FoxO3 Coordinately Activates Protein Degradation by the Autophagic/Lysosomal and Proteasomal Pathways in Atrophying Muscle Cells

Jinghui Zhao,1 Jeffrey J. Brault,1 Andreas Schild,1 Peirang Cao,2 Marco Sandri,3,4,5 Stefano Schiaffino,3,4,6 Stewart H. Lecker,2 and Alfred L. Goldberg1,*

1Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA
2Renal Unit, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02115, USA
3Venetian Institute of Molecular Medicine, 35129 Padova, Italy
4Department of Biomedical Sciences, University of Padova, 35121 Padova, Italy
5Dulbecco Telethon Institute, 35129 Padova, Italy
6Institute of Neuroscience, Consiglio Nazionale delle Ricerche, 35121 Padova, Italy
*Correspondence: alfred_goldberg@hms.harvard.edu
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SUMMARY

Muscle atrophy occurs in many pathological states and results primarily from accelerated protein degradation and activation of the ubiquitin-proteasome pathway. However, the importance of lysosomes in muscle atrophy has received little attention. Activation of FoxO transcription factors is essential for the atrophy induced by denervation or fasting, and activated FoxO3 by itself causes marked atrophy of muscles and myotubes. Here, we report that FoxO3 does so by stimulating overall protein degradation and coordinately activating both lysosomal and proteasomal pathways. Surprisingly, in C2C12 myotubes, most of this increased proteolysis is mediated by lysosomes. Activated FoxO3 stimulates lysosomal proteolysis in muscle (and other cell types) by activating autophagy. FoxO3 also induces the expression of many autophagy-related genes, which are induced similarly in mouse muscles atrophying due to denervation or fasting. These studies indicate that decreased IGF-1-PI3K-Akt signaling activates autophagy not only through mTOR but also more slowly by a transcription-dependent mechanism involving FoxO3.

INTRODUCTION

Muscle atrophy is a debilitating process that leads to rapid loss of strength and endurance. It occurs in specific muscles with inactivity and denervation and systematically in fasting and many diseases, including cancer, diabetes, sepsis, and renal failure (Kandarian and Jackman, 2006; Lecker et al., 2006). In these various conditions, the rapid loss of muscle mass occurs primarily through an activation of protein breakdown. We have previously identified a set of atrophy-specific genes or “atrogenes” that are up- or downregulated similarly in muscles in these diverse types of atrophy (Lecker et al., 2004; Sacheck et al., 2007). These observations indicate that common transcriptional adaptations occur in various types of atrophy leading to accelerated protein degradation.

Proteasomes and lysosomes comprise the two major intracellular proteolytic systems in mammalian cells and have generally been assumed to be regulated independently and to serve distinct functions. The ubiquitin-proteasome pathway degrades both cytosolic and nuclear proteins (Glickman and Ciechanover, 2002), as well as myofibrillar proteins (Solomon and Goldberg, 1996), which comprise most of the protein in adult skeletal muscle. The acid hydrolases in lysosomes degrade most membrane and extracellular proteins taken up by endocytosis, as well as cytoplasmic proteins and organelles through autophagy (Scott and Klionsky, 1998). In diverse types of muscle wasting, the ubiquitin-proteasome pathway degrades both cytosolic and nuclear proteins (Glickman and Ciechanover, 2002), as well as myofibrillar proteins (Solomon and Goldberg, 1996), which comprise most of the protein in adult skeletal muscle. The acid hydrolases in lysosomes degrade most membrane and extracellular proteins taken up by endocytosis, as well as cytoplasmic proteins and organelles through autophagy (Scott and Klionsky, 1998). In diverse types of muscle wasting, the ubiquitin-proteasome pathway is activated, as shown by increased sensitivity to proteasome inhibitors; increased levels of ubiquitin conjugates; enhanced rates of ubiquitin conjugation; and induction of genes for ubiquitin, several proteasomal subunits, and two critical ubiquitin ligases (E3s), atrogin-1/MAFbx and MuRF1 (Bodine et al., 2001; Lecker et al., 2004). Induction of these muscle-specific E3s is essential for rapid atrophy (Bodine et al., 2001). Because inhibitors of lysosomal function fail to block the degradation of myofibrillar components in atrophying muscles (Furuno et al., 1990), the possible contributions of lysosomes to atrophy have not attracted much attention. However, an increased capacity for lysosomal proteolysis has been demonstrated in various types of atrophy (Bech et al., 2005; Furuno et al., 1990).

