Posttranslational modifications control FoxO3 activity during denervation

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Bertaggia E, Coletto L, Sandri M. Posttranslational modifications control FoxO3 activity during denervation. Am J Physiol Cell Physiol 302: C587–C596, 2012. First published November 16, 2011; doi:10.1152/ajpcell.00142.2011.—Loss of muscle mass occurs in a variety of diseases including cancer, chronic heart failure, AIDS, diabetes, and renal failure, often aggravating pathological progression. The atrophy process is controlled by a transcriptional program that regulates the expression of a subset of genes named atrophy-related genes. The Forkhead Box O (FoxO) family of transcription factors plays a critical role in the atrophy program being sufficient and necessary for the expression of rate-limiting enzymes of ubiquitin-proteasome and autophagy-lysosome systems. Therefore, a fine regulation of FoxOs is critical to avoid excessive proteolysis and cachexia. FoxO activity can be modulated by different mechanisms including phosphorylation, acetylation, ubiquitination, and glycosylation. Here we show that FoxO3 is progressively acetylated during denervation and concomitantly atrogin-1, the bona fide FoxO3 target, is downregulated. FoxO3 interacts with the histone acetyl-transferase p300, and its acetylation causes cytosolic relocation and degradation. Several lysine residues of FoxOs are known to be acetylated. To identify which lysines are critical for FoxO3 activity we have generated different FoxO3 mutants that either mimic or prevent lysine acetylation. We found that FoxO3 mutants that mimic acetylation show a decrease of transcriptional activity and cytosolic localization. Importantly, acetylation induces FoxO3 degradation via proteasome system. Between the different lysines, lysine 262 is critical for translocation of FoxO3. In conclusion, we provide evidence that FoxO3 activity is negatively modulated by acetylation and ubiquitination in a time-dependent and coordinated manner. This fine-tuning mechanism of FoxO3 regulation may be important to prevent excessive muscle loss and can be used as a therapeutic approach to counteract muscle wasting.

skeletal muscle; atrophy; acetylation; ubiquitination; Mdm2

MUSCLE IS A PLASTIC TISSUE that adapts its mass to different environmental and endogenous stimuli such as nutrition, hormones, energy balance, loading, and physical activity (29). Anabolic conditions promote muscle growth by increasing protein synthesis via insulin-like growth factor (IGF1)/AKT/mammalian target of rapamycin (mTOR) pathway. In catabolic conditions ranging from denervation, inactivity, microgravity, fasting to a multitude of systemic diseases such as cancer, sepsis, AIDS, diabetes, cardiac failure, and renal failure (17), protein breakdown is enhanced and excess protein synthesis resulting in myofiber atrophy (29). In eukaryotic cells most of the proteins are degraded via two proteolytic systems: the ubiquitin-proteasome and the autophagy-lysosome. In cardiac and skeletal muscles the two systems are coordinately regulated to preserve an almost normal composition of proteins and organelles in atrophying cells (20, 28). The activation of the major proteolytic systems requires a transcription-dependent program. In fact, comparing gene expression in different models of muscle atrophy leads to the identification of a subset of genes that are commonly up- or downregulated in atrophying muscle (1, 10, 18, 27). These common genes are thought to regulate the loss of muscle components and were thus designated atrophy-related genes or atrogenes (29, 31). Forkhead Box O (FoxO) family of transcription factors is playing a major role in the regulation of the atrophy program being critical for the expression of several atrophy-related genes (20, 28, 31). Inhibition of FoxOs reduced muscle loss during disuse (25, 33), dexamethasone treatment (31), and fasting (24). Given the physiological relevance of FoxOs in muscle homeostasis, their activation has to be extremely controlled. In fact, excessive skeletal muscle protein degradation is highly detrimental for the economy of the human body and it can lead to death. For instance, excessive muscle atrophy of intercostals and diaphragm muscles can cause insufficient ventilation and hypoxia. Therefore, it is important to understand the mechanisms that control FoxO activity.

