FOXO1 Regulates the Expression of 4E-BP1 and Inhibits mTOR Signaling in Mammalian Skeletal Muscle*

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The mammalian target of rapamycin (mTOR) is regulated by growth factors to promote protein synthesis. In mammalian skeletal muscle, the Forkhead-O1 transcription factor (FOXO1) promotes catabolism by activating ubiquitin-protein ligases. Using C2C12 mouse myoblasts that stably express inducible FOXO1-ER fusion proteins and transgenic mice that specifically overexpress constitutively active FOXO1 in skeletal muscle (FOXO1++,), we show that FOXO1 inhibits mTOR signaling and protein synthesis, including mTOR, mammalian target of rapamycin; Raptor, regulatory-associated protein of mTOR; p70S6K, p70S6 kinase; TAM, 4-hydroxytamoxifen; WT, wild type; ChIP, chromatin immunoprecipitation; FOXO1–215, DNA binding-defective FOXO1; CA-FOXO1, constitutively active FOXO1; MAFbx, atrogin-1.

Loss of skeletal muscle tissue is a clinical manifestation of many conditions and diseases, including aging, cancer, sepsis, human immunodeficiency virus, and diabetes (1), and important progress has been made in understanding the molecular regulation of both catabolism and anabolism within skeletal muscle (2). MAFbx (atrogin-1), an F-box protein, promotes skeletal muscle protein degradation in response to glucocorticoids (3, 4) and contributes toward muscle atrophy by targeting the E3 ubiquitin ligase MAFbx (4). The decreased protein synthesis observed in skeletal muscle in various catabolic conditions is associated with defects in mRNA translation initiation (15, 16). The translation of mRNA is divided into three steps, initiation, elongation, and termination (17), and is facilitated and regulated by eukaryotic initiation factors (18). The stable, heterotrimeric complex consisting of eIF4E, eIF4G, and eIF4A, which form the active eIF4F complex, plays a critical role in peptide chain orientation by regulating recruitment of capped mRNAs to the 40 S pre-initiation complex (19). The eukaryotic initiation factor-4E (eIF4E) and its inhibitory protein, eukaryotic initiation factor-4E-binding protein-1 (4E-BP1) have been suggested to be critical for the regulation of translation initiation (19–21). 4E-BP1 is a phospho-
phoprotein that, in a hypophosphorylated state, forms a tight association with eIF4E and prevents its interaction with eIF4G and recruitment to the 43 S pre-initiation complex. Thus, when hypophosphorylated, as would be the case in the absence of nutrients or growth factors, 4E-BP1 association with eIF4E serves to repress translation (20, 22). In contrast, when phosphorylated, 4E-BP1 dissociates from eIF4E and allows the recruitment of capped mRNA and translation initiation (22, 23). This complex interaction is thought to represent a rate-limiting step in protein synthesis, which can in part be ascribed to the fact that eIF4E is the least abundantly expressed subunit (24). 4E-BP1 contains at least six phosphorylation sites, including two (Thr^{37/46}) that are hierarchical regulatory sites activated by mammalian target of rapamycin (mTOR) signaling (25–28). In response to Akt activation, mTOR forms a complex with the regulatory-associated protein of mTOR (Raptor), a 150-kDa polypeptide (29), and then phosphorylates 4E-BP1 (30). Raptor is critical for mTOR signaling in vivo (29) and is absolutely required for the mTOR-catalyzed phosphorylation of 4E-BP1 in vitro (30, 31). Interaction with Raptor is also important for the ability of mTOR to phosphorylate p70S6 kinase (p70S6K) (32), a critical factor in promoting protein synthesis.

Recent work in Drosophila (33, 34) and C. elegans (35) suggest we show that FOXO1 inhibits mTOR signaling. We also show that FOXO1 increases total (but not phospho) 4E-BP1 abundance, resulting in a net increase in hypophosphorylated 4E-BP1. These observations occur in the face of a decreased abundance of mTOR and Raptor, which are upstream regulators of 4E-BP1 and p70S6K phosphorylation.

