectively. Menadione reduced the viability to 49.9%  $\pm$  5.3% compared to control. Pretreatment of U0126 or SL327 increased the survival to 73.9%  $\pm$  3.7% ( $p < 0.05$ , menadione versus menadione + U,  $n = 3$ , one-way ANOVA, Turkey's test) or 68.3%  $\pm$  1.5% ( $p > 0.05$ , menadione versus menadione + SL,  $n = 3$ , one-way ANOVA, Turkey's test), respectively. Similar results

were obtained with Live/Dead assay (Figures 5B and 5C). One-hundred  $\mu$ M of H<sub>2</sub>O<sub>2</sub> led to 57.5%  $\pm$  3.3% survival compared to control. Pretreatment of U0126 increased viability to 74.4%  $\pm$  3.5% ( $p < 0.05$ , H<sub>2</sub>O<sub>2</sub> versus H<sub>2</sub>O<sub>2</sub> + U,  $n = 4$ , one-way ANOVA, Turkey's test), and SL327 increased survival to 74.1%  $\pm$  3.6% ( $p < 0.05$ , H<sub>2</sub>O<sub>2</sub> versus H<sub>2</sub>O<sub>2</sub> + SL,  $n = 4$ , one-way ANOVA, Turkey's test). Similarly, menadione reduced viability to 52.1%  $\pm$  2.6% compared to control. Pretreatment of U0126 increased viability to 65.8%  $\pm$  3.8% ( $p > 0.05$ , menadione versus menadione + U,  $n = 4$ , one-way ANOVA, Turkey's test), while SL327 increased survival to 69.4%  $\pm$  2.6% ( $p < 0.05$ , menadione versus menadione + 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 upregulating 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



**Figure 6. Reduced Brain Oxidative Damage and Life Span in SirT1<sup>-/-</sup> Mice**

(A and B) Reduced oxidative stress in SirT1 knockout mice brain. In (A), protein carbonyl content; nmol protein carbonyl per mg protein, mean  $\pm$  SEM, and lipid peroxidation, in (B), TBARS assay is shown; nmol malondialdehyde (MDA) equivalent per mg protein was measured in 18-month-old SirT1 +/+ and -/- mice brains.

(C and D) Calorie restriction does not extend life span of SirT1<sup>-/-</sup> mice. In (C), survival curves are shown for SirT1 +/+ (n = 16), +/- (n = 15), -/- mice (n = 14) on ad-lib diet ( $p < 0.05$ , log-rank test). In (D), survival curves are shown for SirT1 +/+ (n = 13), +/- (n = 18), -/- (n = 12) mice on 40% reduced-calorie diet ( $p < 0.001$ , log-rank test). Mice were two to five months old at the onset of CR.

or treated them with MEK1/2 inhibitor SL327 and then exposed the cells to 400  $\mu$ M H<sub>2</sub>O<sub>2</sub> or 7.5  $\mu$ M menadione for 24 hr. We estimated necrosis by measuring LDH (lactate dehydrogenase) release in the media, and we quantified apoptosis by measuring cytoplasmic histone-associated mono- and oligonucleosomes with Cell Death Detection ELISA<sup>PLUS</sup> kit (Figures 5D and 5E). Hydrogen peroxide and menadione increased LDH release to  $5.5 \pm 0.7$ - and  $4.4 \pm 0.4$ -fold of control, respectively. Inhibition of SirT1 or ERK1/2 significantly alleviated the increase in LDH release (Figure 5D;  $p < 0.01$  compared to H<sub>2</sub>O<sub>2</sub>,  $p < 0.05$  compared to menadione;  $n = 3$ ,  $t$  test). Meanwhile, H<sub>2</sub>O<sub>2</sub> and menadione induced apoptosis to  $1.6 \pm 0.2$ - and  $2.0 \pm 0.2$ -fold of control, respectively. Neither SirT1 nor ERK1/2 inhibition effectively reduced H<sub>2</sub>O<sub>2</sub>-induced apoptosis, but SirT1 inhibition had a small yet significant effect in reducing apoptosis induced by menadione (Figure 5E;  $1.6 \pm 0.2$ -fold of control,  $p < 0.05$ ,  $n = 10$ ,  $t$  test).

