SIRT1 Protein, by Blocking the Activities of Transcription Factors FoxO1 and FoxO3, Inhibits Muscle Atrophy and Promotes Muscle Growth*

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Donghoon Lee and Alfred L. Goldberg

From the Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115

**Background:** SIRT1 regulates the activity of FoxO transcription factors and protects tissues from diverse insults.

**Results:** Upon fasting, SIRT1 levels fall in skeletal muscle, but SIRT1 overexpression deacetylates FoxO3 and thus inhibits induction of atrophy-related genes.

**Conclusion:** SIRT1 overexpression blocks muscle atrophy induced by fasting and denervation and in fed mice promotes hypertrophy.

**Significance:** SIRT1 activity is important in the regulation of muscle size.

In several cell types, the protein deacetylase SIRT1 regulates the activities of FoxO transcription factors whose activation is critical in muscle atrophy. However, the possible effects of SIRT1 on the activity of FoxOs in skeletal muscle and on the regulation of muscle size have not been investigated. Here, we show that after food deprivation, SIRT1 levels fall dramatically in type II skeletal muscles (tibialis anterior), which show marked atrophy, unlike in the liver (where SIRT1 rises) or heart or the soleus, a type I muscle (where SIRT1 is unchanged). Maintenance of high SIRT1 levels by electroporation in mouse muscle inhibits markedly the muscle wasting induced by fasting as well as by denervation, and these protective effects require its deacetylase activity. SIRT1 overexpression reduces muscle wasting by blocking the activation of FoxO1 and 3. It thus prevents the induction of key atrogenes, including the muscle-specific ubiquitin ligases, atrogin1 and MuRF1, and multiple autophagy (Atg) genes and the increase in overall proteolysis. In normal muscle, SIRT1 overexpression by electroporation causes rapid fiber hypertrophy without, surprisingly, activation of the PI3K-AKT signaling pathway. Thus, SIRT1 activation favors postnatal muscle growth, and its fall appears to be critical for atrophy during fasting. Consequently, SIRT1 activation represents an attractive possible pharmacological approach to prevent muscle wasting and cachexia.

An important survival mechanism in mammals in times of food scarcity, injury, and systemic disease (including cancer cachexia, sepsis, diabetes, and renal failure) is to increase protein degradation and reduce protein synthesis in skeletal muscle to provide the organism with a supply of amino acids for hepatic gluconeogenesis, direct oxidation, or repair processes (1, 2). It is now well established that these various types of muscle wasting, although induced by diverse physiological stimuli, involve similar adaptive changes in the transcription of a common set of atrophy-related genes (atrogenes). These adaptations lead to a general activation of protein degradation through the ubiquitin proteasome pathway (3), as well as increased autophagy (3–5). It is now clear that activation of FoxO3 in muscles plays an essential role in transcription of these atrogenes (such as the muscle-specific ubiquitin ligases, atrogin1, and MuRF1) (6, 7) and various atrophy genes (4, 5) in these diverse catabolic conditions and stimulates proteolysis to cause rapid atrophy (3, 5, 8).

Several additional transcriptional regulators have been shown to retard atrophy by inhibiting FoxOs such as JunB (9) or the exercise-induced coactivators, PGC-1α, and its homolog PGC-1 (10–12). In multiple types of atrophy, these factors fall rapidly and thus help signal the atrophy process. This antagonism between PGC-1α and FoxOs in skeletal muscle is surprising because in liver, PGC-1α cooperates with FoxO3 in the induction of gluconeogenic genes during fasting (13). Thus, these key pathways can be regulated in opposite fashions and serve different functions in different tissues, especially muscle.

Among the seven members of the sirtuin family in mammals, SIRT1, a NAD⁺-dependent protein deacetylase, has been the most studied and shown to protect against a surprisingly wide number of pathological processes (14). In a variety of disease models, SIRT1 mediates beneficial effects in many, perhaps all, tissues. Furthermore, small molecule activators of SIRT1 also show many of the beneficial effects seen in transgenic mice (15–18). However, the possible regulatory roles of SIRT1 in skeletal muscle have received surprisingly little attention, although early in fasting, SIRT1 activation has been reported to increase dramatically endurance exercise through the activation of PGC-1α in muscle, which stimulates fatty acid oxidation (16, 17, 19, 20). This rapid switch from carbohydrate to lipid oxidation in muscle occurs within 12 h after food deprivation and thus precedes the acceleration of protein breakdown and the development of muscle wasting (21), which is pronounced in mice only 36–48 h after food deprivation. The possible influence of SIRT1 on muscle size—on the atrophy or hypertrophy

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†To whom correspondence should be addressed: Dept. of Cell Biology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115. Tel.: 617-432-1855; Fax: 617-432-1144; E-mail: alfred_goldberg@hms.harvard.edu.
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process—and on the regulation of FoxO transcription factors have not been studied.

