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Foxo Transcription Factors Induce the Atrophy-Related Ubiquitin Ligase Atrogin-1 and Cause Skeletal Muscle Atrophy

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Summary

Skeletal muscle atrophy is a debilitating response to fasting, disuse, cancer, and other systemic diseases. In atrophying muscles, the ubiquitin ligase, atrogin-1 (MAFbx), is dramatically induced, and this response is necessary for rapid atrophy. Here, we show that in cultured myotubes undergoing atrophy, the activity of the PI3K/AKT pathway decreases, leading to activation of Foxo transcription factors and *atrogin-1* induction. IGF-1 treatment or AKT overexpression inhibits Foxo and *atrogin-1* expression. Moreover, constitutively active Foxo3 acts on the *atrogin-1* promoter to cause *atrogin-1* transcription and dramatic atrophy of myotubes and muscle fibers. When Foxo activation is blocked by a dominant-negative construct in myotubes or by RNAi in mouse muscles in vivo, *atrogin-1* induction during starvation and atrophy of myotubes induced by glucocorticoids are prevented. Thus, forkhead factor(s) play a critical role in the development of muscle atrophy, and inhibition of Foxo factors is an attractive approach to combat muscle wasting.

Introduction

Muscle atrophy occurs systemically in fasting and a variety of diseases (e.g., cancer, diabetes mellitus, AIDS, sepsis, and Cushing's Syndrome) and in specific muscles upon denervation or disuse (Lecker et al., 1999). In these diverse conditions, the atrophying muscles show increased rates of protein degradation primarily through activation of the ubiquitin-proteasome pathway (Jagoe and Goldberg, 2001) and a common series of transcriptional adaptations that together constitute an “atrophy program” (Jagoe et al., 2002; Lecker et al., 2004). The protein induced most dramatically during atrophy is the muscle-specific Ub-ligase, atrogin-1 (MAFbx) (Gomes et al., 2001). mRNA for *atrogin-1* increases 8–40-fold in all types of atrophy studied, and this increase precedes the onset of muscle weight loss. Moreover, knockout animals lacking *atrogin-1* show a reduced rate of muscle atrophy after denervation (Bodine et al., 2001a).

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Since various types of atrophy share a common set of transcriptional adaptations, it seems likely that the diverse stimuli triggering atrophy act through common signaling mechanisms and influence the same transcription factor(s). Low levels of insulin and probably IGF-1, together with elevated levels of glucocorticoids, trigger the loss of muscle protein after food deprivation and in diabetes (Mitch et al., 1999), and insulin resistance is a characteristic feature of many systemic diseases that appears to contribute to muscle wasting. Glucocorticoids are necessary for the activation of proteolysis in fasting, diabetes, and sepsis, and in large doses by themselves cause muscle wasting (Hasselgren, 1999). However, the signal transduction pathways by which glucocorticoids and low insulin trigger muscle protein loss have not been defined.

In addition to stimulating myoblast fusion to form myotubes during embryogenesis, IGF-1 and insulin promote net protein accumulation (i.e., hypertrophy) of mature myotubes and adult muscle fibers. In skeletal muscle, the binding of IGF-1 or insulin activates two major signaling pathways: the Ras-Raf-MEK-ERK pathway and the PI3K/AKT pathway. The Ras-Raf-MEK-ERK pathway affects fiber type composition but has no effect on muscle fiber size (Murgia et al., 2000), while activation of the PI3K/AKT pathway induces muscle hypertrophy apparently by stimulating translation via regulation of GSK and mTOR kinases (Bodine et al., 2001b). In addition to its activation by insulin and AKT, mTOR is controlled by the supply of amino acids and glucose (Kim et al., 2003). Low levels of amino acids lead to inhibition of mTOR and S6K and during fasting, the fall in insulin and IGF-1 decrease nutrient transport into muscle, which must further reduce mTOR activity. mTOR can thus integrate signals from the IGF-1/PI3K/AKT pathway with information about the cell's nutritional status, both of which influence mTOR activity.

Several recent findings suggest that decreased activity of the IGF-1/PI3K/AKT signaling pathway can lead to muscle atrophy (Bodine et al., 2001b). Inhibition of PI3K and expression of a dominant-negative AKT reduce the mean size of myotubes in culture (Rommel et al., 2001). Muscles from mice lacking *Akt1* and *Akt2* are small (Peng et al., 2003). Conversely, activation of AKT in rat muscle can prevent denervation atrophy (Pallafacchina et al., 2002; Bodine et al., 2001b). Much of the growth promotion by IGF-1, insulin, and activated AKT results from a general increase in protein synthesis, and reduced AKT activity probably causes the decrease in protein translation seen in many types of muscle atrophy. However, in related studies, using glucocorticoid-treated myotubes as a simple in vitro model of muscle atrophy, we showed that dexamethasone (Dex) increases protein degradation and *atrogin-1* expression, and these responses could be rapidly suppressed by IGF-1 or insulin (Sacheck et al., 2004). Moreover, inhibitors of PI3K stimulated protein degradation and expression of *atrogin-1* in these myotubes. These experiments strongly suggest that the IGF-1/PI3K/AKT pathway, in addition to affecting translational initiation, suppresses protein degradation, especially of myofibrillar proteins, and expression of *atrogin-1*.

One downstream target of the PI3K/AKT pathway that could mediate these IGF-1 effects is the Forkhead box O (Foxo) class of transcription factors, a subfamily of the large group of forkhead transcription factors. Mammalian cells contain three members of this family, Foxo1 (*FKHR*), *Foxo3* (*FKHRL1*), and *Foxo4* (*AFX*) (Tran et al., 2003). AKT blocks the function of all three by phosphorylation of three conserved residues, leading to their sequestration in the cytoplasm away from target genes (Brunet et al., 1999). Dephosphorylation of Foxo factors leads to nuclear entry and growth suppression or apoptosis (Ramaswamy et al., 2002). Foxo gene expression is also tightly regulated. Fasting and glucocorticoids induce the expression of Foxo factors in mouse liver and muscle, while refeeding suppresses Foxo transcription (Furuyama et al., 2003; Imae et al., 2003). Foxo factors are also necessary for the development of insulin resistance in type II diabetes

(Nakae et al., 2002; Puigserver et al., 2003). Furthermore, we found recently that *Foxo1* is an atrophy-related gene ("atrogene") that is induced in many, perhaps all, types of muscle atrophy (Lecker et al., 2004). These atrophy-related genes (formerly termed "atrogins") are here called "atrogenes" to prevent confusion with the ubiquitin-ligase, atrogin-1.

We have used two simple in vitro models of muscle atrophy, cell starvation, and treatment with the synthetic glucocorticoid, dexamethasone, to identify the downstream targets of the IGF1/PI3K/AKT pathway that are important for the induction of *atrogin-1* and the development of muscle wasting. We demonstrate that IGF-1 acts through AKT and Foxo to suppress *atrogin-1* transcription, and that the mTOR/S6K, GSK, and NFκB pathways are not important in regulating *atrogin-1* expression. Further studies show that Foxo3 activation also causes transcription of the *atrogin-1* promoter and a dramatic decrease in fiber size. Our surprising discovery of the key role of Foxo in triggering the atrophy program should lay the basis not only for further understanding of the mechanisms of muscle wasting in diverse diseases, but also for developing novel therapies for these debilitating conditions.