The FoxO family have been originally found to be under AKT control (3). Growth-promoting conditions lead to activation of the IGF1/ phosphatidylinositol 3-kinase (PI3K)/AKT pathway that induces FoxOs phosphorylation in three conserved residues causing cytosolic localization and inhibition of expression of FoxO target genes. However, several other mechanisms have been found to control FoxO activity and localization independently of AKT. Posttranslational modification like phosphorylation, acetylation, and mono- or polyubiquitination as well as protein-protein interaction affect the localization and the transcriptional activity of FoxOs (5). In skeletal muscle few mechanisms of FoxOs regulation have been identified. We and others have recently found that AMP-activated protein kinase (AMPK) can activate FoxO3 in skeletal muscle and consequently protein breakdown to produce alternative energy substrates to restore energy balance (11, 12, 26). Conversely, JunB interaction with FoxO3 exerts an inhibitory effect on FoxO3 recruitment on atrogin-1 promoter and similarly PGC1α and β suppress FoxO3 activity in atrophying muscle during denervation, fasting, and heart failure (2, 9, 30).

Acetylation has been shown as another important mechanism of regulation that enhances or represses FoxO function on target genes. The final outcome, activation, or inhibition of transcription depends on the cell type and on the context (4). Several lysine residues, which are mainly localized on DNA binding and on the nuclear localization signal domains, are acetylated by histone acetyl-transferase (HAT) in the nucleus (4, 5). Indeed HATs, such as p300 and cAMP-response element binding protein (CREB) binding protein (CBP), regulate gene transcription by inducing histone acetylation and chromatin relaxation but also by acetylating and therefore modu-

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lating transcription factors like p53, MyoD, and FoxOs. Recent findings have confirmed the negative action of p300 and CBP on FoxOs in skeletal muscle. In fact overexpression of HAT in soleus muscle inhibits FoxO3 and FoxO4 action while has no effect on FoxO1 (35). Indeed atrogin-1 and muscle RING finger 1 (MuRF1) expression is reduced once p300 is overexpressed in atrophying muscles. Interestingly, the upregulation of other atrophy-related genes like LC3 and cathepsin L is not
affected by p300 expression, while activation of 4E-BP1 and GADD45α is further enhanced by the presence of the HAT. Genetic evidences have shown that, in skeletal muscle, cathepsin L and GADD45α are primary targets of FoxO1 (15), whereas atrogin-1 and MuRF1 are mainly under FoxO3 control (2). Interestingly, Senf and co-workers (35) reported that acetylation represses FoxO3 and FoxO4 action on FoxO reporter, whereas it shows no effect on FoxO1 activity. Moreover, HAT induces cytosolic relocalization of FoxO3 but not of FoxO1 during nutrient deprivation. Thus acetylation seems to differentially regulate FoxO family members. However, despite these important results a genetic evidence that acetylation of FoxO3 inhibits its transcriptional activity and localization is lacking. Here, by using genetic tools we defined the lysine residues that are critical for FoxO3 transcriptional activity on target genes and for its localization and stability. Moreover, we defined Mdm2 as the ubiquitin ligase that causes FoxO3 ubiquitination and degradation once FoxO3 moves from the nucleus to the cytosol.

MATERIALS AND METHODS

Mice. C57BL/6j were ordered from Harlan Laboratories. All data were obtained in 8-week-old female mice. Mice were housed in an environmentally controlled room (23°C, 12 h light/12 h dark cycle) and provided food and water ad libitum. Denervation was performed by cutting the sciatic nerve of the left limb while right limb was used as control. To block proteasome function, mice were injected intra-peritoneally with MG262 (5 μmol/kg) or with vehicle (DMSO) 24 h before being euthanized. Mouse procedures were approved by the competent authority and the Ethics Committee of the University of Padova.

Cell culture and transient transfections. Murine embryonic fibroblast (MEF) cells were cultured in DMEM (Lifesciences) supplemented with 10% fetal calf serum and 1% penicillin-streptomycin mixture at 37°C and 5% CO2 until cells reached confluence. MEF were transfected using Lipofectamine2000 (Lifesciences) according to the manufacturer’s instructions.