By itself, activation of FoxO3 in muscles can trigger dramatic atrophy of muscle (8). In several tissues, FoxO1 and 3 are known to be deacetylated by SIRT1 (22–25). Because this SIRT1-regulated transcription factor plays critical roles in the induction of atrophy and PGC-1α can block this process (10–12), it seemed likely that SIRT1 may also be critical in controlling the atrophy program. Specifically, we hypothesized that SIRT1 activation in muscle might provide protection against muscle wasting induced by disuse, fasting, and disease by inhibiting FoxOs and activating PGC-1α. We therefore examined whether changes in SIRT1 activity may play a role in the increase in muscle proteolysis and atrophy upon food deprivation (which is signaled by decreases in IGF1 and insulin levels and enhanced glucocorticoids) and also by denervation (which prevents normal contractile activity). We show here that SIRT1 overexpression, by inhibiting FoxO1 and 3, is a major regulator of muscle mass, and not only can prevent the rapid loss of muscle mass upon fasting or denervation, but also can induce rapid hypertrophy of normal muscle.

EXPERIMENTAL PROCEDURES

Antibodies—The following antibodies were used: anti-SIRT1 (07-131; Millipore), anti-SIRT3 (5490; Cell Signaling), anti-SIRT6 (S4322; Sigma), anti-FLAG (F1804; Sigma), anti-acetyl-lysine (ICP0380; Immunechem), anti-JunB (sc-73; Santa Cruz), and anti-ATP5B (sc-55597; Santa Cruz). The following antibodies were purchased from Cell Signaling: anti-FoxO1 (2880), anti-FoxO3 (9467), anti-phospho-FoxO (9464), anti-AKT (4691), anti-phospho-akt(308) (9257), anti-S6K (9202), anti-phospho-S6K (9205), anti-mTOR (2972), anti-phospho-mTOR(S2448) (2971), and anti-Tubulin (2146).

Reagents—Acetylated histone H4 (12–353) was purchased from Millipore.

Animal Studies and Electroporation of Adult Muscle—Adult male CD1 mice (28–30 g) were used in all experiments. Tibialis anterior muscles were transfected by electroporation as described previously (8, 10). To study fasting, 4 days after electroporation, mice were either fed or deprived of food for 2 days. Denervation was performed at the same time as the electroporation, and muscles were collected at the times indicated. In reporter experiments, 10 μg of firefly luciferase constructs containing multiple binding sites for FoxOs (hereafter referred to as FoxO1 and 3) (Addgene plasmid 1789) and 5 μg of Renilla luciferase plasmid (Promega) together with either FLAG-SIRT1 (5 μg) or FLAG-SIRT1H355A (5 μg) (kindly provided by Dr. Pere Puigserver) were co-electroporated. A construct for IxBa-α-SR was purchased from Addgene (plasmid 15264). In electroporation experiments, no microscopic evidence for inflammation was observed. All mouse experiments were performed with the approval of Institutional Animal Care and Use Committee and are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Measurement of Cross-sectional Area—Electroporated muscles were cryosectioned (10 μm) and fixed with 4% paraformaldehyde as described previously (8, 10). Fibers stained with a FLAG antibody (Sigma) or expressing GFP and an equal number of nontransfected fibers were used for measurement of cross-sectional areas using Metamorph (Molecular Devices).

Protein Degradation—After differentiation for 72 h, C2C12 myotubes were infected with adenoviruses expressing GFP or SIRT1 for 4 h and then washed with fresh DMEM plus 2% horse serum and incubated for additional 48 h. Twenty-four hours later, the cells were incubated with [3H]tyrosine (4 μCi/ml; PerkinElmer) for 24 h to label long-lived proteins. Subsequent procedures were performed as described previously (5, 10).

RNA Extraction and Quantitative Real Time PCR—Total RNA was extracted with TRizol (Invitrogen), and reverse transcription was performed to synthesize cDNA. Quantitative real time PCR was performed with mouse gene specific primers (the sequences of the primers used are available upon request) and DyNaMo HS SYBR Green qPCR kit (Finnzymes) using a C100 Thermal Cycler (Bio-Rad).

Deacetylase Assay—FLAG-SIRT1 or -SIRT1H355A was affinity-purified from electroporated muscles and used for deacetylase assay. Acetylated histone H4 peptide (0.2 μg, Millipore) was incubated with the purified FLAG-SIRT1 with or without 5 mM NAD+ (Sigma) at 37 °C for 30 min. Each reaction was blotted on nitrocellulose membrane and visualized by acetyl-lysine antibody (Immunechem) in Western blot. Intensity of dots was quantified using ImageJ (National Institutes of Health).

Statistical Analysis—The analysis was performed using Student’s t test, and significant differences are demonstrated by asterisks. The data are presented as means ± S.E.