MATERIALS AND METHODS

C2C12 Cells and Cell Culture—C2C12 skeletal muscle myoblasts stably expressing FOXO-ER fusion proteins were previously described (36). In brief, C2C12 cells were stably transfected with the empty pBABE retrovirus or pBABE vectors expressing fusion proteins containing either a constitutively active form (where three Akt phosphorylation sites (Thr^{24}, Ser^{256}, and Ser^{319}) are replaced by alanines (37)) or a transcriptionally inactive form (where His and Ser are replaced with arginine to disrupt DNA binding) of human FOXO1 in-frame with a modified (tamoxifen-specific) version of the murine estrogen receptor-α ligand binding domain (38). Cells were selected with puromycin and colonies pooled for studies, as previously described (36). Fusion proteins are restricted to the cytoplasmic space until activation by treatment with TAM (36).

Cells were maintained in proliferation media consisting of Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin mixture at 37 °C and 5% CO₂ until confluent. Confluent myoblasts cultures were rinsed in 1× phosphate-buffered saline, dissociated with 0.05% trypsin, pelleted (1600 rpm for 5 min), rinsed in warm 1× phosphate-buffered saline, and seeded into experimental plates pre-coated with 0.2% gelatin (100-mm plates for protein extraction and immunoprecipitation, 6-well plates for RNA extraction, and 12-well plates for protein synthesis experiments). When experimental cultures were 80% confluent, proliferation medium was changed to differentiation medium consisting of Dulbecco's modified Eagle's medium supplemented with 2% horse serum and 1% penicillin/streptomycin mixture. Day 4, fully fused myotube cultures were supplemented with 10% fetal bovine serum for 24 h to induce the fed state, ameliorate effects of endogenous FOXO proteins, and drive protein synthesis. Following 24 h of fetal bovine serum treatment, cells were treated with 1 μM 4-hydroxytamoxifen to activate FOXO1-ER fusion proteins and promote their translocation to the nucleus (38) or with vehicle (Me₂SO) for 0, 8, 12, and 24 h.

Animal Experiments—The generation and phenotype of the FOXO1^{+/−} mice have been extensively reported previously (39). FOXO1^{+/−} and WT mice were anesthetized via 2.5% Avertin (0.017 ml/g body weight, intraperitoneally). Gastrocnemius muscles were then excised bilaterally using an aseptic technique, weighed, and stored at −80 °C until further processes, exposed to a cold lysis buffer (20 μM NaF, 1% glycerol, 2 μg/ml aprotinin). Gastrocnemius muscles from the animals were homogenized in the same buffer as the C2C12 cultures with a Polytron homogenizer. Homogenates were spun at 16,000 × g for 60 min at 4 °C, and the supernatant was removed and rapidly frozen in liquid nitrogen. Protein concentration of the muscle lysates was subsequently determined.

Lysates were solubilized in Laemmli sample buffer and boiled for 5 min, resolved by SDS-PAGE on 6% (mTOR and Raptor), 12% (p70S6K1), and 18% (4E-BP1 and eIF4E) polyacrylamide gels, transferred to a nitrocellulose membrane, blocked with 5% milk, and immunoblotted overnight with total-Raptor, total-4E-BP1, phospho-4E-BP1 (Thr^{37/46}), total-mTOR, phospho-mTOR (Ser^{2448}), total-p70S6K, phospho-S6K1 (Thr^{389}) antibodies (1:1000, Cell Signaling, Beverly, MA), and total-eIF4E (BD Biosciences). After incubation with horseradish peroxidase-conjugated secondary antibody (1:2000, Amersham Biosciences), the immunoreactive proteins were detected with enhanced chemiluminescence (PerkinElmer Life Sciences) and quantified by densitometry.

Isolation of Nuclear Proteins—Nuclear proteins were extracted from the gastrocnemius muscle of WT and FOXO1^{+/−} mice as previously described (40). Briefly, muscles were homogenized in 500 μl of pre-chilled Buffer A (250 mM sucrose, 10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 μl/30
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mg of tissue protease inhibitor mixture). The homogenate was spun at 500 × g for 5 min at 4 °C. The supernatant containing cytosolic materials (crude fraction) was then removed and stored at −80 °C until required. Then 500 μl of pre-chilled Buffer B (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM MgCl₂, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 ml/30 mg ⁻¹ tissue protease inhibitor mixture) was added to re-suspend the remaining pellet. After 10-min incubation on ice with occasional mixing, the pellet mixture was spun for 5 min at 3000 × g at 4 °C. The supernatant representing the nuclear fraction was extracted and stored at −80 °C until required. Fraction purity was assessed by Western blotting on Histone H1 as described previously (40).