Our prior work showed that activation of FoxO transcription factors is essential for fiber atrophy and atrogin-1 induction upon denervation, fasting, and glucocorticoid treatment (Sandri et al., 2004, 2006). Moreover, expression of constitutively active FoxO3 (ca-FoxO3) induces expression of multiple atrogenes, including the critical E3 atrogin-1, and causes dramatic atrophy of mouse muscles and myotubes (Sandri et al., 2004). The
phosphorylation of FoxO transcription factors by Akt leads to their inactivation through binding to 14-3-3 proteins in the cytosol (Tran et al., 2003), but in ca-FoxO3, the three Akt phosphorylation sites are mutated to alanines, allowing free entry into the nucleus. Through activation of the PI3K-Akt pathway, IGF-1 stimulates protein synthesis and can induce hypertrophy of skeletal muscle (Glass, 2005). In addition, IGF-1/insulin inhibits overall protein breakdown, degradation of myofibrillar proteins (Sacheck et al., 2004), and expression of atrogin-1 and MuRF1 (Sacheck et al., 2004; Stitt et al., 2004). This reduced proteolysis appears to contribute to muscle growth and to result from the inactivation of FoxO by Akt phosphorylation (Sacheck et al., 2004; Stitt et al., 2004), while the accelerated degradation in atrophying cells has been attributed to FoxO-dependent induction of key E3s (Bodine et al., 2001; Sandri et al., 2004). The present studies were undertaken to analyze how FoxO3 affects protein degradation and to assess the contributions of lysosomal and proteasomal pathways to the loss of muscle protein during atrophy. We demonstrate here a marked ability of FoxO3 to stimulate lysosomal proteolysis by activating autophagy via a transcriptional mechanism in muscle cells (as well as in hepatocytes and neuronal cells). In muscle, this process is activated coordinately with the proteasomal pathway and contributes importantly to atrophy.

RESULTS

FoxO3 Causes Atrophy by Stimulating Proteolysis

To determine the effects of FoxO3 on protein degradation, we infected C2C12 myotubes with an adenovirus expressing ca-FoxO3, which within 2 days caused a dramatic reduction in myotube diameter (Figure 1A) and a reduction in the protein:DNA ratio (data not shown) to a level below that observed in control cells infected with an adenovirus expressing GFP only. Thus, viral expression of ca-FoxO3 in myotubes mimics the effects of FoxO3 overexpression in adult muscle (Sandri et al., 2004, 2006).

To measure overall rates of protein degradation, the majority of the myotube proteins were labeled by a 20 hr exposure to [3H]tyrosine prior to viral infection. After infection, overall rates of protein degradation were determined by measuring [3H]tyrosine release from the prelabeled proteins into the medium, which contained a large excess of nonradioactive tyrosine to prevent reincorporation (Gronostajski et al., 1984). After 16 hr exposure to the ca-FoxO3-expressing adenovirus, the overall rate of protein degradation in the myotubes was significantly increased (Figure 1B, p < 0.01), and by 24 hr, the rate was at least 50% greater than in cells infected with control adenovirus (Figure 1B and Figure 2A). If maintained, this enhancement of proteolysis is sufficient to cause marked atrophy in 2 days. When expressed at a level similar to ca-FoxO3, wild-type FoxO3 (wt-FoxO3) increased the rate of proteolysis by only 15% (Figure 1B, p < 0.01), presumably because most of the wt-FoxO3 was phosphorylated by Akt and inactive (Brunet et al., 1999; Sandri et al., 2004).

FoxO3-Induced Increase in Proteolysis in Myotubes Is Mediated Mainly by Lysosomes

To determine to what extent proteasomes contribute to FoxO3-induced proteolysis, we used two specific proteasome inhibitors, Velcade (PS-341) and lactacystin (Kisselev and Goldberg, 2001). To evaluate the contributions of lysosomes, we used a specific inhibitor of the lysosomal proton pump, concanamycin A (Woo et al., 1992), or chloroquine and NH4Cl, which accumulate in lysosomes and raise intralysosomal pH (Seglen, 1983). At concentrations used, the inhibitors caused maximal inhibition of proteolysis, and residual rates of degradation were linear for up to 5 hr with no detectable cell death. The actual contribution of proteasomes or lysosomes was then determined as the amount of proteolysis sensitive to the proteasomal or lysosomal inhibitors, by subtracting the rates of proteolysis in
cells treated with inhibitors from that in untreated cells (Figure 2A, middle panel). Similar rates were found using the two proteasome inhibitors or the three inhibitors of lysosomal acidification (Figure 2A). In control myotubes, breakdown of long-lived proteins appeared to be ~50% proteasomal and ~40% lysosomal (Figure 2A, top panel). This large contribution of lysosomes to total proteolysis is much higher than that estimated previously in well-nourished HeLa cells (Rock et al., 1994) and nutrient-deprived rodent muscles (Furuno et al., 1990).

By 24 hr after infection in cells expressing ca-FoxO3, overall rates of degradation increased by 66%. Surprisingly, this increase was reduced only modestly after treatment with proteasome inhibitors but was markedly inhibited by the lysosomal inhibitors (Figure 2A, top panel). In fact, approximately 70% of the increase in proteolysis required lysosomal function, while only 20% required proteasomal activity (Figure 2A, bottom panel). Thus, FoxO3 stimulates both degradative pathways coordinately but has a substantially greater effect on lysosomal proteolysis in C2C12 myotubes. This increase in lysosomal proteolysis is independent of proteasomal activity, since similar increases were seen in cells pretreated with Velcade for 4 hr (see Figure S1 in the Supplemental Data available with this article online), and is also independent of atrogin-1. In primary muscle cultures derived from wild-type or atrogin-1-deficient mice, ca-FoxO3 expression caused similar large increases in overall proteolysis, and most of this increase was lysosomal (Figure S2).