RESULTS

SIRT1 Content Decreases during Fasting When Atrogenes Are Induced—To determine whether SIRT1 is involved in muscle atrophy during fasting for different periods, we analyzed the expression of SIRT1 and two atrophy-associated ubiquitin ligases, atrogin1 and MuRF1, in mouse tibialis anterior (TA)2 muscles. Although no change was seen in SIRT1 content at 12 or 24 h after food was removed (Fig. 1A and Table 1), when SIRT1 is known to stimulate PGC-1α and lipid oxidation (20) (Fig. 1, A, C, and D), by 48 h, SIRT1 levels had decreased dramatically in the muscles (Fig. 1A). This delayed fall in SIRT1 protein coincided with the induction of atrogin1 and MuRF1 and the loss of muscle mass (which was not significant until this time).

The tibialis anterior is composed of type II (pale) muscle fibers, which tend to show marked wasting after food deprivation (21). By contrast, type I (dark) fibers are relatively resistant to wasting in fasting. Interestingly, the dark soleus muscle showed little or no change in SIRT1 content after a 2-day fast (data not shown).

In contrast to these changes in type II muscle, in the livers of these fasted mice, SIRT1 content had increased by 48 h, and in heart, its level did not change (Fig. 1E). To determine whether the large reduction in SIRT1 protein in the TA muscle resulted from decreased gene expression, we assayed the levels of SIRT1 mRNA by real time PCR but found no significant change (Fig. 1B), as had been reported in liver (26). Thus, the decline in

2 The abbreviation used is: TA, tibialis anterior.
muscle SIRT1 during fasting most likely occurred by enhanced protein degradation or perhaps reduced translation. To determine whether other sirtuins also decrease in muscles during fasting, we also analyzed by Western blot the content of another nuclear sirtuin SIRT6 and the mitochondrial sirtuin, SIRT3. Although there was no change in the level of SIRT6, SIRT3 level increased during fasting, as was reported previously (27) (Fig. 1F). Thus, muscle SIRT1 is regulated in a distinct fashion, and during fasting, its level decreases specifically in muscle at times when the atrogeine program is induced, and there is rapid wasting.

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We therefore hypothesized that the reduction in SIRT1 protein is important in the activation of FoxO3 and the induction of atrogeneS upon fasting. If so, overexpression of SIRT1 might
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inhibit the fasting-associated rise in atrogene expression and thus reduce atrophy (just as the beneficial effects of SIRT1 in other tissues were observed only upon its pharmacological activation or genetic overexpression (15–17, 28, 29)). To test these possibilities, we electroporated into TA muscle of adult mice (8) plasmids encoding FLAG-tagged mouse SIRT1 or a mutant lacking deacetylase activity, FLAG-SIRT1H355A. Control vectors were electroporated into the contralateral TA muscles. After 4 days, mice were either fed ad libitum or deprived of food for 2 days (Fig. 2A). We measured the cross-sectional area of

![Diagram A: Electroporation and Collect TA](image)

![Diagram B: 2 days fasting](image)

![Diagram C: % weight loss](image)

![Diagram D: Atrogin1 and MuRF1](image)

![Diagram E: Atg4b, Gabarap1, Bnip3](image)

![Diagram F: Protein degradation](image)

![Diagram G: FLAG IP with fasting TA lysates](image)

![Diagram H: Control vector, F-SIRT1, PGC-1α](image)
individual fibers in the TA muscle using an anti-FLAG antibody to stain electroporated SIRT1, which as expected was found only in muscle nuclei (Fig. 2B). Remarkably, in the fasted animals, the median cross-sectional area of fibers overexpressing SIRT1 was ~48% larger than that of nonelectroporated fibers in the same muscle.

Because SIRT1 null mice show clear developmental abnormalities (30) and skeletal-muscle specific SIRT1 knock-out mice have not been reported, we generated plasmids encoding shRNA against SIRT1 and electroporated them into TA muscles. Although SIRT1 overexpression inhibited fiber atrophy, the knockdown of SIRT1 did not further increase the extent of atrophy in mice fasted for 48 h, presumably because SIRT1 levels were already markedly reduced at this time (data not shown). In addition, it may be impossible to detect a further decrease in median fiber size, because the rate of muscle wasting is already very rapid between 24 and 48 h (21) (see below). Knockdown of SIRT1 in fed control mice had no effect on muscle size (data not shown). Thus, although the fall in SIRT1 seems to be important for fiber atrophy in fasting, reducing its content in a fed animal per se is not sufficient to activate the atrophy program (see below), which must require additional catabolic signals (most likely, the decreased activity of the Akt pathway and FoxO dephosphorylation, whose importance is well established).