Results

Activity of the PI3K/AKT Pathway Decreases in Myotubes Undergoing Atrophy

To identify the signaling pathway(s) that activate *atrogin-1* expression during atrophy, we defined the changes in *atrogin-1* mRNA content and the phosphorylation levels of intermediates in the PI3K/AKT pathway in two experimental conditions that we found induce atrophy of C2C12 myotubes. Since food deprivation leads to rapid muscle wasting and an 8–10-fold increase in *atrogin-1* mRNA (Gomes et al., 2001), we studied the effects of starving cultured myotubes of serum, glucose, and amino acids. After 6 hr, these myotubes showed a 60% decrease in diameter (Figure 1A), and contained 2.5-fold more *atrogin-1* mRNA than control cells (Figure 1A). These changes were completely reversed by resupplying serum and nutrients for 12 hr (Figure 1A). In related studies, we found that treatment of differentiated myotubes with the glucocorticoid, dexamethasone (Dex) induced *atrogin-1*, stimulated breakdown of myofibrillar proteins, and caused a loss of protein and RNA content (Sacheck et al., 2004). After 24 hr treatment with Dex, there was a 2–3-fold increase in *atrogin-1* mRNA (Figure 1C) and a 40% reduction in mean myotube diameter (see Figure 3D).

Since these rapid responses mimic the major features of atrophy in vivo and since inhibition of the PI3K/AKT/mTOR pathway can induce muscle wasting (Bodine et al., 2001b), we tested whether the phosphorylation of components of this pathway change upon Dex treatment for 24 hr or deprivation of serum and nutrients for 6 hr. Both these treatments reduced levels of phosphorylated AKT below levels in control cultures, as revealed by immunoblot and densitometric analysis of the ratio of phospho-AKT to total AKT (Figures 1B and 1D). Densitometric analysis revealed that the ratio of phospho-AKT to total AKT fell consistently when *atrogin-1* mRNA increased.

A reduction in AKT activity would be expected to lead to decreased phosphorylation of Foxo1, 3, and Foxo4 and in fact, the levels of phosphorylated Foxo1 and 3 decreased in the starved and Dex-treated cultures. Forkhead factors shuttle between the nucleus and cytoplasm and when phosphorylated, are sequestered in the cytoplasm in association with 14-3-3 proteins. We measured the amount of Foxo in nuclear extracts of myotubes after starvation. A marked increase in nuclear Foxo1 and Foxo3 content was seen after starvation for 2 hr (Figure 1E, left image). To test if these nuclear Foxo species had DNA binding activity, we used an electrophoretic mobility shift assay (Figure 1E, right image). Factors in the nuclear extract from starved (but not control) myotubes bound to an oligonucleotide containing a known forkhead binding site. A 100-fold molar excess of this unlabeled

oligonucleotide (competitor) prevented formation of the complex, but an unrelated oligonucleotide (non competitor) containing an SP1 binding site did not. In addition, we analyzed the same nuclear extracts with oligonucleotides containing binding sites for NFκB, AP1, and CREB. Unlike the Foxo factors, neither NFκB, AP1, nor CREB, showed more binding activity in the starved extract. Together, these data strongly suggest that Foxo factors are activated by dephosphorylation and nuclear entry in the atrophying myotubes.

Since S6K activity is modulated by both AKT and the nutrient-sensitive mTOR/RAPTOR/GßL complex (Kim et al., 2003), the removal of serum and nutrients led to an almost complete dephosphorylation of S6K (Figure 1B). The readdition of cell culture medium to the starved cells increased the phosphorylation of AKT, Foxo factors, and S6K and decreased *atrogin-1* mRNA to control levels. Although S6K was markedly dephosphorylated in the starved cells, after Dex treatment, the level of phosphorylated S6K decreased only slightly (Figure 1D). Thus, in these two conditions, the dephosphorylation of Foxo factors, but not of S6K, correlated with *atrogin-1* induction.

Atrogin-1 Expression Is Suppressed by IGF-1 through the PI3K/AKT Pathway

To learn if activation of the PI3K/AKT pathway suppresses expression of *atrogin-1*, we measured whether the addition of IGF-1 could prevent dephosphorylation of AKT and Foxo1, 3, and 4 and block the high level of *atrogin-1* expression in both starved and Dex-treated cells. Indeed, addition of IGF-1 to either model of atrophy suppressed *atrogin-1* mRNA to the level in untreated myotubes (Figure 2A) and increased the levels of phosphorylated Foxo1, 3, and 4. However, in the starved cells, S6K remained largely dephosphorylated after IGF-1 addition, while in the Dex-treated cultures, IGF-1 restored S6K phosphorylation. Thus, S6K is unlikely to be regulating *atrogin-1* expression.

To test directly whether IGF-1 suppresses *atrogin-1* expression through its effect on AKT, we used adenoviral vectors to introduce into myotubes a constitutively active (c.a.) AKT and dominant-negative (d.n.) form of AKT (Skurk et al., 2004). Like IGF-1 treatment, expression of c.a. AKT prevented *atrogin-1* induction by Dex (Figure 2B) and blocked the reduction in phosphorylation of Foxo1, 3, and 4 (Figure 2C). On the other hand, expression of d.n. AKT slightly enhanced the Dex-dependent induction of *atrogin-1* (Figure 2B) and did not affect the dephosphorylation of the forkhead transcription factors (Figure 2C). These findings further support the involvement of PI3K, AKT, and Foxo factors in mediating the effects of IGF-1 on *atrogin-1* expression.

Foxo3 Dephosphorylation Activates the *atrogin-1* Promoter

To further explore the involvement of Foxo factors in *atrogin-1* regulation, we utilized adenoviral constructs that express wild-type Foxo3 (FOXO3A) or used a constitutively active form (c.a.FOXO3A) that is mutated at the three AKT phosphorylation sites, T32A, S253A, and S315A (Brunet et al., 1999). Each construct also expressed GFP, which enabled us to measure the efficiency of infection and to follow the morphological changes in the myotubes that result from expression of active Foxo3. In the myotubes, Foxo3 and the constitutively active mutant both caused a large increase in *atrogin-1* mRNA (Figure 3A). The addition of IGF-1 to the cells expressing the Foxo3 reduced sharply *atrogin-1* mRNA content, presumably because IGF-1 induced AKT-mediated phosphorylation and inactivation of the wild-type Foxo3. Accordingly, IGF-1 addition did not reduce the high level of *atrogin-1* mRNA in cells expressing the c.a. Foxo3, which cannot be phosphorylated.

Further studies explored the effects of these treatments on the activity of the *atrogin-1* promoter. To prepare an *atrogin-1* reporter gene construct, we cloned 3.5 kb of the mouse

atrogin-1 5′ untranslated region behind the firefly *luciferase* gene (*3.5AT1*) and then made a further truncation of this region to create a 1.0 kb promoter fragment (*1.0AT1*). Foxo3 stimulated the activity of both the promoters (Figure 3B). However, the 3.5 kb construct showed greater activity than the 1.0 kb construct, presumably because *3.5AT1* contains 14 potential forkhead binding sites while *1.0AT1* contains only 3 (see below, Figure 6C). These findings indicate that Foxo3 increases *atrogin-1* mRNA content through enhanced transcription rather than some indirect effect on mRNA stability.

Foxo3 Reduces Myotube Size

Since *atrogin-1* induction plays a key role in atrophy and is regulated by Foxo factors, we tested whether activation of Foxo3 might trigger "atrophy" in vitro. Myotubes were infected with adenoviral vectors expressing various forms of Foxo3 four days after differentiation, when the myotubes are of constant size (data not shown) and myoblast fusion no longer occurs (Rommel et al., 2001). Morphological examination of the cells expressing c.a. Foxo3 for 48 hr demonstrated a 50% reduction in mean myotube diameter compared with those expressing only GFP, which did not change diameter during this time (Figure 3C). Furthermore, when the myotubes were infected with a dominant-negative adenoviral construct for Foxo3 (d.n. FOXO3A) which lacks the transactivation domain, there was a 30% decrease in the expression of *atrogin-1* and the induction of *atrogin-1* mRNA by Dex was prevented (Figure 3D). Alone, the d.n. Foxo3 had no effect on myotube size, but it blocked completely the decrease in cell size caused by Dex. These findings demonstrate a key role of dephosphorylated Foxo3 in both the transcription of *atrogin-1* and in the myotube atrophy caused by glucocorticoids.