Evidence has been presented that caspases (Du et al., 2004) and/or calpains (Kramerova et al., 2005) might also play an essential role in enhancing muscle proteolysis during atrophy by promoting conversion of myofibrillar proteins to forms easily digested by the ubiquitin-proteasome pathway. However, addition of both proteasomal and lysosomal inhibitors gave about 90% inhibition of total proteolysis (data not shown). Thus, if caspases or calpains, which are not affected by these inhibitors, do contribute to overall proteolysis in myotubes, their contributions must be minor. Furthermore, treatment with the general caspase inhibitors DEVD-CHO or cpm-VAD-CHO did not significantly reduce overall proteolysis or proteasomal proteolysis in either control or ca-FoxO3-expressing myotubes (Figure S3). Thus, the great majority of the protein degradation by proteasomes is independent of caspases in these cells.

In Other Cell Types, FoxO3 Enhances Lysosomal but Not Proteasomal Proteolysis

Additional experiments tested whether the stimulation of lysosomal and proteasomal proteolysis by FoxO3 is specific to muscle cells. ca-FoxO3 expression using the same adenovirus in H4IIE rat hepatoma cells and PC12 rat pheochromocytoma cells also caused an increase in...
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lysosomal proteolysis (Figure 2B). However, in these cells, the rates of proteasomal degradation were unchanged, presumably because these nonmuscle cells do not express the FoxO-induced ubiquitin ligases and do not contain a pool of myofibrillar proteins. These results also indicate that the activation of these two proteolytic processes by FoxO3 is not necessarily linked.

**FoxO3 Activates Autophagy**

The major route for delivery of cytoplasmic proteins or organelles into lysosomes is by macroautophagy, which involves de novo formation of autophagosomes. These vesicles sequester cytoplasmic constituents and then fuse with lysosomes (Levine and Klionsky, 2004; Ohsumi, 2001). Changes in macroautophagy have long been known to correlate with rates of proteolysis in liver (Mortimore et al., 1983) and cultured cells (Knecht et al., 1984), and rapid changes in macroautophagy occur in response to changes in nutrient supply, mTOR, and insulin (Meijer and Codogno, 2006). To test whether FoxO3 increases lysosomal proteolysis by activating macroautophagy, we used the macroautophagy inhibitor 3-methyladenine (Seglen and Gordon, 1982). This agent reduced basal lysosomal proteolysis by 0.14% ± 0.03%/hr in control cells but caused a much larger decrease (0.50% ± 0.08%/hr) in the lysosomal proteolysis in ca-FoxO3-expressing cells (Figure 3A), strongly suggesting a marked activation of macroautophagy. This inhibition by 3-methyladenine appears to result from its capacity to block class III PI3-kinase (Petiot et al., 2000) since LY294002, an inhibitor of both class I and class III PI3-kinases, failed to inhibit and actually enhanced lysosomal and total proteolysis in these cells (Sacheck et al., 2004). Another mode of delivery of cytosolic proteins to lysosomes is chaperone-mediated autophagy (Dice, 2007), which is not sensitive to 3-methyladenine. ca-FoxO3 does not seem to activate chaperone-mediated autophagy under these conditions since it did not affect the expression of Lamp2a (Cuervo and Dice, 2000), although ca-FoxO3 induced many autophagy-related genes (see below). Another possible source of substrates for lysosomal degradation could be endocytosis of membrane components, but they cannot account for the large fraction of cell proteins digested by lysosomes in the atrophying myotubes. These findings suggest that macroautophagy (hereafter referred to as autophagy) is required for most of the increased lysosomal proteolysis by ca-FoxO3.

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**Figure 3. FoxO3 Activates Autophagy in Myotubes**

(A) Most of the ca-FoxO3-induced increase in lysosomal proteolysis is sensitive to 3-methyladenine. After labeling cell proteins and adenoviral infection, myotubes were treated with 10 mM 3-methyladenine for 1 hr and concanamycin A for another 1 hr before measurement of lysosomal proteolysis. Error bars represent SEM.

(B) ca-FoxO3 induces lipidation of LC3 and increases Gabarapl1 protein levels. LC3, Gabarapl1, and beclin1 protein levels were determined by western blot over 36 hr after infection. β-actin was used as a loading control.

(C) ca-FoxO3 enhances lysosomal degradation of LC3 and Gabarapl1 proteins. Myotubes were infected with control or ca-FoxO3 viruses for 22 hr and then treated with concanamycin A or Velcade for an additional 6 hr before lysis and western blotting. Quantified protein levels of LC3 and Gabarapl1 (relative to β-actin) were plotted. Values shown are the means of duplicates.
To confirm the activation of autophagy, we performed western blots for LC3, the homolog of yeast Atg8, which is cleaved and conjugated to phosphatidylethanolamine during autophagic vacuole formation to generate a faster-migrating form, LC3-II (Ohsumi, 2001). Expression of ca-FoxO3 was associated with a shift of some LC3-I to LC3-II that began 16 hr after infection (Figure 3B), the same time that protein degradation increased (Figure 1B). In addition, the level of Gabarap1 protein (another homolog of yeast Atg8) was increased by ca-FoxO3, but without any change in its migration on SDS-PAGE (Figure 3B).