To confirm the muscle-sparing effect of SIRT1 upon fasting, we measured muscle mass. In the fasted mice, the mass of the TA electroporated with control plasmids (pFLAG-CMV4) decreased by ~20% below that of TA muscle in fed mice (electroporated with the same vector). However, in the muscles overexpressing SIRT1, the weight loss upon fasting was half as great ($p < 0.01$) as in muscles transfected with a control vector (10% loss versus 20%) (Fig. 2C). Because only 60–80% of the fibers in these muscles were transfected, SIRT1 must have caused a much larger reduction in weight loss in the SIRT1-expressing fibers. This inhibition of weight loss required the deacetylase activity of SIRT1 because overexpressing the inactive mutant SIRT1H355A had no protective effect upon fasting (Fig. 2C), unlike the wild type SIRT1. In fact, the mutant increased slightly the fasting-associated weight loss in the muscles ($p < 0.05$) (Fig. 2C), presumably by acting as a dominant negative inhibitor of basal SIRT1 activity.

SIRT1 Blocks Atrogene Induction and Proteolysis in Fasting by its Deacetylase Activity—Because induction of atrogin1 and MuRF1 is critical for various types of rapid muscle wasting (3, 31), we measured their mRNA levels by real time PCR to determine whether SIRT1 overexpression inhibited wasting by blocking their expression. Indeed, the induction of these two atrophy-related ubiquitin ligases upon fasting was significantly decreased by electroporation of SIRT1 (Fig. 2D). In contrast, electroporation of the inactive mutant SIRT1H355A failed to inhibit the expression of atrogin1 or MuRF1 mRNAs (Fig. 2D). Thus, this effect, like the inhibition of fiber atrophy and weight loss, requires the deacetylase activity of SIRT1.

In addition to the stimulation of proteolysis by the ubiquitin-proteasome pathway, largely through induction of MuRF1 and other ubiquitin ligases and the suppression of protein synthesis from the induction of atrogin1 (32), autophagy also is activated and is critical in muscle wasting upon fasting and denervation (4, 5). Therefore, we investigated the inhibition of three key autophagy (Atg) genes: Atg4b, Gabarap1, and Bnip3, by SIRT1 upon fasting. Expression of all three was up-regulated upon fasting, as reported previously (5), but SIRT1 overexpression prevented their induction (Fig. 2E). This inhibition of autophagy gene induction also required the deacetylase activity of SIRT1 (Fig. 2E) and, together with the inhibition of atrogin1 and MuRF1, seems to account for the blockage of muscle wasting in the fasted animals. Because all these atrogens are FoxO3 targets (4, 5, 8), these findings also indicate that SIRT1 overexpression prevents FoxO3 activation upon fasting.

Because SIRT1 overexpression inhibited the induction of major atrogens (Fig. 2, D and E), we measured the mRNA levels of atrogens to determine whether the SIRT1 knockdown can accelerate expression of atrogens upon fasting. Most of our study were performed 48 h after food removal by which time there was marked wasting and a large induction of atrogenes in the muscles (as reported previously). At 24 h, however, muscle wasting is not yet clear. Atrogin1 and MuRF1 were induced, but the electroporation of shRNA against SIRT1 prior to the onset of the fasting caused a small but reproducible increase in the expression of MuRF1 ($p = 0.05$) and Gabarap1 ($p = 0.039$) (data not shown). These findings further suggest that SIRT1 inhibits transcription of atro gene genes.