C2C12 Immunoprecipitation Analysis—Given that C2C12 cells expressing the DNA binding-defective form of FOXO1 (FOXO1−215) appeared to act as a good control for comparison with the constitutively active FOXO1 (CA-FOXO1) cells, we opted to utilize these cell lines for immunoprecipitation studies. Cell cultures were treated in exactly the same way as those for whole cell lysate analysis, but the experiments were only conducted over a 12-h period. Following 24 h of 10% fetal bovine serum supplementation, cultures were treated for 0 or 12 h with/without TAM or Me₂SO (−), cells were lysed in buffer pre-substituted Raío A constituents are as follows, buffer A (20 mM Tris, 20 mM NaCl, 1 mM EDTA, 20 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 ml/30 mg ⁻¹ tissue protease inhibitor mixture) was added to re-suspend the remaining pellet. After 10-min incubation on ice with occasional mixing, the pellet mixture was washed twice with buffer A and twice with buffer B (10 mM Hepes, 50 mM β-glycerophosphate, 50 mM NaCl (pH 7.4)) on ice. The pellet was resuspended in 2 × SDS sample buffer, mixed with a vortex, centrifuged for 1 min, and heated to 85 °C for 5 min and placed on ice. Samples were resolved on 4% SDS-PAGE gels, transferred to a nitrocellulose membrane, blocked with 5% milk, washed five times with Tris-buffered saline with Tween (Tris, NaCl, Tween) and incubated overnight with anti-Raptor (or 4E-BP1) antibody (Cell Signaling). After overnight incubation in the primary antibody, membranes were incubated in horseradish peroxidase-conjugated secondary antibody (1:2,000, Amersham Biosciences), and the immunoreactive proteins were detected with enhanced chemiluminescence (PerkinElmer Life Sciences) and quantified by densitometry.

Gene Expression Studies—Day 4, differentiated, myotube cell cultures were treated with 1 μM TAM for 0, 8, 12, and 24 h or vehicle (Me₂SO). Animal tissues were homogenized with a Polytron homogenizer. Following treatments (cell culture) and homogenization (animal tissue), RNA was extracted via an on-column RNA isolation kit (Absolutely RNA Miniprep Kit, Stratagene) according to the manufacturer’s instructions. RNA samples were reverse transcribed using a thermal cycler (GeneAmp PCR 2400, PerkinElmer Life Sciences) with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) in 40-μl reaction mixtures containing 1 × TaqMan RT buffer, 5.5 mM MgCl₂, 500 μM 2’-deoxynucleoside 5’-triphosphate, 2.5 μM random hexamers, 0.4 unit of 1 μM RNase inhibitor, 1.25 units of μl⁻¹ Multiscribe reverse transcriptase. Control (reverse transcriptase-negative) samples were also made and included in all PCR analyses to check for genomic DNA contamination.

Amino Acid Incorporation Studies—Amino acid incorporation into protein experiments was performed as described by Franch et al. (41) with modifications. Briefly, day 4 myotube cultures were incubated in differentiation media supplemented with 0.6 mM phenylalanine (cold Phe) and 10% serum for 2 h prior to the addition of 0.5 μCi of [¹⁴C]phenylalanine (hot Phe) per well for 2, 8, 12, and 24 h. The appropriate constructs (pBABE-empty vector, FOXO1−215, and CA-FOXO1) were activated (or not) with 1 μM TAM upon addition of [¹⁴C]phenylalanine. Following the time course, cells were washed 3 × with cold (1 ×) phosphate-buffered saline, and lysed in 600 μl of 10% trichloroacetic acid and placed on ice for 60 min. The plates were thoroughly scrapped, and the lysate spun in a centrifuged sherd three bumulbized over night for 1 ml of scin-e incorpo-sessed via gins total