Constructs and reporter activity assay. The FoxO3 expression plasmid has previously been described in Ref. 31. Mutants of FoxO3 are generated by Quik Change Multi Site-Directed kit (Stratagene). The DBE-FoxO reporter and atrogin-1 promoter was described in Ref. 31. pRL-null Renilla was from Promega. RNA interference (RNAi)-mediated knockdown was carried out by transfection of short hairpin RNA (shRNA) constructs targeting Mdm2. The target sequence for Mdm2 gene. For the validation of shRNA constructs MEF cells were transfected with shRNA constructs using Lipofectamine 2000 (Lifesciences). All data were normalized to GAPDH expression. Oligonucleotide primers used for quantitative PCR analyses are the following: atrogin-1, forward GCA AACACTGCGGATCTTCCTCTC and reverse CTTCAGGGGAAAGGT-GAGACG; GAPDH, forward CACCATCTCCAGGAGCGAG and reverse CCTTCTCATGGTGGTGAAGAC; Fox3, forward AC- CTTGCTTCGAGACTTCTTGTG; and reverse GTGCTGTCAGTGGCTGAG.

Immunohistochemistry and fluorescence microscopy. Cryosections of muscle transfected with FoxO3 or mutated FoxO3 and Ub76V-GFP were fixed in ice-cold 4% paraformaldehyde, mounted with fluorescence mounting medium (DAKO), and examined using a Leica DM500B fluorescence microscope. Immunohistochemistry with anti-FoxO3 polyclonal antibody (Cell Signaling) was as previously described (20, 21).

Western blotting and immunoprecipitation. Murine frozen muscles were pulverized by grinding in liquid nitrogen, lysed, and immunoprecipitated as previously described (20, 21). When needed, membranes were stripped using a stripping buffer (25 mM glycine, 1% SDS, pH 2.0) and reprobed. Antibodies used from Cell Signalling Technologies were the following: rabbit polyclonal anti-acetyl-lysine, rabbit polyclonal anti-Akt; rabbit monoclonal (clone 193H12) anti-phospho-Akt (Ser473); Rabbit polyclonal anti-FoxO3 and rabbit polyclonal anti-HA were from Santa Cruz, and monoclonal anti-ubiquitin was from Biomol. Mouse monoclonal anti-GAPDH was from Abcam. Western blots were performed in at least three independent experiments. For immunoprecipitation assay: proteins, 500 μl and 1 mg of total proteins, respectively, from cells and muscles were incubated overnight with 4 μg of anti-HA antibody and 4 μg of anti-FoxO3 or anti-acetyl-lysine or anti-ubiquitin and A/G plus agarose (Santa Cruz). The following day, precipitated proteins were washed and loaded in a denaturing gel.

Statistical analysis. Data are expressed as means ± SE or as means ± SD. Statistical significance was determined by unequal
Fig. 2. FoxO3 mutants that mimic or block acetylation differentially regulate FoxO3 activity and localization. A: scheme of FoxO3 protein that depicts the Forkhead domain, which contains the DNA binding domain, the nuclear localization signal (NLS), and the nuclear export sequence (NES). Underneath are reported the lysines that are conserved and acetylated in FoxO family. Red arrow shows the three lysine residues that are mutated in the 3KQ and 3KR constructs while green arrow depicts the six lysines that are changed in the 6KQ and 6KR plasmids. B: Western blot showing the protein levels of FoxO3 and mutants overexpressed in MEF cells. MEF cells were transfected and 24 h later were lysed and blotted for FoxO3. C: transcriptional activity of FoxO3 mutants. A FoxO-dependent reporter, in which the FoxO binding site is repeated six times (DBE-reporter), was transfected into adult TA muscle in presence or absence of FoxO3 mutants. A Renilla luciferase vector was cotransfected to normalize for transfection efficiency. Seven days later, firefly/Renilla luciferase activity was determined (***P < 0.001; n = 5). D: activity of the atrogin-1 promoter is inhibited by acetylation. Atrogin-1 promoter was transfected into adult TA muscle in the presence or absence of FoxO3 (6KQ) or FoxO3 (6KR). A Renilla luciferase vector was cotransfected to normalize for transfection efficiency. Seven days later, firefly/Renilla luciferase activity was determined (***P < 0.001; n = 6). E: localization of FoxO3 is affected by acetylation. Adult muscle fibers were transfected with FoxO3 and the different mutants and examined 2 wk later. Transfected fibers were identified by anti-FoxO3 immunofluorescence. F: cross-sectional area (CSA) of transfected fibers identified by anti-FoxO3 immunofluorescence was measured (**P < 0.01; n = 400).
ACETYLATION OF FoxO3 AND CONTROL OF ATROPHY PROGRAM