To confirm that these changes in lysosomal proteolysis are due largely to changes in autophagy, we used mouse embryonic fibroblasts (MEFs) that lack the Atg5 gene and are therefore defective in autophagy (Mizushima et al., 2001). When we applied a variety of agents or conditions known to activate autophagy, including rapamycin (an inhibitor of mTOR), API-2 (an inhibitor of Akt), LY294002 (an inhibitor of PI3K), or serum deprivation, all stimulated lysosomal proteolysis in wild-type MEFs, as in myotubes (see below). However, in Atg5-deficient MEFs, these treatments had little effect on lysosomal proteolysis (Figure S5). Thus, these measurements of change in lysosomal proteolysis provide a sensitive and quantitative measure of change in autophagic activity.

**FoxO3 Increases Expression of Many Autophagy-Related Genes in Myotubes**

Studies in yeast have identified many autophagy-related (Atg) genes, most of which have counterparts in higher eukaryotes (Klionsky et al., 2003), but regulation of their expression has not been demonstrated in either system. To clarify how FoxO3 might stimulate autophagy, we tested whether FoxO3 alters the expression in myotubes of the Atg genes LC3b, Atg12l1, Atg4b, and Beclin1 (homolog of yeast Atg6) as well as Ulk2 (possible homolog of yeast Atg1), Gabarap1 (homolog of yeast Atg8), and PI3Kα (homolog of yeast Vps34, which is required for autophagy). Previously, we found that LC3 and Gabarap1 are induced in muscles in diverse types of atrophy (Lecker et al., 2004). Using quantitative real-time PCR, we demonstrated that these seven genes are induced by the expression of ca-FoxO3 (Figure 4A). mRNAs for LC3b, Gabarap1, Vps34, Ulk2, and Atg12l1 were clearly increased by 20 hr after infection with the FoxO3 adenovirus. Those for Atg4b and Beclin1 increased less, and did so more slowly.

FoxO transcription factors share a characteristic DNA-binding domain that recognizes the specific consensus sequence (C/G)(A/T)AAA(C/A)A in the promoters of target genes (Barthel et al., 2005). To determine whether FoxO3 binds directly to the promoters of Atg genes or whether it might activate their transcription by an indirect mechanism, we performed chromatin immunoprecipitation (ChIP) to test whether ca-FoxO3 associates with the putative FoxO-binding sites in the proximal promoter regions of the three most induced genes, LC3b, Gabarap1, and Atg12l1. We analyzed five consensus sequences within the 5 kb genomic region upstream of the LC3b coding sequence, six for Gabarap1, and four for Atg12l1. From the ca-FoxO3 immunoprecipitates, we were able to enrich DNA fragments that cover three of these consensus sequences in the promoter of LC3b (L1, L2, and L4), three for Gabarap1 (G2, G3, and G4), and two for Atg12l1 (A2 and A4) (Figure 4B). These results thus demonstrate that FoxO3 binds directly to these promoters and also allow us to identify its specific binding sites.

After expression of ca-FoxO3, no increase in the levels of beclin1 and LC3 proteins was evident on western blots, unlike Gabarap1 protein, which increased up to 30 hr after infection (Figure 3B). Interestingly, in ca-FoxO3-expressing cells, LC3 and Gabarap1 proteins declined 30 hr after infection (Figure 3B). Most likely, FoxO3 enhances the production and also degradation of these proteins, since when these cells were exposed to concanamycin A to block lysosomal proteolysis, both proteins accumulated to higher levels in FoxO3-expressing myotubes than in controls (Figure 3C), indicating enhanced lysosomal degradation. Thus, FoxO3, through direct binding to the LC3b and Gabarap1 promoters, increases the production of these Atg8 homologs, which are then consumed by lysosomal proteolysis.

**FoxO3 Increases Autophagosome Formation in Isolated Adult Mouse Muscle Fibers**

Several findings indicate that this FoxO-dependent induction of autophagy and autophagy-related genes also occurs during muscle atrophy in vivo. Long before FoxOs were discovered or shown to be necessary for muscle wasting, increased autophagy (Schiavino and Hanzlikova, 1972) and a greater capacity for lysosomal proteolysis (Furuno et al., 1990) were reported in denervation atrophy. Also, increased formation of autophagosomes labeled by GFP-LC3 has recently been demonstrated in mouse muscles upon fasting (Mizushima et al., 2004) and denervation (Mammucari et al., 2007 [this issue of Cell Metabolism]). To determine whether these responses might be due to FoxO3, we examined whether overexpression of ca-FoxO3 activates autophagy in adult muscle fibers. To monitor autophagosome formation, we delivered a GFP-LC3 plasmid together with a control or ca-FoxO3 construct into isolated mouse flexor digitorum brevis (FDB) fibers by electroporation. Two days later, we measured the number of GFP-positive puncta. The fibers expressing ca-FoxO3 had 6-fold more puncta than the control fibers, indicating much greater autophagosome formation (Figure 5A). By this approach, we also demonstrated that the activation of autophagy upon starvation of the muscle requires FoxO3. When the isolated GFP-LC3-expressing fibers were deprived of nutrients and serum, they showed a dramatic increase in the formation of GFP-positive puncta. However, in fibers where FoxO3 was knocked down by electroporation of RNAi for FoxO3, there was a much smaller increase in these GFP-tagged autophagosomes (Figure 5B).