Chromatin Immunoprecipitation Analysis—Again, given that the C2C12 myotubes expressing FOXO1−215 appeared to act as good controls for comparison with the CA-FOXO1 cells (CA-FOXO1), we opted to only utilize these cell lines for ChIP studies. ChIP studies were carried out using the EZ-ChIP kit (Upstate) according to manufacturer guidelines. Briefly, cell cultures were incubated with appropriate treatments in the fed state (10% serum). Proteins were cross-linked to DNA with the addition of 37% formaldehyde. Cells were washed and lysed in SDS lysis buffer and sonicated for 15 s and allowed to recover at 80 °C until extraction. Following incubation with antibody, protein-DNA complexes were eluted, and the cross-links were reversed. DNA was purified using spin filter columns. Primers were designed to flank predicted FOXO binding sites located between residues 261 and 293 bp upstream of exon 1 in the 4E-BP1 promoter (42) or an upstream segment of the promoter with no identified FOXO1 binding sites. Primer information is available upon request.

Statistical Analysis—Data were analyzed using a one-way or two-way analysis of variance with repeated measures (Statis-
RESULTS

4E-BP1 Is a Transcriptional Target of FOXO1 in Skeletal Myotubes—To study the effects of FOXO1 on 4E-BP1 signaling in muscle cells, we employed C2C12 cells stably expressing either a constitutively active form of FOXO1 (CA-FOXO1), or a transcriptionally inactive form of FOXO1 (FOXO1–215) in-frame with a modified form of the estrogen receptor ligand binding domain that responds selectively to TAM (43). Previous studies with these cells have shown that fusion proteins are restricted to the cytoplasmic space in the absence of ligand and then rapidly translocate to the nucleus upon treatment with TAM (36). Although FOXO proteins can prevent myocyte differentiation and fusion (44), this model allowed us to maintain FOXO-ER fusion proteins in an inactive state during myoblast proliferation, differentiation, and fusion, and then examine the effects of acutely activating the CA-FOXO1 fusion protein in mature myotubes by addition of TAM (herein referred to as CA-FOXO1(+) and CA-FOXO1(–), indicative of the presence or absence of TAM treatment, respectively).

Consistent with previous studies (3), treatment with TAM resulted in a significant induction of MAFbx in CA-FOXO1 cells (data not shown) at 8, 12, and 24 h post treatment confirming that MAFbx is a direct target of FOXO1. These data confirmed that TAM treatment successfully promoted the transcriptional activity of our CA-FOXO1 C2C12 myotubes. As shown in Fig. 1a, treatment with TAM also markedly elevated the mRNA abundance of 4E-BP1 at 8, 12, and 24 h post activation, indicating 4E-BP1, like MAFbx, is transcriptionally regulated by activation of FOXO1 in muscle cells. No changes in 4E-BP1 mRNA expression were observed in CA-FOXO cells in the absence of TAM or in control C2C12 cells stably transfected with empty vector (pBABE). Furthermore, TAM had no effect on 4E-BP1 mRNA levels in cells expressing a DNA binding-defective form of FOXO1 where a point mutation has been introduced into helix 3 of the DNA binding domain of FOXO1 (FOXO1–215), indicating that DNA binding is required for the effect of FOXO1 on the expression of 4E-BP1. In contrast to the increased 4E-BP1 mRNA in TAM-treated CA-FOXO1 myotubes, no changes were observed in the level of eIF4E mRNA (Fig. 1b), indicating that the effects of FOXO1 on the expression of 4E-BP1 are specific.

Given that our mRNA data suggested that 4E-BP1 is a transcriptional target of FOXO1 in muscle cells, and that direct interaction with the DNA binding domain of FOXO1 is required for this effect, we considered whether FOXO binding
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sites may be present in the promoter of the 4E-BP1 gene. Using a consensus sequence for FOXO binding sites \((C/T)(G/A)-AAA(C/T))\) \((45)\), we identified several potential FOXO binding sites in the 5’-promoter region located between 261 and 293 upstream of exon 1 of the mouse 4E-BP1 gene \((42)\). Based on this analysis, we performed ChIP experiments in myotubes expressing fusion proteins containing FOXO1–215 and transcriptionally active (CA-FOXO1) FOXO1. All ChIP experiments were performed in the presence of 10% serum to suppress the function of endogenous FOXO proteins. As can be seen in Fig. 1, treatment of myotubes that express CA-FOXO1 with TAM stimulates the binding of FOXO1 to the region of the 4E-BP1 promoter containing predicted FOXO binding sites. This binding occurs only in the region of the 4E-BP1 promoter proposed to contain FOXO1 binding sites in TAM-treated CA-FOXO1 myotube cultures and was not observed in myotubes expressing FOXO1–215. These data provide evidence that 4E-BP1 is a bone fide transcriptional target of FOXO1 in skeletal muscle cells in culture.