RESULTS

FoxO3 is acetylated and ubiquitinated during denervation-mediated muscle atrophy. Denervation in rodents is a well-known and characterized approach to activate the atrophy program, resulting in 40–50% muscle loss within 2 wk. However, in mice the highest rate of protein degradation occurs during the first 7 days, whereas during the next 7 days protein breakdown slows down as well as muscle atrophy (27). When we monitored the expression of the atrophy-related gene atrogin-1 in denervated muscles, we confirmed that it reaches a peak of expression at 3 days after the cut of sciatic nerve and then decreases to the basal level of expression after 14 days (Fig. 1A). We and others have found that FoxO3 is recruited on atrogin-1 promoter and is sufficient and required for atrogin-1 transcription. Thus we immunoprecipitated endogenous FoxO3 from control and denervated muscles, and we checked its changes both in expression and in posttranslational modifications. Interestingly, when we monitored the level of acetylation we found a progressive increase of acetylated FoxO3 that starts at day 10 and reaches the highest level at day 14 (Fig. 1B). Since nuclear HATs can acetylate both histones and transcription factors, including FoxOs, we checked whether p300 binds to FoxO3. Cells were transfected with plasmids encoding p300 and a FoxO3 mutant that has a prominent nuclear localization. Both of these plasmids encoded for HA-tagged proteins, and therefore, FoxO3 was immunoprecipitated with an anti-FoxO3 antibody. Western blot for HA tag confirmed that p300 interacts with FoxO3 (Fig. 1C). Immunoprecipitation experiments for acetylated proteins showed that p300 increases acetylation of FoxO3 confirming that p300 and possibly other HATs can acetylate histones as well as FoxO3 (Fig. 1D).

We then monitored the level of expression of endogenous FoxO3 in denervated and control muscles. Interestingly, concomitant to FoxO3 hyperacetylation there was a progressive decrease of FoxO3 protein in denervated muscles from day 3 to day 14. It is worth to underline that after 3 days of denervation, FoxO3 protein was increased due to the transcriptional upregulation of the gene, as previously reported (27, 34, and Fig. 1A). The decline of FoxO3 protein during the following days was not due to inhibition of transcription since FoxO3 mRNA remained higher than control innervated muscles both at day 10 and 14 (Fig. 1A). Therefore, FoxO3 protein is reduced by day 10 probably because of an increased degradation or a decreased synthesis. Since FoxO3 has been found to be ubiquitinated and degraded via proteasome system (5), we checked the level of FoxO3 ubiquitination during denervation. Indeed starting at day 10, FoxO3 was highly ubiquitinated and, concomitantly, acetylated (Fig. 1B).

To test whether these changes of acetylation and ubiquitination affect FoxO3 localization, we transfected adult muscles with wild-type (wt) FoxO3 and we monitored its localization in innervated and denervated muscles. We utilized our established transfection technique to express wtFoxO3 only in terminally differentiated muscle fibers and not in satellite or interstitial cells. This approach allows a high transfection efficiency without interfering with physiological homeostasis of adult fibers (20, 24, 26, 31, 32). Moreover, transfection of mock control plasmids does not affect myofiber size. As expected expression of wtFoxO3 in innervated muscles showed a cytosolic localization. However, 3 days of denervation were sufficient to induce nuclear relocation of wtFoxO3. Importantly, FoxO3 nuclear localization was greatly reduced in 10 days denervated myofibers, while the cytosolic localization was increased (Fig. 1E). Altogether these findings suggest that acetylation can play a role in FoxO3 localization and stability.