**Induction of Autophagy-Related Genes in Atrophying Mouse Muscles**

In prior work, we found that LC3, Gabarap1, and the lysosomal protease cathepsin L are among the set of
atrogenes that are induced similarly in muscles atrophying due to fasting, renal failure, diabetes, or cancer (Lecker et al., 2004). Therefore, we examined by real-time PCR whether all of the autophagy-related genes induced by ca-FoxO3 in myotubes (Figure 4A) are also induced in mouse muscles after denervation or food deprivation, where FoxO3 appears to be activated. Indeed, these genes were induced in both types of atrophy (except Ulk2 and Beclin1, which were not induced in fasting) (Figures 6A and 6B). LC3b and Gabarapl1 showed the greatest induction (6- to 8-fold 1 week after denervation), while Beclin1, which has been viewed as a key regulator of autophagy (Liang et al., 1999), was induced least. Additionally, the Atg mRNAs induced most by FoxO3 in the myotubes also changed most in atrophying muscles. Surprisingly, although autophagy is activated in muscle upon food deprivation (Mizushima et al., 2004), the induction of these genes in muscle of animals fasted for 1 day was consistently less than following denervation. Presumably, the enhancement of autophagy in muscle of fasted animals occurs largely via a nontranscriptional mechanism (perhaps decreased mTOR activity), unlike that in disuse atrophy, where there is no nutrient deficiency. Thus, activation of autophagy and induction of many autophagy-related genes occur in multiple types of atrophy, although it is unclear from these experiments whether these FoxO3-dependent transcriptional changes drive the enhanced lysosomal proteolysis or only support the maintenance of this process by replacing components consumed during autophagy (e.g., LC3 and Gabarapl1).

Figure 4. FoxO3 Induces Autophagy-Related Genes in Myotubes
(A) ca-FoxO3 increases mRNA levels for many autophagy-related genes in myotubes (as shown by real-time PCR). Error bars represent SEM.
(B) ca-FoxO3 directly binds to the promoters of LC3b, Gabarapl1, and Atg12i. Five consensus (C/G)(A/T)AAA(C/A)A sequences for FoxO3 were identified in the 5 kb promoter region of LC3b, six for Gabarapl1, and four for Atg12i. Myotubes were infected with control or ca-FoxO3 adenoviruses for 12 hr, chromatin immunoprecipitation was performed using an anti-HA antibody (ca-FoxO3 is HA tagged), and pairs of primers that cover these sites were used to amplify the related DNA fragments from the immunoprecipitates by PCR. We were unable to amplify the DNA fragments that cover the two tentative sites between G1 and G2 in the promoter of Gabarapl1. Asterisks indicate tentative sites that are positive for binding of ca-FoxO3 in ChIP assays.
Two Distinct Mechanisms for Regulating Autophagy via the PI3K-Akt Pathway

IGF-1 or insulin can rapidly inhibit proteolysis in muscle and other cells, and this effect is generally assumed to result from an inhibition of autophagy by the PI3K-Akt-mTOR pathway. Accordingly, treatment of C2C12 myotubes with IGF-1 for 2 hr decreased overall proteolysis, while treatment with the Akt inhibitor API-2 (Yang et al., 2004) stimulated this process, and these rapid responses reflected mainly changes in the lysosomal pathway (Figure 7B).

mTOR is a kinase downstream of Akt that can inhibit autophagy in most cells (Levine and Klionsky, 2004). In yeast, mTOR has been reported to phosphorylate Atg13, thus inactivating the Atg1 complex (Levine and Klionsky, 2004). The mTOR inhibitor rapamycin stimulates autophagy, although in C2C12 myotubes, rapamycin at maximally effective concentrations caused only a small but consistent increase (10%–12%, p < 0.05) in lysosomal proteolysis. This response was much smaller than the 50% increase (p < 0.01) in this process induced by API-2 (Figure 7C). Therefore, the inhibition of Akt must stimulate autophagy by an additional mTOR-independent mechanism. Since most of the effect of API-2 is not through mTOR, it most likely occurs through FoxO-dependent transcriptional events. Accordingly, this exposure to API-2 activated FoxO transcription factors in the myotubes, as shown by dephosphorylation of FoxO3 and induction of atrogin-1 expression (Figure 7A).

To test the importance of transcription for this enhancement of autophagy, we blocked mRNA synthesis with actinomycin D. This treatment did not reduce, but rather seemed to enhance, the increase in lysosomal proteolysis caused by rapamycin or its rapid decrease caused by IGF-1, but inhibiting RNA synthesis blocked most of the increase in this process caused by API-2 (Figure 7C). Thus, activation of lysosomal proteolysis by API-2 requires gene transcription either for the autophagy-related genes described here or perhaps for some unidentified activators of this process. Another clear difference in the...
Figure 7. Increase in Lysosomal Proteolysis by Akt Inhibition Is Transcription Dependent

(A) API-2 treatment results in rapid dephosphorylation of Akt and FoxO3 proteins (by western blot) and an increase in mRNA levels of atrogin-1 (by real-time PCR). Myotubes were treated with 1 μM API-2 for the indicated times.