FOXO1 Promotes Increased 4E-BP1 Protein Abundance and Hypophosphorylation of 4E-BP1 and Increases Binding of 4E-BP1 with eIF4E—In the absence of insulin or growth factors, 4E-BP1 is hypophosphorylated and 4E-BP1 association with eIF4E serves to repress translation. When phosphorylated, 4E-\(\text{f}^{-}\) initiation on the pr\(\text{e}-\) translated ulated wi phophorylated 4E-BP1 dissociates from eIF4E and allows translation. When phosphorylated, 4E-\(\text{f}^{-}\) initiation on the pr\(\text{e}-\) translated ulated wi paring CA-FOXO1\((\text{+})\) with other conditions (Fig. 2). These findings were observed in the absence of any differences in either Akt total protein abundance or phosphorylation of Akt (see supplemental Fig. S1).

Next, to test whether the increased abundance of total and hypophosphorylated 4E-BP1 protein results in increased association with eIF4E, we performed immunoprecipitation analyses on cells expressing CA-FOXO1 or FOXO1–215 proteins treated with/without TAM for 12 h using an antibody against eIF4E to immunoprecipitate associated proteins prior to Western blotting. As shown in Fig. 3, activation of CA-FOXO1 associated with immunoprecipitated eIF4E was markedly increased by activation of FOXO1, reflecting the ability of FOXO1 to increase the level of hypophosphorylated 4E-BP1 (Fig. 2). Increased association of eIF4E with 4E-BP1 is expected to limit the availability of eIF4E and thus its ability to promote translation. As shown in Fig. 3b, activation of CA-FOXO1 with TAM did not alter the amount of eIF4E protein in C2C12 cells, indicating that increased association of 4E-BP1 with eIF4E is not due to changes in levels of eIF4E protein.

FOXO1 Decreases Total and Phospho-mTOR Protein Abundance and mTOR-associated Raptor—Given that activation of CA-FOXO1 was associated with increased levels and hypophosphorylation of 4E-BP1, we also examined its effect on components of mTOR signaling. As shown in Fig. 4, activation of FOXO1 results in a significant reduction in the level of total and Ser-2448-phosphorylated mTOR despite cells being maintained in the presence of serum and nutrients. As shown in Fig. 5, activation of CA-FOXO1 also reduced levels of Raptor protein at 8 h and thereafter. The changes in mTOR and Raptor protein levels at 8 h precedes any effect of FOXO1 activation on their mRNA abundance (supplemental Figs. S2 and S3), respectively, indicating that FOXO1 may exert effects on mTOR and Raptor protein expression at least in part at the post-transcriptional level. Of note, Raptor (but not mTOR) mRNA levels were reduced at 12 h (see supplemental Fig. S3) and thereafter, indicating that FOXO1 may contribute to the regulation of mTOR and Raptor protein levels through multiple mechanisms.

In the presence of growth factors, physical association between mTOR and Raptor promotes the access of mTOR to specific downstream targets \((32)\), and the association of mTOR and Raptor is thought to be critical for effective signaling of activated mTOR to downstream targets, including 4E-BP1 and p70S6K. Accordingly, we measured mTOR-as-
associated Raptor as assessed by coimmunoprecipitation. As shown in Fig. 5b, activation of CA-FOXO1 results in a reduction in the physical association between mTOR and Raptor. This reduced mTOR/Raptor association (Fig. 5b) is most likely a manifestation of the reduced protein abundance of these proteins (Figs. 4 and 5a, respectively) and as such the reduced association between mTOR and Raptor is probably not a regulated consequence of FOXO1 activation. However, that being said, the observation of reduced mTOR/Raptor association would be expected to limit signaling to downstream targets.