FoxO3 mutants that mimic or block acetylation show different transcriptional activity and localization. Since the above data cannot discern whether acetylation is a protective mechanism or is a stimulation of FoxO3 degradation, we moved to a genetic approach. To address the role of acetylation on FoxO3 activity, we have generated several FoxO3 mutants that mimic or block acetylation. There are six lysines that have been shown to be conserved between the FoxO members and to be acetylated either in vivo or in vitro (5). These lysines are mainly localized in the DNA binding region and in the nuclear localization signal (NLS) domain (Fig. 2A). Since the acetylation occurs in the nucleus and to study the effect of the p300-mediated acetylation of FoxO3, we used a plasmid encoding a FoxO3 protein whose AKT phosphorylation sites have been replaced with alanine. This form of FoxO3 has been previously characterized and has been shown to localize in the nucleus (31). Initially, we generated two types of FoxO3 mutants. In the first we mutated three lysines while in the second we mutated all the six residues. We changed the lysine either into arginine (KR) to prevent acetylation or into glutamine (KQ) to mimic acetylation. All the mutants were expressed at a level similar to the original FoxO3, suggesting that the mutations did not affect protein stability during the first 24 h (Fig. 2B). We then tested the transcriptional activity of these mutants in vivo by using the FoxO sensor (DBE reporter) that contains a repetition of 6 FoxO binding sites upstream the luciferase gene and that has been previously characterized (31). We transfected adult TA muscles with each FoxO3 mutant together with the DBE reporter, and 7 days later muscles were collected and luciferase activity was determined. Interestingly, the mutation of three lysines into arginine (3KR) improved the transcriptional activity of FoxO3, and this increase is further enhanced when all the six lysines were replaced. These findings suggest that when FoxO3 moves into the nucleus it starts to be acetylated to reduce its transcriptional activity. Consistent with this hypothesis, the mutation of three lysines into glutamine (3KQ) showed similar activity to FoxO3, confirming that at least these three lysines are constitutively acetylated at basal state at 7 days posttransfection. Interestingly, at this time the mutant FoxO3 (6KQ) was completely unable to activate DBE reporter. Thus acetylation of the further three lysines is a major signal to shut down FoxO3 activity and, depending from the context and the time, is modulated (Fig. 2C). We then tested the activity of the FoxO3 (6KQ) and (6KR) mutants on atrogin-1 promoter, the best bona fide FoxO3 target gene. Indeed the mutant that mimic acetylation (6KQ) failed to activate atrogin-1 promoter (Fig. 2D). To understand whether the different transcriptional activity reflects different localization of FoxO3, as suggested by the denervation experiments, we transfected TA with the different mutants, and 12 days later we collected the muscles and analyzed FoxO3 localization (Fig. 2E). While the FoxO3 (3KR) and (6KR) showed a nuclear localization, FoxO3 (6KQ) displayed a prominent cytosolic localization. FoxO3 (3KQ)
was localized in the nucleus. To understand whether FoxO3 acetylation might affect not only the activity of sensors but also the atrophy program and muscle loss, we quantified the cross-sectional area of myofibers expressing FoxO3 (6KR) and (6KQ) (Fig. 2F). Consistently with the data of transcripational activity and of nuclear localization, FoxO3 (6KQ) induced less atrophy than FoxO3 (6KR).