(B) API-2 stimulates, while IGF-1 suppresses, overall proteolysis in myotubes mainly by affecting lysosomal proteolysis. Myotubes were incubated with [3H]tyrosine for 20 hr and switched to chase medium for 2 hr. These cells were then treated with 1 μM API-2, 10 ng/ml IGF-1, or vehicle for an additional 2 hr before proteolysis measurement.

(C) Increase in lysosomal proteolysis induced by API-2 requires transcription, unlike that induced by rapamycin. Experiments were performed as above, but myotubes were pretreated with actinomycin D (10 μg/ml) for 1 hr before the addition of 1 μM API-2, 0.3 μM rapamycin, or 10 ng/ml IGF.

(D) API-2, but not rapamycin, induces mRNAs for LC3b and Gabarapl1 within 4 hr in myotubes. *p < 0.05, **p < 0.01 by two-tailed t test.

(E) Effects of API-2 and rapamycin on lysosomal proteolysis in control and ca-FoxO3-expressing myotubes. Experiments were performed as in Figure 2A, but 22 hr after infection, cells were treated with API-2 or rapamycin for 2 hr before lysosomal proteolysis was determined.

(F) Expression of constitutively active Akt (ca-Akt) reduces both proteasomal and lysosomal proteolysis in myotubes. Myotubes were infected with adenovirus expressing ca-Akt for 24 hr before proteolysis measurement.

(G) Rapamycin treatment prevents the specific phosphorylation of S6K and 4E-BP1, while expression of ca-FoxO3 has no apparent effects on their phosphorylation or that of mTOR. Antibodies indicated were used for western blotting.

(H) Schematic of two different mechanisms of regulating autophagy downstream of PI3K-Akt signaling. Error bars represent SEM.
activation of autophagy by ca-FoxO3 is that it had no apparent effects on the specific phosphorylation of mTOR or that of mTORC1’s substrates, S6K and 4E-BP1. In contrast, rapamycin rapidly and completely abolished the specific phosphorylation of S6K and 4E-BP1 (Figure 7G).

Thus, a reduction in the activity of the PI3K-Akt signaling pathway can activate autophagy by two mechanisms: (1) a rapid transcription-independent mechanism through mTOR, and (2) a slower mechanism independent of mTOR and requiring gene expression, apparently mediated by FoxO3 (Figure 7H). Accordingly, API-2 increased the transcription of Gabarapl1 and to a lesser extent LC3b within 4 hr, but rapamycin did not (Figure 7D), even though both rapamycin and API-2 increased the lipidation of LC3 (Figure S6). Another indication that blocking Akt in myotubes activates autophagy via FoxO3 is that treatment with API-2 caused a much greater increase in lysosomal proteolysis in control cells (0.41% ± 0.03%/hr) than in ca-FoxO3-expressing cells (0.13% ± 0.04%/hr), where activation of autophagy by FoxO3 is maximal and independent of Akt. By contrast, rapamycin, which does not act through FoxOs, caused similar effects in both types of cells (0.11% ± 0.02%/hr versus 0.17% ± 0.03%/hr) (Figure 7E). These findings indicate that IGF-1 or insulin can reduce protein degradation rapidly by suppressing autophagy via mTOR activation rapidly by suppressing autophagy via mTOR and with time can also suppress this process by inactivating FoxOs, which also inhibits proteasomal degradation through the reduction of atrogin-1 and MuRF1 transcription (Sachek et al., 2004). Accordingly, overproduction of Akt by adenoviral expression of constitutively active Akt (ca-Akt) in C2C12 myotubes for 24 hr led to a reduction in overall proteolysis (as was noted previously in HT29 cells [Arico et al., 2001]), and this effect involved coordinate inhibition of both lysosomal and proteasomal processes (Figure 7F).

**DISCUSSION**

The present studies demonstrate that FoxO3-dependent transcription enhances the cell’s capacity for autophagy and thus that in muscle, the lysosomal and proteasomal pathways for protein degradation are coordinately regulated. These results can account for our earlier findings (Furuno et al., 1990) that atrophying muscle shows an increased capacity for lysosomal proteolysis together with a large increase in nonlysosomal ATP-dependent proteolysis (i.e., the ubiquitin-proteasome pathway). While serving complementary functions in promoting protein loss, activation of these two processes seems to result from the coordinate transcription of key genes rather than crosstalk between the two systems since inhibition of either process for many hours (or loss of atrogin-1) did not alter the rate of the remaining degradative process. It has been reported that inhibition of proteasomes leads to a compensatory increase in autophagy (Iwata et al., 2005), but no such change was observed in our studies.

Interestingly, this simultaneous activation of the two proteolytic processes was restricted to muscle cells. In hepatic (H4IIE) cells or neuronal (PC12) cultures, FoxO3-stimulated lysosomal proteolysis but not the proteasomal process, presumably because these cells lack the muscle-specific ubiquitin ligases atrogin-1 and MuRF1 that are induced during atrophy. In muscle, protein degradation serves a physiological role distinct from proteolysis in other tissues since muscle components, especially myofibrillar proteins, are the major amino acid reservoir in the organism. The stimulation of muscle proteolysis is therefore critical in fasting and disease to mobilize amino acids for hepatic gluconeogenesis. In atrophying muscles, these two pathways catalyze the degradation of different cellular components. While proteasomes degrade myofibrillar and most soluble proteins (Solomon and Goldberg, 1996), organelles (especially mitochondria) are degraded primarily in lysosomes (Scott and Klionsky, 1998). The simultaneous activation of these two proteolytic pathways by FoxO3 presumably ensures that the loss of different cell components is coordinated upon fasting or disease and leaves the muscle with relatively normal composition, though reduced in strength due to loss of myofibrillar components and in endurance due to loss of mitochondria.