Other Targets of AKT Have Little Effect on *atrogin-1* Expression

To learn if other AKT-regulated proteins also influence *atrogin-1* expression, we examined several AKT targets that have been proposed to play a role in the regulation of muscle size. NFκB has been suggested to mediate the effects of TNFα in inducing muscle wasting in sepsis and cancer (Li et al., 2003) and AKT has been reported to activate NFκB by triggering IκB degradation (Israel, 2000). We therefore used adenoviral constructs to express components of the NFκB pathway in myotubes and assayed the effects on *atrogin-1* expression. No changes in the levels of *atrogin-1* mRNA were seen upon expression of either a constitutively active form of the NFκB-inducing kinase (c.a. NIK), which activates IKK and causes IκB degradation, or c.a. IκB, which resists degradation (Figure 4A). Thus, activation of NFκB or maintaining NFκB inactive, complexed with IκB, did not influence *atrogin-1* expression.

Since inhibition of GSK3β by AKT leads to hypertrophy of myotubes (Rommel et al., 2001), activation of GSK3β might be a possible mechanism contributing to atrophy. c.a. GSK3β induced a small but reproducible increase in *atrogin-1* expression. However, a d.n. GSK3β mutant had no effect (Figure 4A). Additional experiments demonstrated that altering the activity of NFκB or GSK3β did not influence the induction of *atrogin-1* by Dex (see Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/117/3/399/DC1). In contrast to c.a. NIK or c.a. IκB, expression of Foxo3 and to a much lesser extent, GSK3β, activated the *atrogin-1* promoter (Figure 4B). These findings together argue against a major role of GSK3β or NFκB in regulating *atrogin-1* expression.

AKT also Suppresses *atrogin-1* Expression in Adult Skeletal Muscle

In order to determine whether the IGF-1/AKT/Foxo pathway also reduces *atrogin-1* expression and influences fiber size in adult muscle, as it does in cultured myotubes, we transfected adult mouse skeletal muscles by electroporation with *3.5AT1* and with constructs expressing members of the AKT/Foxo pathway. We have used this technique

previously to introduce multiple DNA constructs into muscle fibers in mice (Pallafacchina et al., 2002). Initial experiments demonstrated that the electroporated *atrogin-1* reporter was regulated in a similar way as the endogenous *atrogin-1* gene since 24 hr after food deprivation, extracts of muscles transfected with the *atrogin-1* reporter contained 3–4-fold more luciferase activity than extracts of muscles of fed animals, and the endogenous *atrogin-1* gene was induced as shown by in situ hybridization (Figure 5A).

To test if AKT activation suppresses *atrogin-1* expression in these muscles, we cotransfected *3.5AT1* with HA-tagged *c.a. AKT* (Brunet et al., 1999). This active form of AKT reduced the *atrogin-1* promoter activity to 10% of its normal level, and completely blocked the large increase in promoter activity that occurs after food deprivation (Figure 5A). Furthermore, in the muscles from food-deprived mice, the AKT-transfected fibers contained little *atrogin-1* message by in situ hybridization, while the surrounding untransfected fibers contained high levels of *atrogin-1* mRNA (Figure 5A). Thus, AKT activity is a key factor suppressing *atrogin-1* expression in vivo and can even block its induction in fasting.

Foxo Activates the *atrogin-1* Promoter in Mouse Muscle during Fasting

Subsequent studies examined whether Foxo3 had similar effects on *atrogin-1* expression in adult mouse muscles. After transfection, HA-tagged wild-type Foxo3 was found by immunohistochemistry in both nuclei and cytoplasm, while the HA-tagged c.a.FOXO3A mutant was present exclusively in nuclei (Figure 5B). Transfection of both *3.5AT1* and these Foxo3 constructs showed that the *atrogin-1* promoter was activated 3-fold by wild-type Foxo3 and more than 20-fold by c.a. Foxo3 (Figure 5C). To ensure that the increased activity of the *atrogin-1* reporter reflected Foxo3 function, the *luciferase* gene driven by 6 Foxo binding sites arrayed in tandem was used as the reporter (DBE promoter, [*D*AF-16 *B*inding *E*lements]) (Figure 5C). This construct was activated to a similar extent by coexpression of wild-type or c.a. Foxo3 as was the *atrogin-1* promoter. Furthermore, the fibers overexpressing Foxo3 also showed increased *atrogin-1* mRNA levels as detected by in situ hybridization (Figure 5D).

To test whether Foxo also induces *atrogin-1* in atrophying muscle during fasting, we expressed an RNAi containing a region that blocks the function of both *Foxo1* and *Foxo3*. Electroporation of the RNAi completely prevented the induction of the *atrogin-1* promoter in these muscles 24 hr after food deprivation (Figure 5E). In myotubes, this RNAi construct reduced Foxo1 and 3 levels by more than 50%. By contrast, a control RNAi (against *GFP*) did not affect the activation of the *atrogin-1* promoter. In addition, RNAi against unique *Foxo1* and *Foxo3* sequences also partially prevented activation of the *atrogin-1* reporter (see Supplemental Figure S2 available on *Cell* website) Thus, Foxo factors are critical in the induction of *atrogin-1* during fasting (Figure 5E) and Foxo inactivation can account for the inhibition of this response by AKT.

Foxo3 Causes Marked Atrophy of Adult Skeletal Muscle

To test if Foxo3 not only promotes *atrogin-1* transcription but might also trigger the entire atrophy program, we transfected HA-tagged c.a. *FOXO3A* into mouse tibialis anterior muscle, and measured fiber size 8 and 14 days later. Muscle fibers expressing c.a. FOXO3A (Figure 5F), were much smaller than the untransfected surrounding fibers. Mean cross-sectional area, determined in more than 1800 fibers from eight muscles, was markedly reduced at both times ($p<0.01$), this 35% decrease in cross-sectional area after eight days resembles the atrophy in tibialis anterior one week after denervation (Bodine et al., 2001b). These changes in fiber size were even more dramatic at 14 days, when c.a. FOXO3A-positive fibers contained minimal cytoplasm, while the peripheral nuclei appeared normal, and no evidence for apoptosis was noted by TUNEL (data not shown). At this time, cross-

sectional area of 1200 fibers from nine muscles was reduced by 58%. Since overexpression of atrogin-1 alone does not cause myotube or muscle atrophy (M.S., C.S., and S.H.L., unpublished data) these findings suggest that Foxo3 also induces other atrophy-related transcriptional changes that lead to the enhanced protein breakdown necessary to account for such marked fiber shrinkage.