FOXO1 Does Not Alter p70S6K Abundance but Decreases Phosphorylation of p70S6K and Impairs Incorporation of Amino Acids into Protein in Skeletal Muscle Myotubes—In addition to 4E-BP1, p70S6K also is a major target of Raptor/mTOR signaling (32). Given that the abundance of both mTOR and Raptor was decreased, and by virtue of the reduced abundances of these proteins, their association was reduced by FOXO1, we next examined the effect of FOXO1 activation on p70S6K (Fig. 6). We observed no differences in the level of total p70S6K protein when comparing CA-FOXO1(+) with other conditions (Fig. 6). However, phosphorylation of p70S6K on its regulatory residue (Thr-389) was markedly reduced in CA-FOXO1(+) cells at 8, 12, and 24 h. This indicates that activation of FOXO1 not only reduces levels of mTOR and Raptor, but also impacts upon the ability of the mTOR/Raptor complex (via reductions in their respective abundances) to phosphorylate and, therefore, propagate signaling to downstream targets.

Because the phosphorylation of both p70S6K and 4E-BP1 plays a critical role in promoting protein synthesis (46), we next examined whether activation of FOXO1 might impair the incorporation of amino acids into protein. As shown in Fig. 6b, the incorporation of ¹⁴C-labeled phenylalanine into protein was impaired by activation of CA-FOXO1 in C2C12 cells at 8, 12, and 24 h. In contrast, we observed no change in [¹⁴C]phenylalanine incorporation in CA-FOXO1 myotubes treated with carrier alone or in control (pBABE or FOXO1–215) cells treated with TAM.

Effects of CA-FOXO1 on 4E-BP1 and mTOR Signaling in Vitro Are Observed in FOXO1+/− Mice—We have previously observed that muscle mass is markedly reduced in transgenic

FIGURE 3. Effect of FOXO1 on the binding of 4E-BP1 to eIF4E in skeletal muscle cells.

a, association of 4E-BP1 and eIF4E. Myotubes expressing fusion proteins containing FOXO1–215 or CA-FOXO1 were treated without (+) or without (-) TAM for 0, 8, 12, or 24 h prior to lysis and analysis of eIF4E levels by Western blotting and densitometry.

b, wild-type C2C12 myotubes (pBABE), or myotubes expressing fusion proteins containing FOXO1–215 or CA-FOXO1 were treated with (+) or without (-) TAM for 0, 8, 12, or 24 h prior to lysis and analysis of eIF4E levels by Western blotting and densitometry.

FIGURE 4. FOXO1 decreases total mTOR and phospho-mTOR abundance. The top panel shows representative Western blot analysis, the middle panel shows quantification of total mTOR protein, and the bottom panel shows quantification of phosphorylated mTOR (Ser-2448) protein. Total and Ser-2448-phosphorylated mTOR were analyzed in C2C12 wild-type (pBABE) myotubes and myotubes expressing fusion proteins containing DNA binding-defective (FOXO1-215) or constitutively active (CA-FOXO1). Myotubes were treated without (-) or with (+) TAM for 0, 8, 12, or 24 h prior to lysis and analysis of protein abundance by Western blotting and densitometry. *, difference (p < 0.05) compared with 0 h.
mice expressing CA-FOXO1 in skeletal muscle (FOXO1\(^{+/+}\)) (39). Although it is known that FOXO1 promotes catabolism, our in vitro data suggested that it might also impair anabolism via effects on the expression of 4E-BP1, and mTOR signaling. We therefore examined the muscles from FOXO1\(^{+/+}\) mice relative to wild-type littermate control mice (herein referred to as WT) to see if FOXO exerts similar effects in vivo. As can be seen in Fig. 7\(a\), the muscles from the transgenic mice are much smaller and paler in color, as previously reported (39), and the total weights of the muscles are significantly reduced when comparing FOXO1\(^{+/+}\) to WT mice (Fig. 7\(a\)). The nuclear abundance of FOXO1 was markedly higher in the FOXO1\(^{+/+}\) mice (Fig. 7\(a\)). Consistent with our in vitro data, the FOXO1\(^{+/+}\) mice exhibit decreased Raptor and mTOR protein abundance and decreased mTOR phosphorylation (Ser-2448) as well as increased 4E-BP1 protein abundance and decreased 4E-BP1 phosphorylation (Thr-37/46) in the presence of unchanged total or phosphorylated Akt (Ser-473). These changes were accompanied by a reduction in the phosphorylation of p70S6K on its regulatory residue (Thr-389, Fig. 7\(b\)). At the mRNA level we found that, like our CA-FOXO1(+) myotubes, FOXO1\(^{+/+}\) mice displayed increased 4E-BP1 expression (Fig. 8\(a\)). Also consistent with our in vitro analyses, eIF4E mRNA abundance was not different when comparing FOXO1 decreases Raptor total protein abundance and reduces mRNA, bx mRNA,