Because of the importance of the ubiquitin-proteasome pathway in degrading myofibrillar proteins, there has been appreciable interest in developing inhibitors of the atro-phy-specific ubiquitin ligases or the proteasome to retard muscle atrophy and cachexia (Beehler et al., 2006; Glass, 2005). Our findings argue that such approaches would not prevent the loss of those muscle components digested by lysosomes. Unlike in adult muscle, in which they comprise 60%–70% of cell protein, in cultured myotubes, myofibrils comprise only a small fraction of total protein. This difference probably explains why proteasome-dependent proteolysis comprises only a small fraction of the FoxO3-induced proteolysis in myotubes, while this system accounts for most of the accelerated proteolysis in normal and atrophying adult muscles (Lecker et al., 2006).

The present findings that FoxO3 induces the expression of many autophagy-related genes in culture and that similar changes in their expression occur upon denervation and fasting in mice are in agreement with our previous finding that FoxO activation is essential for muscle wasting upon denervation and fasting (Sandri et al., 2004). In adult muscle, in which FoxO3 causes profound atrophy, it also induces these same genes and causes a large increase in the number of autophagic vacuoles (Figure 5A), as well as occurs in mouse muscles atrophying due to food deprivation (Mizushima et al., 2004) or denervation (Mammucari et al., 2007). These in vivo responses appear to depend on FoxO3 since RNAi against FoxO3 blocked the enhanced autophagy induced by starvation of isolated muscle fibers (Figure 5B).

Elucidation of the pathway for autophagy in yeast has greatly advanced our knowledge of this process in mammalian tissues, but how autophagy in specific tissues is regulated is still poorly understood. For example, mTOR, the major nutrient sensor in eukaryotic cells, is an important regulator of autophagy, and its inhibitor rapamycin is widely used to activate this process, although how it stimulates autophagy remains unclear. In myotubes,
rapamycin induces only a small increase in lysosomal proteolysis (<15%), less than that induced in myotubes by the Akt inhibitor API-2. Thus, most of Akt’s effects on lysosomal proteolysis must be through an mTOR-independent mechanism, as has been suggested previously (Sarkar et al., 2007; Tassa et al., 2003; Yamamoto et al., 2006). The activation of autophagy by FoxO3 provides an additional mechanism by which the cell’s capacity for autophagy can be enhanced. In muscle, FoxO3 causes transcription of a large number of autophagy-related genes, seven of which were found to be increased in these studies. FoxO3 seems to directly activate their transcription since ca-FoxO3 was shown to bind directly to the promoters of LC3b, Gabarapl, and Atg12/ (Figure 4B).

These studies, however, have not resolved precisely how FoxO3 activates autophagy or the consequences of enhanced expression of Atg genes. Possibly the increased levels of multiple Atg proteins per se lead to greater autophagy if the levels of one or more of them are rate limiting for formation of autophagic vacuoles. Alternatively, FoxO3 may also induce one or more other proteins to enhance this process. Surprisingly, expression of Beclin1, which had been proposed to be critical in the activation of autophagy in other cells, changes much less than expression of other autophagy-related genes in response to ca-FoxO3 and denervation in mouse muscle. Possibly, the FoxO3-dependent expression of many autophagy-related genes may be important not for the enhancement of autophagy but to enable high levels of autophagy to be maintained for extended periods. The activation of autophagy by FoxO3 provides an additional mechanism for regulation of autophagy by the IGF-1/insulin-Pi3K-Akt pathway must function together in the suppression of proteolysis during growth (as shown here when Akt was overexpressed) and also in enhancing protein loss in nutrient- or insulin-deficient conditions (as shown here when Akt was inhibited). It is noteworthy that in myotubes, the increase in lysosomal proteolysis seen with the Akt inhibitor was severalfold larger than that seen with rapamycin. Presumably, in atrophying muscle, both modes of activating autophagy function together with the enhancement of the ubiquitin-proteasome pathway to cause protein loss.

The capacity of FoxO3 to stimulate autophagy in muscle cells is likely to be important in other physiological or pathological processes and other cell types (e.g., hepatic H4IE and neuronal PC12 cells). Accordingly, it was recently reported that Drosophila FoxO is required for starvation-induced autophagy in fat bodies (Juhasz et al., 2007). In many cells, autophagy is activated during apoptosis, which is often triggered by FoxOs (Tran et al., 2003). Additionally, FoxO-induced autophagy may be important in the extension of life span induced by the reduction in the Pi3K-Akt signaling pathway in worms and flies. In such mutants (daf-2) in C. elegans, the prolonged life span requires the FoxO ortholog (daf-16) or an Atg gene (Beclin1), and cells exhibit enhanced autophagy (Melen dez et al., 2003). Autophagy also helps protect cells by enhancing their capacity to destroy toxic protein aggregates, viruses, or intracellular bacteria. Therefore, activation of FoxO3, like treatment with rapamycin, may offer promise as a strategy to stimulate autophagy and help cells withstand such threats to their viability.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Materials**