Forkhead Binding Sites Are Located at 5′ End of the *atrogin-1* Gene

Since forkhead factors appear to catalyze transcription of the *atrogin-1* promoter, we made a series of promoter truncations to define the important binding sites (Figure 6A), and then transfected adult mouse muscles by electroporation with the *atrogin-1* reporters with or without constructs expressing c.a. Foxo3. The extent of reporter induction by Foxo3 appeared to correlate roughly with the length of the promoter construct (i.e., presumably with the presence of multiple forkhead-sensitive regions throughout the 5′ untranslated region; Figure 6B). Surprisingly, even the smallest 5′ *atrogin-1* fragment, which reduced the basal activity of the promoter, responded to Foxo3. A detailed analysis of that short sequence revealed two potential forkhead binding sites, one located just past the transcription start site ($Foxo_1$, +2), and a second partially overlapping the putative TATA box at −94 relative to the transcription start site ($Foxo_2$) (Figure 6C). The ability of forkhead to bind to these sites was tested in an electrophoretic mobility shift assay using a purified forkhead fusion protein (Figure 6D). FoxoGST, bound to a known forkhead site from the *IGFBP1* promoter. A 100-fold molar excess of unlabeled IGFBP1 oligonucleotide prevented formation of the FoxoGST-IGFBP1 complex. Formation of this complex was also markedly reduced by an unlabeled oligonucleotide containing one of the putative forkhead binding sites in the *atrogin-1* promoter, ATAAATA (AT Foxo 1). By contrast, a mutated version of this site, AT*C*A*C*TA (AT Foxo 1mut), and an unrelated oligonucleotide containing an SP1 binding site, did not prevent complex formation. FoxoGST also bound to the oligonucleotide containing the $Foxo_1$ site but did not bind to the mutated version. Finally, in a competition assay, increasing amounts of the unlabeled AT Foxo 1 oligonucleotide, but not of the mutated version, were able to block FoxoGST-AT Foxo 1 complex formation. These results confirm that forkhead factors can bind directly to the *atrogin-1* 5′ untranslated region close to both the putative TATA box and the initiation site of transcription. While unusual, there are other examples when elements involved in transcriptional regulation are found in untranslated regions downstream the TATA box (Gilley et al., 2003).

Finally, we investigated whether both forkhead binding sites in the short 0.4 kb fragment of the promoter are necessary for the activation by Foxo3. We used site-directed mutagenesis to introduce into the 0.4 kb reporter, mutations in $Foxo_1$, and both $Foxo_1$ and $Foxo_2$ (Figure 6E). Adult muscles were transfected with the mutated reporters and c.a. *Foxo3*, and luciferase activity determined in muscle extracts four days later. The single mutation in $Foxo_1$ reduced Foxo3-mediated activation by 70% below the level with the 0.4 kb *atrogin-1* promoter, and almost no activation was observed with the double-mutant lacking both binding sites. These experiments show by a combination of approaches that Foxo3 binds to the *atrogin-1* promoter and activates its transcription.

Discussion

Foxo and Atrophy

The present studies have provided several important new insights concerning the control of muscle fiber size and the mechanisms of atrophy. Specifically, we have shown that: (1) in normal, growing myotubes and adult muscle fibers, the IGF-PI3K-AKT pathway suppresses the expression of the key ubiquitin ligase *atrogin-1* by inactivating Foxo factors; (2) activation of Foxo can by itself cause dramatic atrophy of myotubes and mature muscle

fibers; and (3) inhibiting Foxo function reduces the induction of *atrogin-1* by glucocorticoids in vitro and by fasting in vivo. Thus, activation of Foxo 3 is both necessary and sufficient for rapid atrophy, as presumably is activation of Foxo 1 and 4, since these three family members appear to be coordinately regulated. In muscles atrophying due to fasting, diabetes, uremia, or cancer, *Foxo 1* and *3* mRNA levels rise (Furuyama et al., 2003; Lecker et al., 2004). In our two models of myotube atrophy, Foxo 1, 3, and 4 appeared to be phosphorylated and dephosphorylated in a similar manner. It is noteworthy that the d.n. FOXO3A constructs used here also reduce function of Foxo 1 and 4.

These experiments are the first to implicate a specific transcription factor in atrophy of any cell and demonstrate an unexpected role for the Foxo family in the control of muscle cell size. In many cells, transcription by Foxo retards cell cycle progression and activates apoptosis (Tran et al., 2003). Consequently, to avoid causing apoptosis or other problems that are likely when Foxo3 is transduced into myoblasts (Bois and Grosveld, 2003), we used viral infection of differentiated myotubes and gene transfer into adult muscles to analyze the effects of Foxo3 in postmitotic tissue where it did not cause apoptosis.

To elicit profound atrophy, Foxo factors must stimulate the expression of many atrophy-related genes and enhance protein degradation. Because *MuRF-1* is induced by glucocorticoids and suppressed by IGF-1 (Sacheck et al., 2004; Stitt et al., 2004) it seems very likely that Foxo also transcribes the gene for *MuRF-1*, the other atrophy-related muscle-specific E3. However, it will be important to determine which of the other recently identified atrogenes are controlled directly or indirectly by Foxo.

During atrophy, Foxo 1- and 3-dependent transcription appears to be activated by two mechanisms: (1) their mRNA levels rise, presumably through increased transcription (Lecker et al., 2004), and (2) these factors are hypo- or dephosphorylated due to the reduction in AKT activity (and/or perhaps by action of a phosphatase). Amongst the genes most strongly induced in our microarray analyses of atrophy due to fasting, diabetes, uremia, and cancer (Jagoe et al., 2002, Lecker et al., 2004) are *PDK4*, *p21*, *Gadd45*, *4E-BP1*, all of which can be controlled by Foxo factors (Nakae et al., 2003; Puig et al., 2003; Tran et al., 2003). Moreover, Foxo family members have been recently implicated in the development of insulin resistance (Nakae et al., 2002), a prominent feature of muscles in all of these catabolic states. Resistance to insulin should also reduce the inhibition of expression of *atrogin-1*, *MuRF-1*, and other atrogenes, as well as the inhibition of protein degradation by insulin and IGF-1. In these atrophying muscles, mRNA for *IGF binding protein 5*, an enhancer of IGF-1 function also decreased (Lecker et al., 2004), which may additionally contribute to IGF-1 resistance and Foxo activation.

In a variety of organisms, AKT and its downstream targets affect muscle size. In *Drosophila*, the loss of AKT leads to smaller organs due to a reduction in cell size and number (Verdu et al., 1999), and mice deficient in *AKT1/2* have very little muscle mass (Peng et al., 2003). In addition, overexpression of the Foxo homolog in *Drosophila*, dFoxo, leads to small flies with reduced cell numbers and structural changes resembling those in starvation (Kramer et al., 2003; Puig et al., 2003). However, final muscle mass is determined by the initial rate of formation and growth of myotubes and subsequently by the postnatal hypertrophy of muscle fibers. Both myotube fusion and the net accumulation of proteins are stimulated by IGF-1 and AKT, and thus may be inhibited by Foxo3. In fact, inhibition of Foxo family members by phosphorylation may be required for muscle cell differentiation and fusion of myoblasts into myotubes (Hribal et al., 2003). However, the atrophy of adult muscle and of myotubes in the models studied here is a distinct process unrelated to myoblast fusion. In these differentiated myotubes, which are no longer replicating, and in adult muscle where growth is not occurring, Foxo action does not affect cell number, and apoptosis is not evident by

TUNEL. Instead, Foxo, like disuse atrophy or muscle wasting during disease, is causing shrinkage and marked loss of preexistent cell protein. Furthermore, it may be misleading to extrapolate from observations concerning the control of myoblast fusion (e.g., by NFκB or TNFα) to possible mechanisms for atrophy or cachexia in vivo.