**DISCUSSION**

The importance of intact mTOR signaling and downstream targets in linking nutritional and hormonal cascades to the regulation of cell size is well established (27, 47, 48). Importantly, recent evidence suggests that 4E-BP1, together with p70S6K, regulates animal growth and/or size (48, 49). Here we report that activation of the FOXO1 construct in C2C12 myotubes and the expression of CA-FOXO1 in the skeletal muscles of mice result in increased binding of FOXO1 to the promoter of 4E-BP1, which results in increased mRNA and protein expression. The increases in 4E-BP1 protein and mRNA are concomitant with a reduction in the abundance of Raptor, an important mediator of the mTOR signaling pathway, reduced phosphorylation of the downstream protein p70S6K, and associated with reduced incorporation of labeled amino acid into protein as measured by incorporation of \(^{14}\)C]phenylalanine into C2C12 skeletal muscle myotube cells. These data suggest that FOXO1, previously known to activate catabolic pathways via transcriptional activation of atrogenes such as MAFbx, may also play an important role in negatively regulating anabolic pathways.

Raptor acts as an essential scaffold protein with mTOR (26, 27), and, in the presence of growth factor and nutrients; the
Raptor-mTOR complex phosphorylates and inhibits 4E-BP1 from binding to eIF4E. Suppressing the binding of 4E-BP1 to eIF4E allows the formation of the eIF4F complex and subsequent initiation of translation (30, 31). Conversely, increased levels of hypophosphorylated 4E-BP1 would be expected to inhibit the ability of eIF4E to form the eIF4F complex, and thereby limit translation, although we have not assessed eIF4F formation. Also, in the presence of reduced fully functional mTOR, increased levels of 4E-BP1 may interact with Raptor and further limit the ability of mTOR-Raptor complexes to signal to other downstream targets, including p70S6 kinase.

Interestingly, the mTOR-catalyzed phosphorylation of 4E-BP1 in vitro is entirely dependent on the presence of Raptor (30, 31), whereas the mTOR-mediated phosphorylation of p70S6K (another downstream target of mTOR signaling essential for anabolism) in vitro is less dependent on the presence of Raptor (31). Our data support the concept that Raptor contributes to p70S6K phosphorylation. When Raptor abundance is reduced, p70S6K phosphorylation is concomitantly reduced but not completely suppressed. Interestingly, Ohanna et al. (50) suggested the presence of a pathway, independent of mTOR, that acts in concert with p70S6K to mediate Akt-induced growth and hypothesized that inhibition of FOXO1 could fulfill such a function. Although FOXO proteins might exert p70S6K-independent effects on growth, our data indicate that FOXO proteins also may play an important role in regulating the phosphorylation (and, therefore, function) of p70S6K phosphorylation (Fig. 6a). A corollary of this finding is that inhibition of FOXO1 may be important in the ability of insulin and growth factors to stimulate p70S6K phosphorylation/activation and thus promote anabolism. Interestingly, while this report was in revision, Cao et al. (51) reported that, under nutrient/hormone replete conditions, phosphorylated FOXO1 may interact with the tuberous sclerosis complex and thereby enhance the ability of Rheb to activate mTOR signaling. Together, these results indicate that FOXO1 may contribute to the regulation of mTOR signaling through multiple mechanisms.

It is of interest that we see no change in the phosphorylation of Akt by the activation of FOXO1 under our experimental conditions. Previous studies have suggested that FOXO proteins can enhance the phosphorylation and activation of Akt, both through increased expression of insulin receptor substrate-2 (52, 53) and by suppressing expression of tribble-3 protein (53). Other studies indicate that p70S6K can suppress the

![FIGURE 7. Phosphorylation of 4E-BP1 and Inhibits mTOR Signaling](image-url)