FIGURE 2. SIRT1 overexpression in adult muscles inhibits muscle wasting upon fasting by repressing induction of atrogens. A, order of electroporation and food deprivation in these experiments. B, SIRT1 attenuates the decrease in fiber size upon food deprivation for 2 days. Frequency histogram showing the distribution of cross-sectional areas of fibers of TA electroporated with plasmids encoding FLAG-tagged SIRT1 (F-SIRT1) or those of nonelectroporated fibers (left image). Fluorescence microscopy of TA muscle shows electroporated fibers stained with FLAG antibody for F-SIRT1 (in red) in nucleus (in blue) (right image). Expression of FLAG-SIRT1 electroporated into TA muscles of fasted mice was examined in Western blots (lower left). Scale bar, 40 $. C$, SIRT1 inhibits weight loss upon fasting. Four days after electroporation of F-SIRT1 or inactive FLAG-tagged SIRT1H355A (F-SIRT1H355A) into TA muscles, mice were fed or deprived of food for 2 days. Shown is the weight loss in TA muscle upon fasting as a percent of the original weight (i.e., mean weights of control muscle electroporated with a control vector in fed mice). Control vector. GFP. *, $p < 0.05$ versus lane 3 ($n = 4$); **, $p < 0.01$ versus lane 1. D, overexpression of SIRT1, but not the catalytically inactive mutant, suppresses the induction of atrogin1 and MuRF1 during fasting. RNA was extracted from muscles electroporated with F-SIRT1, F-SIRT1H355A, or a control vector, and mRNAs for atrogin1 and MuRF1 were determined by RT PCR ($n = 3$). *, $p < 0.01$ versus fasted + control vector. E, SIRT1 also blocks the induction of atrogens (ATGs) in a deacetylase-dependent manner during fasting ($n = 3$). *, $p < 0.05$ versus fasted + control vector. F, increase in protein degradation in serum-deprived myotubes is inhibited by overexpression of SIRT1. After infection with viruses expressing GFP, sh-SIRT1, or SIRT1 at a multiplicity of infection of 30, C2C12 myotubes were labeled with [3H]lysine for 24 h and then deprived of serum to stimulate proteolysis. Protein degradation was measured by determining the rate of conversion of [3H]lysine to acid-soluble materials at the indicated times. The inhibition of proteolysis was evident only after a lag of 4 h, when the FoxO-dependent increase occurs (5) ($n = 5$). G, deacetylase activity of electroporated FLAG-SIRT1 or SIRT1H355A. Affinity-purified SIRT1 or SIRT1H355A and acetylated histone H4 peptides were used to assay deacetylase activity as described previously (33). Western blot shows the same amount of purified SIRT1 proteins. The assays were repeated three times, and the mean values are represented. The error bars represent S.D. ($n = 3$). *, $p < 0.05$. H, SIRT1 overexpression promotes the expression of the mitochondrial protein, ATP5B, by deacetylating PGC-1a. After electroporation of F-SIRT1, F-SIRT1H355A, or a control vector, levels of acetylation of immunoprecipitated PGC-1a and ATP5B during fasting were examined by Western blot. IP, immunoprecipitation; WB, Western blot.
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Because SIRT1 overexpression inhibited the reduction in fiber size and the expression of atrogin1, MuRF1, and autophagy genes upon fasting, it seemed likely that protein degradation would be also diminished. To test this prediction, we measured the overall rate of proteolysis using C2C12 myotubes infected with adenoviruses encoding GFP or SIRT1. To radiolabel long-lived cell proteins (i.e., the fraction whose degradation is stimulated by FoxOs (5)), C2C12 myotubes were incubated with [3H]tyrosine for 1 day. After a chase period, the degradation of proteins was assayed during serum deprivation to maximally activate autophagy. Overexpression of SIRT1 significantly reduced the increase in overall protein degradation, whereas knockdown of SIRT1 (sh-SIRT1) showed no effect (Fig. 2F). This reduction in the increased proteolysis became evident several hours after serum deprivation, which is also consistent with the ability of SIRT1 to inhibit induction of atrogens by FoxO3 and to reduce muscle wasting upon food deprivation.

To confirm that these effects of electroporation resulted from the increase in SIRT1 deacetylase activity, we assayed its activity in the muscles electroporated with SIRT1 or the inactive mutant. We isolated the transfected SIRT1 using an anti-FLAG antibody from extracts of the H355A electroporated muscles and assayed in vitro its ability to decrease acetylation of histone H4 peptides. Although strong deacetylase activity was observed after electroporation of the WT gene, SIRT1H355A electroporation had no effect on acetylation of histone H4 peptide (Fig. 2G). (Some residual acetylation of the substrates was evident in this assay, probably because SIRT1 deacetylates the various lysine residues in histone H4 to different extents (33).) It is nevertheless clear that the ability of SIRT1 to decrease fasting-induced muscle wasting correlates tightly with changes in deacetylase activity.

One important substrate of SIRT1 whose level falls upon nutrient deprivation and that inhibits atrophy is the exercise-induced transcriptional coactivator, PGC-1α (10, 11). In addition to stimulating mitochondrial expression, overexpression of PGC-1α in muscles can inhibit muscle wasting primarily by repressing the activity of FoxO3 and NF-κB (10–12). To confirm that SIRT1 overexpression deacetylates and activates PGC-1α in muscle, we monitored acetylation of PGC-1α and expression of one of its mitochondrial target genes, ATP5B, a subunit of the F1-ATPase complex. In the fasted mice, ATP5B expression was greatly stimulated in muscles overexpressing SIRT1 (Fig. 2H). This enhanced PGC-1α activity should also contribute to the capacity of SIRT1 to block atrophy upon fasting.

SIRT1 Overexpression Also Inhibits Denervation Atrophy—If SIRT1 blocks atrogin expression by FoxOs generally, it should also inhibit atrophy induced by denervation/disuse. Muscles of one hind limb in adult mice were therefore denervated by severing the sciatic nerve, and a FLAG-SIRT1 construct was electroporated into the contralateral innervated and denervated TA muscles. Measurement of cross-sectional areas in the denervated muscles 10 days later showed that the median size of fibers overexpressing SIRT1 was 46% larger than that of the denervated fibers in the same muscle (Fig. 3). Remarkably, the cross-sectional areas of fibers overexpressing SIRT1 in the denervated muscle were even larger than nonelectroporated innervated fibers in the contralateral TA (Fig. 3, A and B). Thus, overexpressing SIRT1 not only prevented weight loss, but even seemed to induce net fiber growth despite the denervation (see Below).