C2C12 myoblasts were maintained and differentiated to myotubes as described previously (Sachek et al., 2004). Adenoviral infection was conducted on the sixth or seventh day of differentiation. Adenoviruses expressing ca-FoxO3, wt-FoxO3, and ca-Akt were described previously (Sandri et al., 2004). Wild-type and Atg5-deficient MEFs were provided by Y. Ohsumi (National Institute for Basic Biology, Okazaki, Japan). Velcade (bortezomib) was provided by Millennium Pharmaceuticals. Lactacystin, API-2, rapamycin, and actinomycin D were purchased from Calbiochem. Concanamycin A, 3-methyladenine, IGF-1, and chloroquine were from Sigma-Aldrich.

**Determination of Proteasomal and Lysosomal Proteolysis**

C2C12 myotubes were incubated with [3H]tyrosine (5 Ci/ml) for 20 hr to label cell proteins and then switched to chase medium (containing 2 mM unlabeled tyrosine to prevent reincorporation of [3H]tyrosine) for 2 hr. Fresh chase medium containing proteasomal or lysosomal inhibitors (1 μM Velcade, 8 μM lactacystin, 0.1 μM concanamycin A, 50 μM chloroquine, or 10 mM NH4Cl) was added. Starting 1 hr after addition of these inhibitors, medium samples were collected for 3–4 hr and combined with 10% TCA (final concentration) to precipitate proteins. The acid-soluble radioactivity reflects the amount of prela beled, long-lived proteins degraded at different times and was expressed relative to the total radioactivity initially incorporated. Plotting these values versus time gave the total rates of proteolysis. Proteasomal or lysosomal proteolysis was determined by subtracting the rates of proteolysis in cells treated with proteasomal or lysosomal inhibitors from that in untreated cells. When the inhibitors used are not indicated, proteasomal and lysosomal proteolysis were determined with Velcade and concanamycin A, respectively. All measurements were performed in triplicate.

**RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR**

Total RNA was isolated with TRIzol (Invitrogen). Reverse transcription was performed using TaqMan reverse transcription reagents (Applied Biosystems). Mouse gene-specific primers were selected with Primer 3 software. PCR reactions were performed using a DyNAme HS SYBR Green qPCR kit (Finzymes) and an ABI 7900HT Fast Real-Time PCR system (Applied Biosystems). Genes were quantified as described previously (Sachek et al., 2004), using GAPDH as the internal control. Primers for Lamp2a were provided by A.M. Cuervo (Albert Einstein College of Medicine, New York). Sequences of primers used for real-time PCR are listed in Table S1.

**Western Blotting**

Cells were solubilized in lysis buffer (1% Triton X-100, 10 mM Tris [pH 7.6], 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 mM...
EDTA, 0.1 mM Na3VO4, and protease inhibitor cocktail (Roche)]. Thirty micrograms of total proteins was separated by SDS-PAGE, transferred to PVDF membranes, and analyzed by western blot using the ECL method (Amersham). We used polyclonal antibodies against FoxO3, p-FoxO3(Thr32) (Upstate), 4E-BP1, S6K, p-S6K(Thr24), mTOR, p-mTOR(Ser2448) (Cell Signaling), β-actin (Abcam), LC3, beclin1 (Santa Cruz), Gabarap1 (Proteintech Group, Inc.). Protein levels were quantified by using Quantity One software.

Chromatin Immunoprecipitation Assay
Myotubes were infected with control or ca-FoxO3 adenoviruses for 12 hr before analysis using the chromatin immunoprecipitation (ChiP) assay kit (Upstate). Cellular chromatin was crosslinked by formaldehyde and then sheared by sonication. The soluble chromatin was immunoprecipitated with an anti-HA polyclonal antibody (Santa Cruz). After decrosslinking, the immunoprecipitates were subjected to PCR. Primers used are listed in Table S2.

Generation of atrogin-1-Deficient Mice and Primary Muscle Cultures
Atrogin-1 knockout mice (Bodine et al., 2001) were provided by Regeneron. Myoblasts were isolated using the procedure of Rando and Blau (1994). Muscles were removed from the hindlimbs of 2-week-old mice. After treatment with 0.1% collagenase D and dispase II (Roche), the isolated cells were plated on collagen-coated dishes. Myoblasts were cultured in F-10 nutrient medium with 20% fetal calf serum, 2.5 ng/ml basic fibroblast growth factor (Invitrogen). Myotubes were cultured in F-10 nutrient medium, when myotubes formed.

Single-Fiber Analyses of Adult Mouse Muscle
Flexor digitorum brevis muscles from adult mice were digested in type I collagenase at 4°C for 1 hr, at 37°C for 2 hr, and dissociated into single fibers. The fibers were electroporated using a BTX porator (482 Cell Metabolism). The specific sequence used against shRNA construct used for knocking down FoxO3 was described previously (Sandri et al., 2004). The specific sequence used against FoxO3 was 5′-GGATAAAGGCAGCACAGCAAC-3′.

Supplemental Data
Supplemental Data include six figures and two tables and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/6/6/472/DC1/.

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