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RESEARCH ARTICLE

Muscle Wasting: Cellular and Molecular Mechanisms

Ubiquitin E3 ligase Atrogin-1 protein is regulated via the rapamycin-sensitive mTOR-S6K1 signaling pathway in C2C12 muscle cells

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Abstract

Atrogin-1 and Muscle-specific RING finger protein 1 (MuRF1) are highly expressed in multiple conditions of skeletal muscle atrophy. The phosphoinositide 3-kinase (PI3K)/Akt/forkhead box (FoxO) signaling pathway is well known to regulate Atrogin-1 and MuRF1 gene expressions. However, Akt activation also activates the mechanistic target of rapamycin complex 1 (mTORC1), which induces skeletal muscle hypertrophy. Whether mTORC1-dependent signaling has a role in regulating Atrogin-1 and/or MuRF1 gene and protein expression is currently unclear. In this study, we showed that activation of insulin-mediated Akt signaling suppresses both Atrogin-1 and MuRF1 protein contents and that inhibition of Akt increases both Atrogin-1 and MuRF1 protein contents in C2C12 myotubes. Interestingly, inhibition of mTORC1 with a specific mTORC1 inhibitor, rapamycin, increased Atrogin-1, but not MuRF1, protein content. Furthermore, activation of AMP-activated protein kinase (AMPK), a negative regulator of the mTORC1 signaling pathway, also showed distinct time-dependent changes between Atrogin-1 and MuRF1 protein contents, suggesting differential regulatory mechanisms between Atrogin-1 and MuRF1 protein content. To further explore the downstream of mTORC1 signaling, we employed a specific S6K1 inhibitor, PF-4708671. We found that Atrogin-1 protein content was dose-dependently increased with PF-4708671 treatment, whereas MuRF1 protein content was decreased at 50 μM of PF-4708671 treatment. However, MuRF1 protein content was unexpectedly increased by PF-4708671 treatment for a longer period. Overall, our results indicate that Atrogin-1 and MuRF1 protein contents are regulated by different mechanisms, the downstream of Akt, and that Atrogin-1 protein content can be regulated by the rapamycin-sensitive mTOR-S6K1-dependent signaling pathway.

mTORC1; skeletal muscle; ubiquitin proteasome system

INTRODUCTION

Atrogin-1 (also known as Muscle atrophy F-box protein: MAFbx or *FBXO32*) and Muscle-specific RING finger protein 1 (MuRF1 or *TRIM63*) are muscle-specific E3 ligases, and their expression is highly associated with various skeletal muscle atrophic models (1, 2). A plethora of studies have confirmed that Atrogin-1 and MuRF1 mRNA expression are useful molecular biomarkers of skeletal muscle atrophy (3, 4). Although the phosphoinositide 3-kinase (PI3K)-Akt dependent signaling pathway is known to regulate Atrogin-1 and MuRF1 gene expression, the posttranslational processes (5, 6) that regulate protein content and stability of these two E3 ligases remain to be elucidated. Many studies have assumed that mRNA expressions implicitly reflect the corresponding changes of protein content, but in reality the expression levels of individual mRNA and its corresponding protein are indeed poorly correlated (7, 8). As protein is the final product executing gene function, direct measurement of protein content is therefore more relevant to biological functions (6, 9).

Studies have shown that treatment with IGF-1 or the introduction of constitutively active Akt prevents both Atrogin-1 and MuRF1 mRNA transcription in C2C12 myotubes (10, 11). Mechanistically, Akt phosphorylates the transcription factor forkhead box (FoxO) to induce FoxO nuclear exclusion, which downregulates FoxO-dependent gene transcription (12). A study has confirmed that overexpression of FoxO3a in mouse skeletal muscle is able to induce Atrogin-1 mRNA expression and an atrophic phenotype (11). In contrast, siRNA knockdown of FoxO1–3 inhibits Atrogin-1 promoter activity during fasting-induced muscle atrophy (11). These findings have evidenced that the Akt-FoxO axis is critical for regulating Atrogin-1 and MuRF1 mRNA transcription. However, some contradictory results have been reported. For example, a

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study showed that deletion of Akt1 or Akt2 did not alter Atrogin-1 mRNA and protein expressions in mouse skeletal muscle (13), suggesting that there may be a redundancy between Akt isoforms. Furthermore, Atrogin-1 and MuRF1 mRNA expression, including Atrogin-1 protein content, were shown to be unchanged in aging-induced muscle atrophy, despite the elevation of Akt activity and FoxO3a phosphorylation (14). Also, Smad3 and NF-κB have been reported to regulate Atrogin-1 and/or MuRF1 expression, depending on the atrophy model (3, 15). These contradictory findings raise the question of whether the Akt signaling pathway is a primary pathway regulating Atrogin-1 and MuRF1 expression.