Furthermore, SIRT1 electroporation into denervated muscles also inhibited the induction of the key atrogens, including atrogin1, MuRF1, and Gabarap1, and Bnip3 (Fig. 3C). By contrast, electroporation of the SIRT1 mutant lacking deacetylase activity had no inhibitory effects. The similar findings upon fasting and denervation strongly suggest that enhanced SIRT1 activity can prevent muscle wasting in various disease states, where the atrogen program is activated (31).

SIRT1 Binds FoxO3 and Reduced Its Activity and Content in Muscles of Fasted Mice—Because atrogin1, MuRF1, and several autophagy genes (Atg4b, Gabarap1, and Bnip3) are all targets of FoxOs, the inhibition of their induction by SIRT1 suggested that it attenuated FoxO3 transcription activity in muscle. To directly measure the FoxO-dependent activity, vectors encoding luciferase under the regulation of multiple FoxO-responsive elements (23) were electroporated with or without FLAG-SIRT1 or FLAG-SIRT1H355A into TA muscles. Four days later, the mice were fed or deprived of food for 2 days. In the muscles transfected with control vectors of fasted mice, FoxO activity increased (Fig. 4A, lanes 1 and 2) over levels in fed controls. However, overexpression of wild type SIRT1, but not a SIRT1 mutant lacking deacetylase activity, completely blocked this activation of FoxOs (Fig. 4A) by deacetylation (Fig. 4B). Moreover, immunoprecipitation of the electroporated FLAG-SIRT1 also pulled down FoxO3, indicating a direct association between these proteins (Fig. 4C).

Because FoxO1 expression increased and phosphorylation of FoxO1 and 3 decreased in atrophying muscles (8, 31, 34), we tested whether SIRT1 also influenced FoxO1 and 3 contents. In addition to inhibiting the transcriptional activity of FoxO, SIRT1 overexpression reduced the contents of both FoxO1 and 3 (Fig. 4D). However, SIRT1 did not affect FoxO3 mRNA level (Fig. 4E) in either fed or fasted mice. Also, SIRT1 overexpression did not alter the contents of other components of the AKT-mTOR pathway or their phosphorylation (Fig. 4D; see Fig. 6A). Thus, SIRT1 appeared to down-regulate both the activity and also the levels of FoxOs, probably by enhancing its degradation, as others also suggested recently (35).

In Fed Mice, SIRT1 Overexpression Induces Rapid Hypertrophy without Activating PI3-Akt Signaling—To our surprise, the cross-sectional areas of fibers in the innervated TA that were electroporated with FLAG-SIRT1 were consistently greater than those of nonelectroporated control fibers (Fig. 3B). Thus, SIRT1 activation can also signal fiber hypertrophy. Furthermore, in our experiments on fasting mice, during the 4 days preceding food deprivation, the muscles electroporated with SIRT1 showed a small (7%) but reproducible (p < 0.05) gain in muscle mass (Fig. 5D). We therefore examined more systematically this apparent ability of SIRT1 overexpression to influence muscle size in normal adult mice (Fig. 5). Remarkably, analysis of muscle fiber areas showed clear increases by 3 days after electroporation (Fig. 5A). By 6 days after SIRT1 electroporation, the mass of the TA muscle (p < 0.05) was greater than that of control, even though, at most, only 70% of the fibers were transfected (Fig. 5B). Furthermore, no change in size was seen...
upon electroporation of SIRT1H355A (Fig. 5C), and thus, the rapid increase in muscle mass was dependent upon protein deacetylation. Interestingly, muscle growth then ceased, and muscle mass and content of SIRT1 did not change further between 6 and 12 days (Fig. 5D). Thus, although SIRT1 can induce rapid hypertrophy, the extent of the induced growth appears to be limited (Fig. 5D).

To probe the mechanisms of this rapid growth, we assayed levels and phosphorylation of AKT, which is typically activated in hypertrophying muscles; e.g., in response to IGF-1 and insulin (3, 36). Surprisingly, the growth of muscle induced by SIRT1 was not accompanied by enhanced signaling of the PI3K-AKT pathway (Fig. 6A). In addition, we did not observe an increase in overall protein synthesis rate upon overexpression of SIRT1 in C2C12 myotubes (Fig. 6B). It was recently reported that SIRT1 can cause deacetylation of AKT in animals deprived briefly of food and treated with insulin (37). However, in a variety of experiments, we have not observed any change in the extent of acetylation of AKT in muscle upon overexpression of SIRT1 (Fig. 6C). These divergent results presumably are due to the different experimental conditions used in the two studies. Also a lack of effect of SIRT1 on AKT is consistent with their different subcellular localizations (SIRT1 being nuclear and AKT being cytosolic) (Figs. 2B and 6D).