IGF-1 and Insulin Suppress an Atrophy Program

The ability of insulin and IGF-1 to suppress the activation by Foxo of *atrogin-1* expression and the transcriptional program for atrophy constitute important new actions of these hormones that must contribute in a major way to their capacity to stimulate muscle growth. While their ability to stimulate muscle protein synthesis through activation of PI3K and AKT is widely appreciated, IGF-1 and insulin also reduce expression of *atrogin-1* and protein breakdown, especially of myofibrillar proteins, and this effect correlates with the decreased expression of *atrogin-1* (Sacheck et al., 2004). Thus, in promoting growth, IGF-1 and insulin not only enhance overall synthesis but also suppress proteolysis and the expression of atrophy-related ubiquitin ligases. Under these anabolic conditions, general protein synthesis rises at the translational level due to AKT-induced phosphorylation and activation of mTOR and S6K, while the phosphorylation of Foxo factors leaves them inactive in the cytosol. Together, these adaptations lead to protein accumulation and fiber hypertrophy (Figure 7, left). By contrast, in catabolic conditions, where IGF-1 and insulin are low (e.g., fasting or diabetes) and there is resistance to their actions (e.g., cancer, uremia, sepsis, Cushing's syndrome), or after denervation and disuse (Bodine et al., 2001b), AKT is dephosphorylated and its activity reduced below control levels, leading to activation of Foxo and to transcription of *atrogin-1*, *MuRF1*, and other genes that promote wasting (e.g., genes for insulin resis tance). Consequently, all these states should involve reduced protein synthesis, because of the dephosphorylation of GSK, mTOR, and S6K. Together, these adaptations lead to a dramatic decrease in cell protein and myofiber size (Figure 7, right).

Glucocorticoids are also required for enhanced proteolysis and muscle wasting in many systemic diseases (Hasselgren, 1999). The sensitivity of rat muscles and myotubes to the catabolic actions of glucocorticoids is decreased by insulin apparently because AKT can maintain Foxo in an inactive state. Because the *atrogin-1* promoter lacks glucocorticoid-response elements, glucocorticoids must act indirectly by inducing or activating proteins that stimulate *atrogin-1* transcription. The obvious candidates are Foxo family members. In fact, after administration of glucocorticoids, *Foxo1* and *3* mRNA rise in muscle (Furuyama et al., 2003), as occurs in various types of atrophy (Lecker et al., 2004). Foxo members and glucocorticoids function together in controlling transcription in liver cells (Nakae et al., 2001; Nasrin et al., 2000). In fasting, when glucocorticoids stimulate proteolysis and net release of amino acids from muscle, they enhance the liver's capacity to convert amino acids to glucose, and some of these gluconeogenic actions require Foxo1 and 3 (Nakae et al., 2001). Foxo1 and 3 work as cofactors with glucocorticoids perhaps by recruiting the p300/CBP/SRC coactivator complex to the forkhead binding site (Nasrin et al., 2000). Similarly, glucocorticoids may promote *atrogin-1* expression and atrophy not only by inducing Foxo, but also by recruiting other factors to act with Foxo1 or 3 on the *atrogin-1* promoter.

Kinases downstream of AKT, including GSK3β, as well as the MEK and calcineurin systems, have been implicated in the regulation of muscle fiber size (Bodine et al., 2001a; Murgia et al., 2000; Musaro et al., 1999). However, in contrast to AKT, none of these factors were found to have direct roles in the regulation of *atrogin-1* expression. Our findings with Foxo3 can account for the prior observation that inhibition of AKT by a dominant-negative mutant induces myotube atrophy (Bodine et al., 2001b; Pallafacchina et al., 2002). By contrast, the inability of dominant-negative GSK3β (Supplemental Figure S1 available on *Cell* website) or LiCl treatment (data not shown) to inhibit *atrogin-1* induction by Dex seems to rule out a major role for GSK3β in this process. *Atrogin-1* induction also

appears to occur independently of mTOR/S6K since IGF-1 could suppress *atrogin-1* expression even in cells starved for nutrients and serum, where there was almost a complete inhibition of mTOR/S6K (Figure 2A). Most likely, in these starved cells, as in fasting in vivo, protein translation decreases through mechanisms involving mTOR/S6K and GSK3β dephosphorylation, while the concomitant activation of *atrogin-1* transcription involves Foxo dephosphorylation. Accordingly, inhibition of PI3K (with LY294002, dominant-negative AKT, or constitutively active SHIP) leads to atrophic myotubes, but inhibition of mTOR with rapamycin does not induce atrophy in culture or in vivo (Bodine et al., 2001b; Pallafacchina et al., 2002; Rommel et al., 2001).

The proinflammatory cytokine TNFα has also been proposed to stimulate muscle atrophy in sepsis and certain types of cancer, and in many cells, TNFα triggers activation of the transcription factor NFκB (Li et al., 2003). Based on its ability to inhibit myoblast differentiation, which is a very different process from atrophy of adult muscle fiber, NFκB had been proposed to be a key factor causing muscle wasting and cachexia (Guttridge et al., 2000). Our findings upon transfection of an activator of NFκB or a dominant-negative IκB mutant exclude an important role for NFκB or TNFα in the con trol of *atrogin-1* transcription, although they may promote wasting by other mechanisms.

While clearly advancing our understanding of the mechanisms of atrophy, the present findings have raised many important questions concerning the role of different Foxo family members, what genes they transcribe in muscle to trigger atrophy and the precise functions of atrogin-1 and MuRF1 in muscle wasting. While our understanding remains limited, the identification of Foxo3 as a major activator of *atrogin-1* expression and muscle atrophy suggests several new approaches for pharmacological interventions to prevent or diminish this debilitating process.

Experimental Procedures

Antibodies and Reagents

See Supplemental Data available on *Cell* website.

Cell Culture, Adenoviral Infection, and Myotube Analysis

C2C12 mouse myoblasts (ATCC) were cultured in DMEM 10% FCS (ATCC) until the cells reached confluence. The medium was then replaced with DMEM 2% horse serum (HS) (ATCC) ("differentiation medium") and incubated for four days to induce myotube formation before proceeding with experiments. For infection, myotubes were incubated with adenovirus at a multiplicity of infection (MOI) of 250 in differentiation medium for 18 hr, and then medium was replaced. The infection efficiency was typically greater than 90%. Myotube diameter was quantified by measuring a total of >160 tube diameters from five random fields at 100× magnification using IMAGE software (Scion, Frederick, MD) as described (Rommel et al., 2001). All data are expressed as the mean ± SEM.

Adenoviral Vectors

The d.n. AKT, the c.a. AKT, the c.a. GSK3, d.n. SK3, c.a. NIK, c.a. IκB, human Foxo3 (FOXO3A), c.a. FOXO3A, d.n. FOXO3A, control GFP, and the control β-gal adenoviral vectors have all been described previously (Kim et al., 2002; Skurk et al., 2004).

Protein Extraction, Western Blotting, RNA Extraction, and Northern Blot Analysis

See Supplemental Data available on *Cell* website.

atrogin-1 Promoter Cloning

See Supplemental Data available on *Cell* website.

Transient Transfections and Luciferase Assays in Muscle Cell Cultures

Myoblasts were transfected with different reporters using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. When the cells reached confluence, the medium was shifted to differentiation medium to induce myotube formation. After four days, myotubes were infected with adenovirus as described above. 36 hr later, myotubes were lysed and analyzed using the Dual-Luciferase reporter assay system (Promega). Detailed experimental procedures are described in Supplemental Data available on *Cell* website.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA technique and oligonucleotides are described in Supplemental Data available on *Cell* website.

RNAi in Myotubes

The siRNA for *Foxo1–3* (see below) was cloned into Lentilox RNAi vector *pLL3.7* (Rubinson et al., 2003) and was cotransfected with the lentivirus packaging mix (ViraPower Packaging Mix, Invitrogen) into 293FT cells. Supernatant from three 150 cm^2 dishes were collected, ultrancentrifuged at 25,000 rpm in SW28 Beckman rotor for 1.5 hr, and resuspended in 100 μl of cold PBS. C2C12 myoblasts were infected (50 μl virus/9.4 cm^2 dish) and differentiated for eight days. Myotubes were lysed in Trizol and protein level analyzed by Western blot. At least 70% of myotubes were infected in these experiments.