#### Reduced Oxidative Damage in SirT1<sup>-/-</sup> Mouse Brain

We have shown in neurons that SirT1 inhibition protects against exogenous oxidative insult of H<sub>2</sub>O<sub>2</sub> 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 (Figure 6A) and lipid peroxidation with TBARS method (Figure 6B). Compared to wild-type (WT) mice ( $2.3 \pm 0.14$  nmol/mg),

KO brains showed 17% decrease in protein carbonyl content ( $1.9 \pm 0.1$  nmol/mg;  $p < 0.05$ ,  $n = 7$ ,  $t$  test). Lipid peroxidation was also reduced in KO brain ( $3.7 \pm 0.2$  nmol malondialdehyde/mg), 20% less than that in WT ( $4.5 \pm 0.2$  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 life span 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 the SirT1 gene (McBurney et al., 2003). The mice were fed ad lib (Figure 6C) or on a 40% reduced-calorie diet for 2 years (Figure 6D). Compared to WT and HET mice, SirT1 KO mice survived shorter on both normal and CR diets. 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 CR 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; 2006; Lee et al., 2006). 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, 2001, 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 indicate that SirT1 increases insulin/IGF-I signaling in part by deacetylating IRS-2, which promotes its phosphorylation. These posttranslational 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<sup>−/−</sup> 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 KO mice under both ad lib or CR 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. (2007) examined different tissues and found SirT1 predominantly localized to the cytosol in mouse brain tissue. 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 antiaging role in mammals by deacetylating and activating FOXO (forkhead transcription factors class O) (Brunet et al., 2004; Kobayashi et al., 2005). Similarly, SIRT1 exhibits antagonistic effects of insulin on PGC-1 $\alpha$  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 S6 kinase 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 (MEFs) 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 overexpression of SirT1 was reported to increase Akt activation under insulin-resistant conditions but not under normal conditions (Sun et al., 2007). In 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 *Irs-2*<sup>−/−</sup> mice live longer (Taguchi et al., 2007).

ERK1/2 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), while it increases the sensitivity to oxidative stress under other conditions. Inhibition of ERK abrogates cell death induced by H<sub>2</sub>O<sub>2</sub> in pancreatic cancer cells (Osada et al., 2008) and protects against glutamate-induced neuronal death (Satoh et al., 2000). Our results suggest that SirT1 inhibition protects neurons by decreasing Ras/ERK signaling and provide evidence for the proaging role of the Ras/ERK pathway downstream of IGF-I, analogously to that of the Ras pathway in *S. cerevisiae*. In agreement with our present results, a reduction in brain IRS-2 increased the life span of mice and stabilized MnSOD (manganese superoxide dismutase) activity after fasting (Taguchi et al., 2007). These results in brain *Irs-2*<sup>+/−</sup> 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 antisurvival



functions depending on the conditions. For instance, SirT1 countered p53-dependent apoptosis caused by etoposide in MEFs (Luo et al., 2001). In an ALS mouse model, SirT1 rescued neurons (Kim et al., 2007). However, SirT1 can also exacerbate cell death. As described earlier, SirT1 KO MEFs showed higher replicative life spans under chronic, sublethal oxidative stress (Chua et al., 2005). SirT1 also sensitized HEK293 cells to TNF $\alpha$ -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. Second, SirT1's subcellular localization may 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 antiapoptotic effect may come only from nuclear-localized SirT1 (Tanno et al., 2007). Third, SirT1 has a wide array of targets which may become preferentially (de)activated in different contexts. Deacetylation of p53 and FOXO contributes to the prosurvival effect of SirT1 (Brunet et al., 2004; Langley et al., 2002; Motta et al., 2004), while that of NF- $\kappa$ B and p19<sup>ARF</sup> mediate the prodeath 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 KO 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. (2007) reported the beneficial effect of SirT1 overexpression in the heart against oxidative stress, although they showed that this antioxidant effect becomes a pro-oxidant effect at a higher overexpression level. 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 CR diets (Figure 6). Thus, differently from our studies in yeast, we did not find that CR further extends 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 can contribute 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), IRS-2 (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 is a plasmid carrying SirT1 siRNA sequence of 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  $\mu$ M glutamate and B-27, and plated at  $3 \times 10^4$ /well in 96-well plates for viability assays, at  $10^5$ /well in 6-well plates for immunostaining or  $5 \times 10^5$  for immunoblotting, at  $3 \times 10^6$  onto 10 cm dishes for activity assay or immunoprecipitation. They were maintained in neurobasal medium supplemented with B-27 and glutamine until 5–7 DIV for transfection or 10–14 DIV for treatments. Neurons for MTT were pretreated with SirT1 inhibitor for 48 hr prior to exposure to H<sub>2</sub>O<sub>2</sub> or menadione for 24 hr in Eagle's minimal essential medium supplemented with 21 mM glucose, 5% fetal bovine serum, and 5% horse serum.