Although muscle growth in the absence of increased AKT phosphorylation is unusual, no change in AKT activation occurs in the rapid fiber hypertrophy induced by JunB overexpression (9). One additional mechanism for fiber hypertrophy reported recently is through the transcriptional co-regulator, PGC-1α4, which increases IGF-1 production in the muscle while repressing myostatin expression (38, 39). We examined whether hypertrophy resulting from SIRT1 overexpression involved increased expression of JunB or PGC-1α4. However, their expression levels do not appear to be altered by SIRT1 overexpression (Fig. 6E). Thus, SIRT1 induces fiber hypertrophy by some novel mechanism that is distinct from those of JunB, PGC-1α4, and growth factors, which primarily affect the cytosolic PI3-AKT-mTOR signaling pathway.
**DISCUSSION**

**SIRT1 in Muscle and Energy Homeostasis**—The marked decrease in SIRT1 content described here in TA muscle during a 48-h fast (Fig. 1) appears to be important for the activation of proteolysis, induction of atrogens, and wasting of muscle. This reduction in SIRT1 in type II fibers is crucial for the activation of FoxOs, which is essential for these catabolic responses. Moreover, decreasing SIRT1 level in type II fibers seemed to accelerate the rise in atrogen expression (data not shown). In contrast to this sharp fall in SIRT1 type II muscles, in the heart and the dark soleus, which shows much less atrophy upon fasting (21), there was no clear change in SIRT1 level. Although this fall in SIRT1 appears essential for the rapid atrophy on fasting and in denervation, reducing SIRT1 content in muscles of fed...
animals did not by itself cause wasting (data not shown). Therefore, additional signals are also necessary to activate the atrogenic program, most likely the decrease in PI3K/AKT signaling whose role in causing muscle wasting in IGF-1-deficient insulin-resistant conditions is well established (40). Surprisingly, the marked decline in muscle content of SIRT1 during fasting did not result from a decrease in its transcription (Fig. 1B) but probably from accelerated degradation, either through activation of a ubiquitin ligase or a modification of SIRT1 that promotes its susceptibility to a pre-existent ubiquitin ligase.

During a brief fast, the activation of AMPK leads to increased NAD$^+$ synthesis, which in turn stimulates SIRT1 to meet cellular energy demands, especially by promoting utilization of fatty acids in muscle and other tissues (20, 41). However, this adaptive response occurs rapidly upon food deprivation and should no longer be activated under the conditions studied here (food deprivation for 2 days), by which time the SIRT1 content of the muscle is very low, and proteolysis is maximal (Fig. 1A). Thus, as fasting progresses, SIRT1 must serve distinct functions in muscle. Initially (at 12 or 24 h in mice), it triggers the PGC-1α-dependent transition from utilization of carbohydrates to fatty acids (20), and subsequently (by 48 h) it causes the activation of muscle protein degradation (21). Accordingly, deacetylation (activation) of PGC-1α clearly increased 12 and 24 h after food deprivation (Fig. 1C), and as expected (20, 42), the expression of genes for utilization of fatty acid, targets of PGC-1α, was up-regulated at these times (Fig. 1D). However, by 48 h, PGC-1α was again acetylated and thus unable to inhibit transcription by FoxOs (Fig. 1C).

SIRT1 Protects against Atrophy—As shown in Fig. 2 (B and C), SIRT1 overexpression can provide remarkable protection from atrophy; e.g., upon fasting, it almost completely blocked the decrease in fiber diameter and reduced by 50% the loss of muscle weight, even though only 60–70% of the fibers contained the transgene. This inhibition of atrophy requires the deacetylase activity of SIRT1 and results largely from a blockage of the increase in protein degradation (Fig. 2, B, D, and F) and induction of the atrogenic program (Fig. 2, D and E), because of

![Figure 5](image_url)
an inhibition of FoxO activity (Fig. 4A). A similar attenuation of atrophy should be achievable through pharmacological manipulations prior to the decrease in SIRT1 level, because a number of SIRT1 activators have been developed, although for other biomedical applications (14, 43). Because SIRT1 also inhibited atrogene induction and atrophy upon denervation (Fig. 3) and promoted growth in normal mice (Fig. 5), SIRT1 activation may be a generally useful therapeutic approach in multiple types of muscle wasting (e.g., cancer cachexia and renal failure) or perhaps to build muscle in frail elderly (“age-associated sarcopenia”).

The levels of SIRT1 in muscle also are reduced in another frequently studied model of disuse atrophy, hind limb suspension (44), and probably contribute to the loss of muscle mass as they do in fasting animals. Surprisingly, however, in the muscles denervated for 10 days, SIRT1 levels were not decreased, even though, at this time, the atrogene mRNAs and overall proteolysis are increased, and loss of myofibrillar components was accelerated (45, 46). Therefore, it is not clear why the endogenous SIRT1, unlike the overexpressed SIRT1 (Fig. 3A), fails to prevent atrophy upon denervation. By 10 days after denervation, atrogene expression is returning to control levels, and possibly, SIRT1 levels are decreased only initially after nerve section, when activation of the atrogene program is maximal (2–5 days after denervation (45)). Because the factors signaling atrophy in denervated muscles clearly predominate over the endogenous SIRT1 deacetylase activity, the prevention of atrophy possibly requires the higher levels of SIRT1 achieved by overexpression or some activating agent. Alternatively, the SIRT1 in these denervated muscles may be inhibited somehow and therefore unable to prevent atrophy. In either case, to elicit profound atrophy, it seems essential for the muscles to exhibit low SIRT1 deacetylase activity for the activation of FoxOs and