Adult Mouse Skeletal Muscle Transfection

Adult female CD1 mice (28–30 g) were used in all experiments. Tibialis anterior muscles were transfected as described previously (Murgia et al., 2000). The muscle was isolated through a small hind-limb incision, and 25 μg of plasmid DNA was injected along the muscle length. In reporter experiments, 10 μg of the expression vector with 10 μg of the *3.5AT1* reporter construct and 5 μg of *pRL-TK* vectors were coinjected. Electric pulses were then applied by two stainless steel spatula electrodes placed on each side of the isolated muscle belly (50 Volts/cm, 5 pulses, 200 ms intervals). Muscles were analyzed 4, 8, or 14 days later. No gross or microscopic evidence for necrosis or inflammation as a result of the transfection procedure was noted. The reporter containing six forkhead binding sites (DBE promoter) (Furuyama et al., 2000) was generated by ligation of the following oligonucleotides to NheI-XhoI double digested *pGL3-basic* vector. DBE oligonucleotides: 5′-TCGAAAGTAAACAACTATGTAAACAACTATAAGTAAACAACTATGTAAACAACTATAAGTAAACAACTATGTAAACAAGATC-3′ and 5′-CTAGGATCTTGTTTACATAGTTGTTTACTTATAGTTGTTTACATAGTTGTTTACTTATAGTTGTTTACATAGTTGTTTACTT-3′.

Immunohistochemistry and Fiber Size Measurements

Mouse muscle fibers expressing HA-tagged proteins were stained in cryocross-sections fixed with 4% paraformaldehyde. Immunohistochemistry with anti-HA polyclonal antibody (Santa Cruz) was as previously described (Pallafacchina et al., 2002). Muscle fiber size was measured in fibers transfected with the Foxo3 mutant and in an equal number of untransfected fibers from the same muscle as described elsewhere (Pallafacchina et al., 2002). Fiber cross-sectional areas were measured using IMAGE software (Scion, Frederick, MD). All data are expressed as the mean ± SEM. Comparison were made by using the student's t test, with $p < 0.05$ being considered statistically significant.

In Situ Hybridization

In situ hybridization was performed as described (Murgia et al., 2000). An ^{35}S-labeled cRNA probe complementary to the *atrogin-1* coding region was prepared by in vitro transcription (Roche) using the full-length mouse *atrogin-1* gene in KS^{+}Bluescript as a template.

RNAi in Adult Skeletal Muscle

A target finder and design tool (Ambion) was used to identify target regions in the mouse *Foxo1* and *3* and *GFP* genes amenable to siRNA. *Foxo1* and *3*: 5′-GGATAAGGGCGACAGCAAC-3′, *Foxo1*: 5′-CCTGTCGTACGCCGACCTC-3′, *Foxo3*: 5′-TGAAGGCACGGGCAAGAGC-3′, *GFP*: 5′-GCAAGCTGACCCTGAAGTTC-3′. These sequences were incorporated into 64 bp self-annealing oligonucleotides (Brummelkamp et al., 2002), synthesized and cloned into BglII-HindIII double-digested *pSUPER* vector. Adult skeletal muscle was cotransfected with 30 μg of the *pSUPER* vector along with 10 μg of the 3.5 kb *atrogin-1*-firefly *luciferase* reporter construct and 5 μg of *pRL-TK* vectors as above described. Seven days after transfection, the mice were fasted for 24 hr and sacrificed.

Acknowledgments

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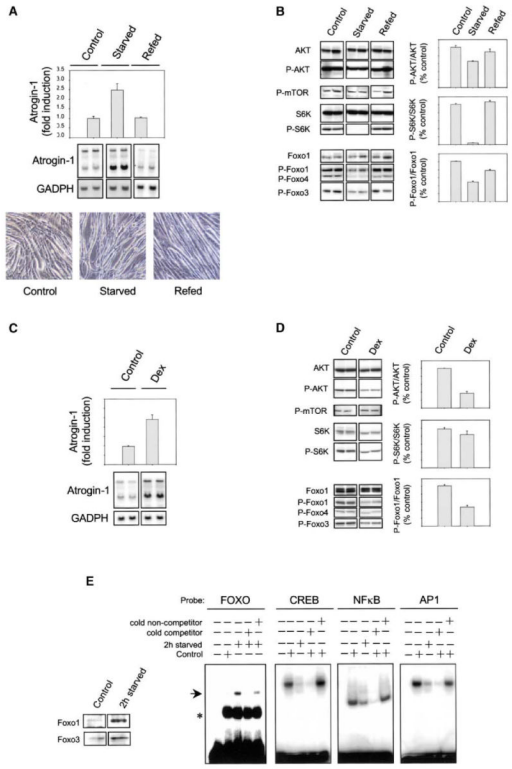

Figure 1.

Starving Cells and Glucocorticoid Treatment Induce *atrogin-1* Expression and Dephosphorylation of Members of the PI3K/AKT-Signaling Pathway

(A and B) C2C12 myotubes were starved by removal of growth medium and incubated in PBS for 6 hr. Medium was replaced in refed samples for 12 hr.

(A) Effect of starvation on *atrogin-1* expression: Northern blots probed for *atrogin-1* and *GAPDH* (middle image) and fold increase in *atrogin-1* mRNA, calculated by dividing the *atrogin-1* band intensity (*atrogin-1/GADPH*) with the *atrogin-1/GADPH* ratio in the control condition (upper image). Both *atrogin-1* bands were used in the analysis. Micrographs of representative control, starved, and refed C2C12 myotube cultures (lower image).

(B) Effect of starvation on components of the PI3K/AKT pathway: proteins were extracted from the same samples as in a, and subjected to immunoblot analysis. Quantitation of the ratio of phosphorylated to total protein was determined as above. Data was normalized to a control of 100%.

(C) Effect of 1 μM Dex treatment on *atrogin-1* expression.

(D) Effect of Dex on PI3K/AKT pathway components.

(E) Foxo factors are nuclear in starved C2C12 myotubes. Left image: nuclear extracts from control and starved myotubes were immunoblotted with anti-Foxo antibodies. Right image: nuclear extracts were tested for binding to double-stranded ^{32}P-labeled oligonucleotides containing Foxo, CREB, NFκB, and AP1 binding sites by electrophoretic mobility shift assay. Arrow, Foxo–oligonucleotide complexes. Asterix, nonspecific band.

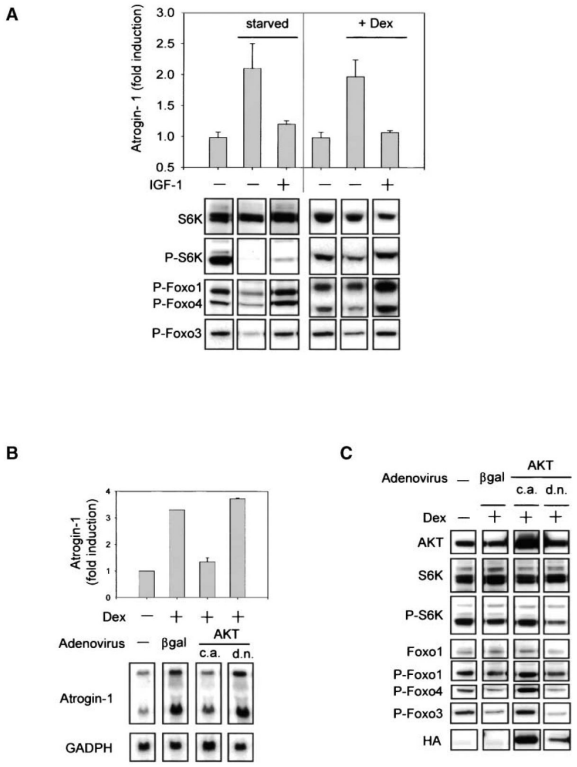

Figure 2.