Relocalization in the cytosol induces FoxO3 degradation via proteasome system. At the time we measured cross-sectional area of myofibers transfected with FoxO3 mutants, we noticed
that there were always less fibers expressing FoxO3 (6KQ) than FoxO3 (6KR) or FoxO3. We decided to perform a time course study to monitor FoxO3 (6KQ) localization and expression. We transfected TA muscles and we collected them at 8, 10, and 12 days after electroporation (Fig. 3A). Immunostaining analysis confirmed that FoxO3 (6KQ) was mainly localized in cytosol. Importantly, the percentage of FoxO3 (6KQ) expressing fibers dramatically decreased between 8 and 10 days, and the reduction of FoxO3 protein occurred preferentially in the cytosol. This finding suggests that the cytosolic localization of hyperacetylated FoxO3 is a signal for its degradation. To determine whether FoxO3 (6KQ) reduction is dependent on proteasome function, we used the proteasome inhibitor MG262. To demonstrate the efficacy of MG262 treatment in blocking proteasome function in vivo, skeletal muscles of control and MG262-treated mice were transfected with the short-lived UbG76V-GFP reporter as previously reported (6, 19). Mice were treated with MG262 24 h before being euthanized, and then muscles were collected and analyzed. As shown in Fig. 3B, green fluorescent protein (GFP) fluorescence was detected in transfected fibers of MG262-treated but not in transfected fibers of control mice, thus confirming that proteasome function was effectively repressed by the inhibitor in vivo. Inhibition of the proteasome significantly increased the FoxO3 (6KQ)-positive fibers at 10 days posttransfection restoring the percentage of fibers detected at 8 days (Fig. 3D). As expected, the rescue of FoxO3 (6KQ) protein mainly occurred in the cytosolic fraction (Fig. 3, C and D). To further prove that FoxO3 (6KQ) is degraded via proteasome, we decided to identify and to block the ubiquitin-ligase involved in FoxO3 ubiquitination and to check whether we rescued FoxO3 (6KQ) expression. Mdm2 is the E3 ligase that has been described to ubiquitinate FoxO3 (8). Interestingly, when we checked the expression of Mdm2 in atrophying muscles we found that Mdm2 is upregulated during denervation (Fig. 3E). This finding suggests that Mdm2 might contribute to the atrophy program in denervation. We used an RNAi approach to inhibit Mdm2 in vivo. To knockdown target proteins in vivo for days/weeks, we used bicistronic vectors that simultaneously encode for shRNAs and GFP protein. Therefore, detection of GFP fluorescence allows to monitor the area of transfection. We designed three shRNAs against Mdm2, and we tested their efficiency in downregulating Mdm2 expression. Between the different oligos we identified one that greatly reduced Mdm2 protein (Fig. 3F). We cotransfected the plasmid encoding the shRNAs against either Mdm2 or unrelated oligos (scramble) and the FoxO3 (6KQ) into TA muscles. Ten days later, FoxO3 (6KQ) was barely detectable in fibers expressing scramble oligos, while it was clearly revealed in myofibers in which Mdm2 was knocked down (Fig. 3, G and H). Quantification of FoxO3 expressing fibers showed a significant increase of FoxO3 (6KQ) when Mdm2 was knocked down (Fig. 3H). Thus inhibition of FoxO3 activity requires an acetylation-dependent translocation of FoxO3 outside the nucleus, which is followed by its degradation via proteasome. To prove that proteasome-dependent degradation is an important step of FoxO3 regulation, we treated control and denervated mice with MG262, and we monitored the expression of atrogin-1. Inhibition of proteasome function maintained a high atrogin-1 level in denervated muscles, preventing the return to basal expression after 14 days from the cut of the sciatic nerve (Fig. 3I). To establish the role of Mdm2 in FoxO3 degradation we performed immunoprecipitation experiments to reveal whether Mdm2 forms a complex with FoxO3. Immunoprecipitation experiments confirmed the interaction between FoxO3 and Mdm2 (Fig. 3J). To further determine whether acetylation is critical for Mdm2 binding to FoxO3, we express FoxO3 and Mdm2 in the presence or absence of p300. The interaction of FoxO3 with Mdm2 is greatly enhanced by the presence of p300 (Fig. 3K). Importantly, the binding of Mdm2 to FoxO3 significantly reduced the total FoxO3 protein (Fig. 3K).