Mechanistic target of rapamycin complex 1 (mTORC1) plays an important role in regulating protein synthesis and the autophagy-lysosome system (16), and its activation has been well associated with skeletal muscle hypertrophy (17, 18). Surprisingly, the involvement of mTORC1 in regulating muscle protein degradation has not been well investigated. A recent study led by Zhao et al. (19) suggested that mTOR (including mTORC1 and mTORC2) is involved in the regulation of proteasome-mediated protein degradation in C2C12 myotubes. Their previous study showed that treatment with rapamycin, a specific mTORC1 inhibitor, increases protein degradation in C2C12 myotubes (20), which led the authors to suggest that mTORC1 may contribute to control protein degradation via the ubiquitin proteasome system. Furthermore, there is also evidence suggesting that Atrogin-1 and MuRF1 mRNA expressions are regulated by distinct signaling mechanisms. Sacheck et al. (20) showed that rapamycin treatment increases Atrogin-1, but not MuRF1, mRNA expression. However, proof at the protein level is currently lacking, and such information is needed to better understand what signaling mechanisms are involved in controlling Atrogin-1 and MuRF1 protein content, which essentially execute the enzymatic ubiquitin E3 ligase activity.

The present study therefore aimed to investigate whether the downstream targets of Akt, such as the mTORC1 and S6K1 signaling pathway, are involved in controlling Atrogin-1 and MuRF1 protein content in C2C12 myotubes. Using small molecules inhibiting mTORC1 or S6K1 activity, we demonstrated that Atrogin-1, but not MuRF1, protein content is regulated in the rapamycin-sensitive mTOR and S6K1-dependent signaling pathways. Our results suggest that the role of Akt-FoxO is not the only signaling pathway regulating Atrogin-1 protein content and that the downstream of Akt, such as the rapamycin-sensitive mTOR and S6K1-dependent signaling pathways, are involved in regulating Atrogin-1 protein content in skeletal muscle.

MATERIALS AND METHODS

C2C12 Cell Culture

Mouse skeletal muscle C2C12 myoblast cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were seeded and maintained in Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific, Loughborough, UK; 31966021) containing GlutaMAX, 25 mM glucose, and 1 mM sodium pyruvate, supplemented with 10% (vol/vol) HyClone fetal bovine serum (FBS; Fisher Scientific, Loughborough, UK; SV30180.03) and 1% (vol/vol) penicillin-streptomycin (10,000 U/mL-μg/mL, Thermo Fisher Scientific, Loughborough, UK; 15140122). Myoblasts were seeded onto six-well multidishes (Greiner Bio-One; 657160), and when confluence was reached at 90% myoblasts were differentiated into myotubes for 6 days in DMEM supplemented with 2% (vol/vol) horse serum (Sigma-Aldrich, Cambridge, UK; H1270) and 1% (vol/vol) penicillin-streptomycin. The medium was changed every 48 h. Cultures were maintained in a humidified incubator at 37°C with an atmosphere of 5% CO_2 and 95% air.

Drug Reconstitution and Cell Treatment

Akt1/2/3 inhibitor MK-2206 dihydrochloride (ApexBio; A3010), Rapamycin (Sigma-Aldrich; 553211), adenosine monophosphate (AMP)-activated protein kinase (AMPK) activator 991 (AOBIOUS, Gloucester, MA; AOB8150), and S6K1 inhibitor PF-4708671 (Sigma-Aldrich, Poole, UK; 559278) were prepared as 10 mM, 100 μM, 20 mM, and 50 mM stocks in DMSO, respectively, and treatment conditions are described in figure legends. Insulin solution human was obtained from Sigma (Sigma-Aldrich, Poole, UK; I9278). The treatment conditions were determined based on our and other previous studies: MK-2206 (10 μM) (21), Rapamycin (100 nM) (22), AMPK activator 991 (20 μM) (23), S6K1 inhibitor PF-4708671 (24), and insulin (100 nM) (25).