IGF-1 and AKT Block the Induction of *atrogin-1* by Starvation and Dex Treatment and Cause Foxo Phosphorylation

(A) Myotubes were incubated in the absence or presence of IGF-1 (10 ng/ml) and concomitantly starved (for 6 hr) or treated with Dex (for 24 hr). Northern blots were performed for *atrogin-1* expression (upper image) and the amount and phosphorylation of AKT pathway members assayed by immunoblot (lower image) as in Figure 1.

(B) Myotubes were infected with adenoviral vectors for c.a. AKT, d.n. AKT, or with control virus (βgal). 36 hr after infection, half the myotubes were treated with 1 μM Dex for 24 hr, and *atrogin-1* expression analyzed by Northern.

(C) Immunoblots for AKT and its downstream targets in the cultures from (B), overexpressed proteins were detected by anti-HA immunoblot.

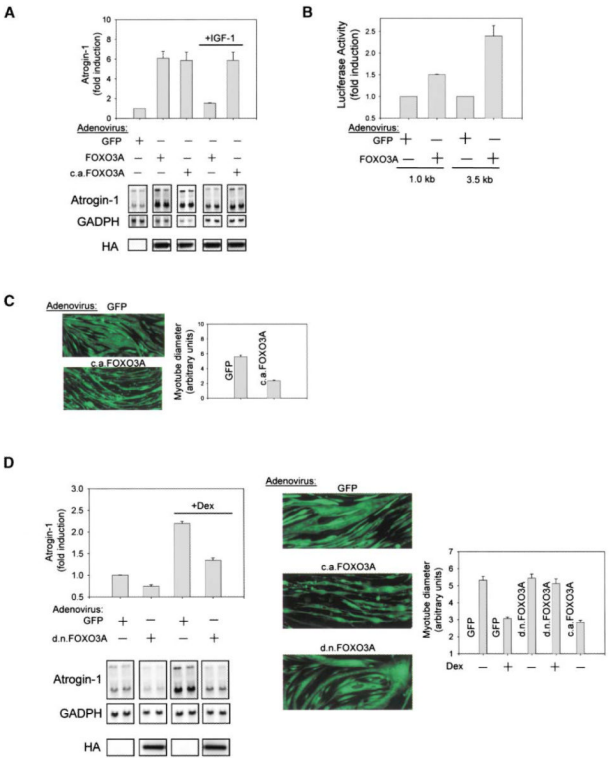

Figure 3.

Foxo3 Induces *atrogin-1* Expression and Causes Reduction in Myotube Size

(A) Foxo3 induces *atrogin-1* expression. Myotubes were infected with adenoviral vectors for FOXO3A and c.a. FOXO3A in the absence or presence of IGF-1 (10 ng/ml) and after 48 hr, *atrogin-1* mRNA levels were assayed and depicted as in Figure 1. Overexpressed proteins were detected by anti-HA immunoblot.

(B) The *atrogin-1* promoter is activated by Foxo3. Myoblasts were transfected with the *atrogin-1* reporter constructs *1.0AT1* or *3.5AT1*, differentiated for four days, and then infected with FOXO3A or with a control (GFP) vector for 24 hr. Extracts were assayed for Firefly and Renilla luciferase activity. Firefly/Renilla activity was normalized to 1.0 in the control (GFP) infection.

(C) Fluorescence microscopy of myotube cultures overexpressing Foxo3. Myotube cultures were infected with control adenovirus (GFP) or c.a. FOXO3A and photographed 48 hr after infection. Mean myotube diameter from each culture was quantified from three independent experiments.

(D) D.n. Foxo3 inhibits dex-induced *atrogin-1* expression and reduction in myotube diameter. Myotubes were infected with adenoviral vectors expressing d.n. FOXO3A, c.a. FOXO3A, or GFP, and incubated in the absence or presence of 1 μM Dex for 24 hr. Overexpressed d.n. FOXO3A was detected by anti-HA immunoblot. Left image: Northern analysis of *atrogin-1* expression. Middle image: fluorescence microscopy of myotube cultures after 48 hr. Right image: quantification of mean myotube diameters in the presence of Dex and Foxo expression.

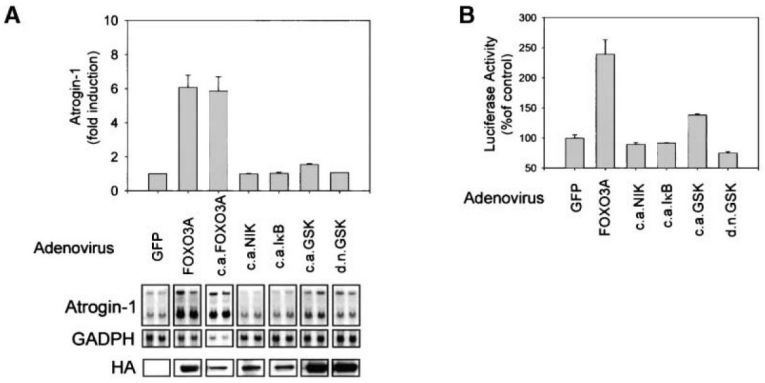

Figure 4.

Foxo3, but Not Other AKT Targets, Activates *atrogin-1* Expression

(A) Myotubes were infected with various adenoviral vectors for 48 hr and then *atrogin-1* expression was analyzed (upper image). Overexpressed proteins were detected by anti-HA immunoblots (lower image).

(B) Myoblasts were transfected with *3.5AT1*, differentiated, and infected with the indicated vectors. Luciferase activity in extracts from these cultures was analyzed as in Figure 3B. Results are normalized to the control GFP infection.

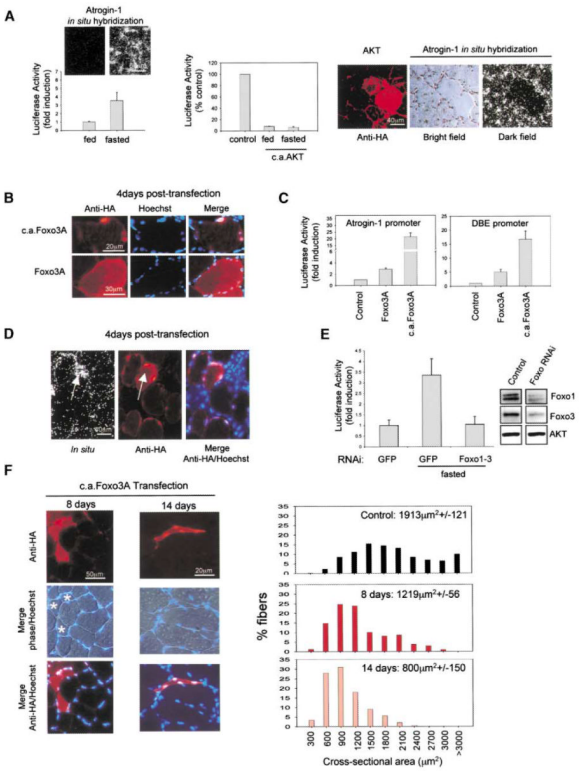

Figure 5.