Lysine 262 is critical for FoxO3 localization but is not sufficient to cause protein degradation. The differences of activity and localization between the mutants 3KQ and 6KQ prompted us to determine which lysine residue beyond the first three is inhibiting FoxO3. Therefore, we generated a FoxO3 (4KQ), and we tested its localization in adult fibers. Interestingly, FoxO3 (4KQ) displayed a cytosolic distribution that well mimicked the FoxO3 (6KQ) mutant. Since the lysine that was changed was the lysine 262, we decided to mutate only this residue on FoxO3 (Fig. 4A). When we expressed the single mutant FoxO3 (K262Q) in adult myofibers, the pattern of distribution resembled that one of FoxO3 (4KQ) (Fig. 4B). However, compared with FoxO3 (6KQ), both of these mutants...
did not show any significant time-dependent decrease of expression, and indeed the mutant proteins were stable in the cytosol. In fact, the percentage of fibers expressing 4KQ and K262Q did not decline with time. To further prove the discrepancy of these mutants with FoxO3 (6KQ) we also monitored their transcription activity. FoxO3 (4KQ) and (K262Q) showed a decrease transcriptional activity compared with FoxO3, but they did not completely abolish transcription as it occurred for FoxO3 (6KQ) (Fig. 4C). This finding supports the concept that the complete inhibition of FoxO3 requires both the relocalization in the cytosol and the degradation via ubiquitin-proteasome system (Fig. 5). The latter aspect is only accomplished when FoxO3 is hyperacetylated.

**DISCUSSION**

Our study identifies a complex cascade of events that modulate FoxO3 activity during muscle atrophy induced by denervation. Indeed we found that acetylation is a regulatory step that reduces FoxO3 transcription capability, and therefore, the activation of the atrophy program. This is the first genetic evidence in adult skeletal muscle, in vivo, that shows that acetylation causes cytosolic relocalization and instability of FoxO3 protein. Our data complement and sustain recent findings that describe the role of HATs as negative regulators of FoxO3 activity in atrophying soleus muscle during immobilization (35). Maintaining high levels of p300 in immobilized soleus was sufficient to block FoxO3 action on target genes, whereas reducing p300 activity showed the opposite effect enhancing FoxO3-dependent transcription. However, the possibility that p300 and in general HATs acetylate cofactors or other transcription factors that affect FoxO3 function cannot be completely excluded. Our genetic manipulation of FoxO3 lysines rules out this possibility and underlines that the degree of inhibition depends on the number of acetylated lysines. The different transcriptional activity of our FoxO3 mutants suggest that a certain level of FoxO3 acetylation normally occurs when FoxO3 is in the nucleus. In fact the changes of three lysines into glutamine to mimic acetylation did not reduce FoxO3 transcriptional activity on DBE sensor, whereas their replacement with arginine to prevent acetylation showed a trend of enhancement of FoxO3 activity. However, the mutation of four lysines reduced FoxO3 activity that was completely suppressed at the time we changed six lysines. It is important to underline that in this work we used a nuclear FoxO3 to avoid interference due to changes in AKT status. Indeed, in our study we focused on the potential role that acetylation can have on FoxO3 activity when FoxO3 is in the nucleus. In fact it has been reported in different systems that FoxOs are acetylated by p300 and CBP but these HATs are mainly localized in the nucleus. Since p300 action takes place in the nucleus, therefore, p300-mediated regulation of FoxOs seems to be mainly related to FoxO3 recruitment and/or detachment from different DNA regions of chromatin. Interestingly, FoxO3 (6KQ) does not display any transcriptional activity on both atrogin-1 promoter and DBE sensor, but it is still able to induce a small degree of atrophy (18%). Therefore, it is also possible that some atrophy-related genes are not directly transcribed by FoxO3 but instead are indirectly affected by a protein complex that contains FoxO3. Indeed, FoxOs have been described to interact with p53 and Cis and to release the repressive action of these transcription factors on target promoters (16, 23). Thus FoxOs can also work as coactivators being able to block transcriptional repressors. Since this repression appears to be mediated by the direct interaction that is independent of DNA binding region, acetylation of the lysines belonging to the DNA binding and NLS sites should not affect this interaction. However, our data do not support this concept.
address the potential role of acetylation in the cytosol, a site where this posttranslational modification can elicit other different functions.