Negative control and SirT1 siRNA (SirT1 target sequence: AAGTGCCT CAAATATTAATAA) were from QIAGEN. The sequence specificity of siRNA against rat SirT1 was verified by a genome-wide search against the rat build four-genome database (8,014 sequences; 5,387,086,425 total letters, NCBI). At least 2 nt differences were found in the nearest match, and no predicted rat sirtuins (SIRT2-7) were in the top 20 hits. On 5 DIV, rat cortical neurons were transfected with 25 nM control or SirT1 siRNA using HiPerFect Transfection Reagent. Two 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

SirT1 deacetylase activity was measured using fluorometric SIRT1 Assay Kit (Sigma CS1040) per manufacturer instructions except that 1  $\mu$ M trichostatin A was also added to the reaction. Fluorescence intensity at 444 nm (excitation 355 nm) was recorded and normalized to micrograms of protein and values represented as percent of control.

### Viability

Cell survival was measured by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Absorptions at 570 nm were normalized to control and three to four wells were used for each condition per experiment. Data from three independent experiments were averaged and shown as mean  $\pm$  SEM. LDH assay was performed with CytoTox 96 Non-Radioactive Cytotoxicity Kit (Promega, Madison, WI). Data from three independent experiments are shown as mean  $\pm$  SEM and normalized to control. Apoptosis was measured with Cell Death Detection ELISA<sup>PLUS</sup> kit (Roche, Indianapolis, IN). Data from 10 wells from three independent experiments are shown as mean  $\pm$  SEM and normalized to control. In Live/Dead Assay (Invitrogen L3224), cells were stained with 2  $\mu$ M calcein AM (green fluorescent due to esterase in living cells) and 2  $\mu$ M 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, two coverslips were used for each condition and four random fields were captured for each coverslip. Live (green) and dead (red) cells were counted from three independent experiments. A portion of a representative image is shown for each condition.

### Immunocytochemistry

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  $\text{Na}_3\text{VO}_4$ , 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  $\mu\text{g}$  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  $\mu\text{M}$  TSA and 5 mM nicotinamide and incubated with IRS-1 or IRS-2 antibodies; 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 an equal amount of IRS-2 was incubated with different amounts of SirT1 deacetylase (0, 1, 2  $\mu\text{g}$ ) in 1 mM NAD, 25 mM Tris (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM  $\text{MgCl}_2$ , and 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<sup>+/+</sup>, SirT1<sup>+/-</sup>, and SirT1<sup>-/-</sup> genotypes have been described previously (McBurney et al., 2003). Mice were deeply anesthetized with isoflurane followed immediately by decapitation, and brains (and hippocampi) were collected right away. In life-span studies, ad-libitum mice were fed NIH-31 standard feed while CR mice were fed daily with NIH31/NIA-fortified food containing 60% of calories consumed by ad-libitum 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. The forebrain was homogenized in chilled buffer containing 0.32 M sucrose, 10 mM HEPES (pH 7.4), 2 mM EDTA, protease inhibitors, and phosphatase inhibitors and centrifuged at 1000 $\times$  g for 15 min to yield the nuclear fraction pellet. The supernatant was then centrifuged at 200,000 $\times$  g for 15 min to yield cytosol in the supernatant. The resulting pellet was resuspended and centrifuged four more times to obtain the membrane fraction.

**Determination of Oxidative Stress**

Half brain minus cerebellum (HFB) was homogenized in 50 mM phosphate buffer (pH 7.4) and centrifuged to yield supernatant for assaying for protein carbonyl content with OxiSelect Protein Carbonyl ELISA Kit (Cell Biolabs 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 two groups. One-way ANOVA was used to compare multiple groups with Turkey's test as posthoc test. Groups were determined significantly different when  $p < 0.05$ .

**SUPPLEMENTAL DATA**

Supplemental Data include seven figures and can be found online at <http://www.cellmetabolism.org/cgi/content/full/8/1/38/DC1/>.

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