AKT Suppresses and Foxo Stimulates *atrogin-1* Expression and Foxo Activation Causes Marked Atrophy of Mouse Muscle Fibers

(A) AKT prevents induction of *atrogin-1* expression by fasting. Left image: mouse tibialis anterior muscles were transfected by electroporation with the *3.5AT1* and *pRL-TK*. Seven days later, the mice were fed or fasted and then sacrificed. Firefly and Renilla luciferase activity was measured in muscle extracts. In situ hybridization on sections from muscles of fed and fasted mice (left image, insert). Scale bar is equal to 60 μm. Middle image: muscles were cotransfected with *3.5AT1, pRL-TK*, and either *c.a. HA-AKT* or the parent vector. Seven days later, the mice were fasted for 24 hr and sacrificed. Firefly/Renilla luciferase activity was measured. Right image: serial cross-sections of these transfected muscles were processed for immunofluorescence with anti-HA antibody or for in situ hybridization with an *atrogin-1* antisense probe. Scale bar is equal to 50 μm.

(B) Nuclear localization of c.a. FOXO3A. Sections of adult tibialis anterior muscles transfected with c.a. *FOXO3A* or *FOXO3A* were prepared and visualized with anti-HA antibodies (for Foxo) and Hoechst staining (for nuclei) four days after infection. Images were merged to demonstrate colocalization. Scale bars are equal to 20 μm for c.a. FOXO3A and 30 μm for FOXO3A.

(C) Foxo3 activates the *atrogin-1* promoter in transfected muscle fibers. Muscles were cotransfected with *3.5AT1, pRL-TK*, and with either *FOXO3A* or c.a. *FOXO3A* as described above. Similarly, a Foxo reporter (DBE promoter) was transfected in place of the *atrogin-1* reporter. Luciferase activity was measured four days after transfection.

(D) *Atrogin-1* mRNA is increased in muscle fibers overexpressing Foxo3. Cross-sections of tibialis anterior muscle transfected with c.a. *FOXO3A* were processed for immunofluorescence with anti-HA antibody or for in situ hybridization for *atrogin-1* four days after transfection. *Atrogin-1* transcripts are increased in Foxo3 overexpressing fibers, in close proximity to the Foxo3-positive nuclei (arrow) Scale bar is equal to 40 μm.

(E) siRNA-mediated knockdown of *Foxo1-3* inhibits *atrogin-1* promoter activity during fasting. Left image: adult skeletal muscle was cotransfected with *pSUPER*-vectors, *pRL-TK* and *3.5AT1*. Seven days later, the mice were fasted and sacrificed. Firefly/renilla luciferase activity was measured as above. Right image: Foxo1 and 3 protein level detected by immunoblot in myotubes.

(F) Foxo3 expression for 8 or 14 days causes marked muscle fiber atrophy. Left images: adult tibialis anterior muscles were transfected with c.a. *FOXO3A* and mice were sacrificed after 8 or 14 days. Atrophic fibers expressing c.a. FOXO3A were detected in transverse sections stained with anti-HA (for Foxo) (asterix in eight day micrograph) For eight days, scale bar is equal to 50 μm. For 14 days, scale bar is equal to 20 μm. Right images: frequency histograms showing the distribution of cross-sectional areas (μm^2) of fibers expressing c.a. FOXO3A for eight days (red bars) or 14 days (pink bars) and surrounding untransfected fibers (control fibers) (black bars).

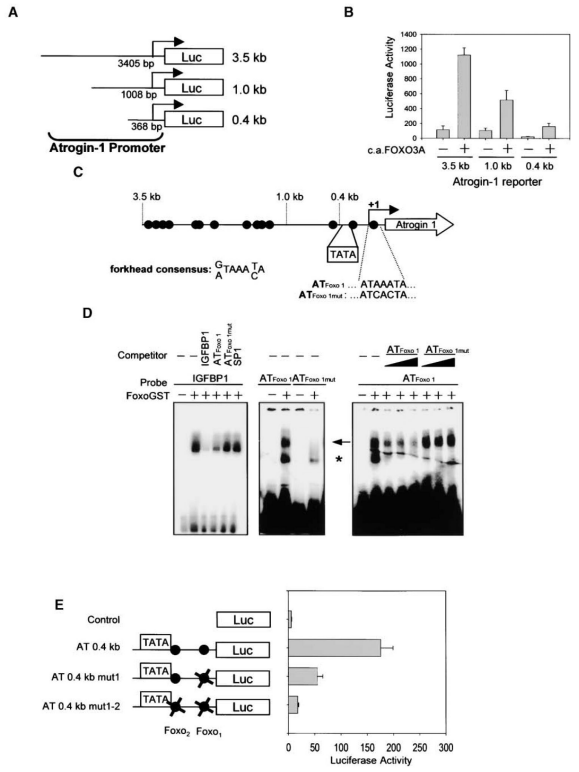

Figure 6.

Foxo Binding Sites Are Required for *atrogin-1* Promoter Activation by Foxo3

(A) Serial truncations of the *atrogin-1* promoter were fused to the firefly *luciferase* gene.

(B) The *atrogin-1* reporters and *pRL-TK* were transfected into adult muscle in presence or in absence of c.a. *FOXO3A* as above. Mice were sacrificed four days after transfection and muscles were assayed for luciferase activity.

(C) The *atrogin-1* promoter. Multiple forkhead consensus binding sites are noted by black circles. Potential forkhead binding site ($Foxo_1$) present in smallest promoter truncation, used in the gel-shift experiment in (D), as well as the mutated version are shown.

(D) Purified FoxoGST protein was tested for binding to double-stranded ^{32}P-labeled oligonucleotides containing the IGFBP1 site, AT Foxo 1 and AT Foxo 1 mut sites by electrophoretic mobility shift assay as described in Experimental Procedures. Arrow, FoxoGST–oligonucleotide complexes. Asterix, nonspecific band.

(E) Left image: mutations in the 0.4 kb *atrogin-1 luciferase* reporter constructs. The two putative Foxo sites are noted by black circles and mutations by X. Right image: adult muscle were transfected with the *atrogin-1* reporters described in the left image together with *pRL-TK* and c.a. *FOXO3A* and luciferase activity measured.

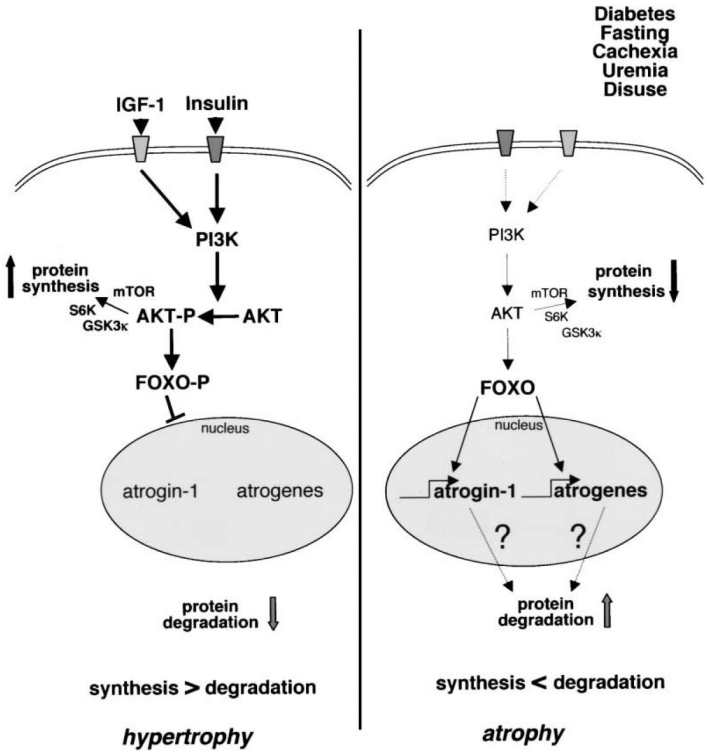

Figure 7.

A Summary of the Roles of the IGF-1/AKT Pathway and Foxo in Muscle Atrophy (right image) and Hypertrophy (left image) Factors and pathways in bold are activated (see text for details).