**FIGURE 7.** Phosphorylation of 4E-BP1 and Inhibits mTOR Signaling.
activation of Akt, apparently by phosphorylating insulin receptor substrate-1, which can suppress its function and promote its degradation (54, 55, 56). In the present study, reduced phosphorylation and activation of p70S6K might also be expected to promote increased phosphorylation of Akt. However, other studies indicate that the phosphorylation of Ser-473 in Akt is mediated by a complex of mTOR and Rictor (56, 57). Because the activation of FOXO1 in C2C12 myotubes and skeletal muscle reduced total mTOR levels, it is possible that decreased abundance of mTOR protein might prevent the phosphorylation of Akt from being increased, despite reduced activation of p70S6K. In any case, the fact that the phosphorylation of Akt is not suppressed by the activation of FOXO1 in C2C12 myotubes suggests that the ability of FOXO1 to suppress signaling by mTOR/Raptor signaling to downstream targets (4E-BP1 and p70S6K) is not due simply to a reduction in Akt activity and reflects direct effects of FOXO1 on this pathway downstream from Akt.

Our in vitro data were mirrored in FOXO1/+/H11001/H11001 transgenic mice, suggesting that FOXO1 is a possible therapeutic target for conditions where muscle cachexia is manifest. As previously reported (39), FOXO1+/+/ mice are characterized by smaller muscles. Given the well known effect of FOXO proteins on atrogin-1 expression, this skeletal muscle phenotype has been thought to be due to the up-regulation of catabolic pathways. The data reported here demonstrate that FOXO1+/+/ mice display markedly reduced mTOR signaling (Fig. 7b), indicating that the skeletal muscle phenotype of these mice may also be mediated, at least in part, by impaired anabolism. However, we Translation of mRNA is a complex (58) process and consists of three phases: initiation, elongation, and termination (58, 59). We have only investigated one aspect of translational control, regulation of initiation by FOXO1, in these studies, and we have not investigated the downstream phases, elongation and termination. However, given the results described here where FOXO1 induces changes in a major regulator of initiation, further studies probing the effect of FOXO1 on elongation and termination are warranted.

Although the exact physiological significance of our data remains to be determined, it is tempting to speculate that the role for FOXO1 is one of providing contextual growth inhibition. For example, when nutrients and insulin/growth factors are available, FOXO1 is transcriptionally inactive, and growth pathways predominate (Fig. 9a). Conversely, when nutrients are depleted and insulin/growth factor levels are low, or under circumstances where insulin signaling is impaired in the presence of adequate nutrients, such as insulin resistance (40), transcriptionally active FOXO proteins may function both to promote the function of catabolic pathways that mobilize nutrient stores, including proteolysis, and to suppress the function of anabolic pathways that promote growth, including protein synthesis (Fig. 9b). This concept is consistent with recent studies in the liver, where FOXO1 exerts positive effects on the activity of metabolic

FIGURE 9. Regulation of mTOR activity and protein anabolism by FOXO proteins. Under conditions of nutrient and growth factor activity (a), Akt phosphorylates and activates p70S6K, which also phosphorylates 4E-BP1, allowing eIF4E to bind eIF4G and promote translation initiation of capped mRNAs. mTOR also phosphorylates p70S6K, which also promotes translation of protein. Transcriptionally active FOXO proteins (b) stimulate the expression of 4E-BP1, which displaces eIF4G from eIF4E. FOXO proteins also suppress the expression of mTOR and Raptor, limiting the phosphorylation of 4E-BP1 and p70S6kinase and incorporation of amino acids into protein.
pathways that are adaptive for periods of nutrient restriction (amino acid catabolism, glycerol transport, and gluconeogenesis) and negative effects on the expression of pathways involved in promoting anabolism in the fed state (glycolysis, the pentose phosphate shunt, lipogenesis, and sterol synthesis) (60). Recent studies suggest that FOXO1 may also interact with the mTOR signaling pathway in other ways (51). Together, these findings suggest that FOXO proteins play an important role in integrating the metabolic adaptation to changes in nutritional status in multiple target tissues and through effects on both anabolic and catabolic pathways.

In conclusion, these studies provide evidence that FOXO1 inhibits the function of anabolic pathways in skeletal muscle via increased expression and reduced phosphorylation of the transrepressor partner 4E-BP1 and impaired signaling via reductions in mTOR and Raptor abundance. FOXO1 may be an important therapeutic target for human diseases where anabolism is impaired.

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