**FIGURE 6. SIRT1 overexpression does not activate AKT.** JunB, or PGC-1α4 in skeletal muscle. A, electroporation and food deprivation experiments were performed as described in Fig. 2, and muscle lysates were used to monitor activity of AKT/mTOR pathway. B, SIRT1 overexpression had no measurable effect on the overall rate of protein synthesis in myotubes. After 48 h of infection with viruses expressing GFP, or SIRT1, C2C12 myotubes were labeled with [3H]tyrosine for 2 h, and the incorporation of [3H]tyrosine into proteins was measured. C, SIRT1 does not deacetylate AKT in skeletal muscles. TA muscles were electroporated with FLAG-SIRT1 or control vector, and 4 days later mice were deprived of food for 2 days. Muscle lysates were prepared for immunoprecipitation (IP) of endogenous AKT, and the level of its acetylation was determined by Western blots (WB) using acetyl-lysine antibody. D, SIRT1 is localized in nucleus, whereas AKT resides in cytoplasm. Subcellular localization of SIRT1 and AKT in C2C12 myoblasts or myotubes was determined. Scale bar, 15 μm. E, SIRT1 overexpression does not increase expression of JunB nor PGC-1α4. The asterisks indicate nonspecific bands, as described by Zhang et al. (39).
possibly other transcription factors and for the inactivation of PGC-1α.

Induction of Muscle Hypertrophy by SIRT1—A highly surprising finding was that SIRT1 overexpression not only reduced atrophy, but in normal muscle caused rapid hypertrophy (Figs. 3B and 5). Although muscles in fed animals are exposed to multiple anabolic factors (e.g., IGF-1) if denervated or inactive, muscles become resistant to their influence (47), and there is net proteolysis. However, as shown here, SIRT1 overexpression can tip the overall balance between protein degradation and synthesis, so that atrophy is diminished or prevented. In fact, the sizes of denervated fibers overexpressing SIRT1 were similar or even greater than those of fibers in the innervated control muscles (Fig. 3, A and B). Thus, in response to SIRT1, the denervated muscles could even be induced to undergo net hypertrophy.

Clearly, SIRT1 is critical in inhibiting the accelerated proteolysis and atrogenic expression by FoxOs, but to induce hypertrophy, it probably also has to stimulate some anabolic processes. Although the induction of ubiquitin ligases promotes breakdown of many proteins, especially myofibrillar proteins (46), by the proteasome and also mitochondria by autophagy (48), atrogin1 induction by promoting destruction of initiation factors also inhibits protein synthesis (32). In fed mice, even though only 60–70% of its fibers were transfected, SIRT1 overexpression enhanced the weight of the TA by 13% within 4–6 days (Fig. 5, C and D), which suggests an approximately 20% growth of transfected fibers. This rapid hypertrophy continued for only ~1 week, and the cellular mechanisms and physiological consequences of this growth will be interesting to study. Despite these dramatic effects on muscle size, and even though SIRT1 was reported to increase signaling by insulin and IGF-1 in muscle in insulin-resistant states (40), we failed, despite appreciable effort, to show that SIRT1 overexpression stimulates the PI3K/AKT/mTOR pathway (Fig. 6, A, C, and D) or to increase levels of JunB or PGC-1α 4 (Fig. 6E), which can also induce rapid growth (32, 35, 47). It thus remains possible that the SIRT1 induces growth in these muscles only through an inhibition of basal protein degradation with little or no change in overall protein synthesis. Although activation of FoxOs is crucial for the enhancement of proteolysis by autophagy and the ubiquitin proteasome system during atrophy, and inhibition of FoxO3 was found to induce hypertrophy by increasing protein synthesis (49), it remains unclear to what extent normal rates of proteolysis and protein synthesis are regulated by the basal activity of the FoxO family. Alternatively, SIRT1 overexpression may increase muscle mass by both decreasing FoxO expression and activity (Fig. 4) or by stimulating protein synthesis by some still unknown mechanism.

The loss of muscle mass in systemic diseases (e.g., cancer cachexia) or in the aged not only reduces the functional capacity of the individual but also decreases the ability to withstand the disease process (50). Consequently, it is of major medical importance to develop new approaches to combat muscle wasting and to promote the restoration of muscle mass after disease, injury, or surgery. This ability of SIRT1 activation to block atrophy and promote rapid growth by inhibiting FoxOs and activating PGC-1α thus may have therapeutic applications.

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