Both our work and the study of p300-mediated inhibition have found that FoxO3 inhibition involves a cytosolic FoxO3 relocalization and degradation. These changes of localization and stability seem to be peculiar of FoxO3, since nuclear translocation of FoxO1 is not affected by acetylation (35). Despite our data showing that replacement of lysine 262 with acetyl group is a critical step for FoxO3 movement from the nucleus to the cytosol, it does not exclude that other posttranslational modification participate in this process. Previous studies have been reported that acetylation of FoxO1 enhances AKT phosphorylation at serine 253 (22). In our experiments we have used a FoxO3 mutant that contains the three Akt phosphorylation sites modified into alanine, and, therefore, we can exclude that AKT activity is critical for FoxO3 relocalization of these mutants. Even if we have demonstrated how the translocation mediated by acetylation can bypass the AKT-dependent phosphorylation system, we cannot exclude that other kinases can phosphorlyate further residues contributing to FoxO3 inhibition.

Our current model of FoxO3 regulation during denervation consider a multistep cascade of posttranslational modifications (Fig. 5). The lack of contractility or trophic factors induces FoxO dephosphorylation and translocation into the nucleus where it interacts with p300/CBP and binds the target regions of the DNA. The recruitment of the HATs on FoxO3 target genes is critical for chromatin remodeling and for transcription during the first 3 days of denervation. However, subsequently to histones modification p300 acetylates FoxO3 as well. The changes of lysine charge induced by acetyl groups weakens the binding of FoxO3 on target promoters. Finally, when lysine 262 is acetylated then FoxO3 is translocated to the cytosol where Mdm2 recognizes the hyperacetylated FoxO3 and ubiquitinates it causing the degradation via proteasome system. AKT can still play a role as a successive step of acetylation to restrain FoxO3 protein in the cytoplasm with much strength to keep it inactive or to degrade it. In fact, AKT has been reported to phosphorylate and activate Mdm2 (8, 36).

This is a dynamic and extremely fast process that fine tunes FoxO action. It is possible that when catabolic conditions persist FoxO is maintained active by the action of histone deacetylase (HDAC) that removes the acetyl groups. Indeed, it has been reported that FoxO3 in catabolic conditions, such as during oxidative stress, interacts with the class III HDAC Sirt1. Sirt1 activity is different from the other HDACs proteins since it is not constitutive but is inducible and is modulated by NAD+/NADH levels. Indeed, elevation of NAD⁺ as a consequence of low nutrients induces SIRT1-dependent deacetylation of FoxO increasing transcription of FoxO target genes (7, 14). Therefore, it is reasonable that in catabolic conditions the presence of energy stress activates Sirt1 to maintain FoxO3 deacetylated and therefore active. Activated FoxO3 coordinates autophagy-lysosome and ubiquitin-proteasome systems to produce both alternative energy sources and metabolic changes to adapt muscles to the situation of stress (14, 20).

Muscle loss aggravates catabolic conditions and impairs therapy of burn injuries, cancer, chronic heart failure, AIDS, sepsis, uremia, and many other pathological conditions, including sarcopenia in aging. Thus muscle loss ultimately aggravates diseases and increases morbidity and mortality. In these conditions, an appropriate therapeutic approach would inhibit the degradation machinery to counteract muscle wasting. However, inhibition of the proteolytic system seems also detrimental for myofiber homeostasis (13, 21) suggesting that a fine negative modulation of the proteolytic machinery is preferable than a strong inhibition. Our findings demonstrate that acetylation is a mechanism of fine tuning of FoxO3 action and therefore can be used as a new therapeutic approach to reduce the atrophy program and to counteract excessive muscle wasting.

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AUTHOR CONTRIBUTIONS
Author contributions: E.B., L.C., and M.S. conception and design of research; E.B. and L.C. performed experiments; E.B., L.C., and M.S. interpreted results of experiments; E.B. and L.C. prepared figures; E.B., L.C., and M.S. drafted manuscript; E.B., L.C., and M.S. edited and revised manuscript; E.B., L.C., and M.S. approved final version of manuscript.

Fig. 5. Time course scheme of the posttranslational modifications that affect FoxO3 activity, stability, and localization during muscle atrophy.
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