Cell Lysis

Cells were lysed in ice-cold sucrose lysis buffer containing 250 mM sucrose, 50 mM Tris-base (pH 7.5), 50 mM sodium fluoride, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 1 mM sodium orthovanadate, 1× complete Mini EDTA-free protease inhibitor cocktail (Roche), 1% Triton X-100, and 100 mM 2-chloroacetamide. Cell lysates were centrifuged for 15 min at 13,000 *g* and 4°C, and the supernatant was stored at −80°C before analysis for total protein concentrations with the Bradford protein assay (Thermo Fisher Scientific, Loughborough, UK; 23200). Protein in each sample was quantified from a standard curve with BSA standards (Thermo Fisher Scientific, Loughborough, UK; 23209).

Western Blot

Cell lysates were prepared in 1× NuPAGE LDS sample buffer (Invitrogen; NP0008) containing 2-mercaptoethanol (final concentration 1.5%) and left to denature overnight at room temperature. Prepared cell lysates (10–15 μg of total protein) were loaded into 8% or 10% Bis/Tris gels before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run in 1× MOPS buffer for ~60 min at 140 V. Proteins were transferred onto 0.2-μm polyvinylidene fluoride (PVDF) membranes (Millipore, Watford, UK) for 1 h at 100 V. Membranes were blocked in 5% milk diluted in Tris-buffered saline-Tween 20 (TBS-T): 137 mM sodium chloride, 20 mM Tris-base pH 7.5, 0.1% Tween 20 for 1 h. After blocking, membranes were washed three times for 5 min in TBS-T before being incubated overnight at 4°C with the appropriate primary antibodies (Table 1). Membranes were washed three

Table 1. *Antibodies for Western blot*

Antibodies	Dilution	Source	Identifier
Phospho-Ser473 Akt	1:1000	Cell Signaling Technology	Cat. No. 4060
Phospho-Thr308 Akt	1:1,000	Cell Signaling Technology	Cat. No. 2965
Akt	1:1,000	Cell Signaling Technology	Cat. No. 4691
Phospho-Thr389 p70 S6 kinase	1:1,000	Cell Signaling Technology	Cat. No. 9234
p70 S6 kinase	1:1,000	Cell Signaling Technology	Cat. No. 2708
Phospho-Ser240/244 S6 ribosomal protein	1:8,000	Cell Signaling Technology	Cat. No. 5364
S6 ribosomal protein	1:8,000	Cell Signaling Technology	Cat. No. 2217
Phospho-Thr172 AMPKα	1:1,000	Cell Signaling Technology	Cat. No. 2535
AMPKα	1:1,000	Cell Signaling Technology	Cat. No. 2532
Atrogin-1	1:1,000	ECM Biosciences	Cat. No. AM3141
MuRF1	1:1,000	Santa Cruz	Cat. No. SC-398608
Phospho-Ser555 ULK1	1:1,000	Cell Signaling Technology	Cat. No. 5869
ULK1	1:1,000	Cell Signaling Technology	Cat. No. 4773
Phospho-FoxO1 (Thr24)/FoxO3a (Thr32)	1:750	Cell Signaling Technology	Cat. No. 9464
FoxO1	1:750	Cell Signaling Technology	Cat. No. 2880
Vinculin	1:2,000	Abcam	Cat. No. Ab129002
Anti-mouse IgG, HRP-linked antibody	1:10,000	Cell Signaling Technology	Cat. No. 7076
Anti-rabbit IgG, HRP-linked antibody	1:10,000	Cell Signaling Technology	Cat. No. 7074
Anti-rat IgG, HRP-linked antibody	1:10,000	Cell Signaling Technology	Cat. No. 7077

AMPK, adenosine monophosphate activated protein kinase; HRP, horseradish peroxidase.

times for 5 min in TBS-T before incubation in horseradish peroxidase-conjugated secondary antibodies (Table 1) at room temperature for 1 h. Membranes were washed a further three times in TBS-T before antibody detection with an enhanced chemiluminescence horseradish peroxidase substrate detection kit (Millipore, Watford, UK). Imaging was undertaken with a G:BOX Chemi-XR5 (Syngene, Cambridge, UK). Band intensities were quantified with ImageJ/Fiji (NIH, Bethesda, MD). Vinculin was used as a loading control, and all data (both phosphorylation and total protein content) were normalized to Vinculin. Phosphorylation data were further calculated by the expression of phosphorylated protein divided by the expression of nonphosphorylated total protein.

Statistical Analysis

The statistical analyses were performed with Prism version 8.1.2 (GraphPad Software, San Diego, CA, www.graphpad.com). Values of $P < 0.05$ were considered statistically significant. For time course and dose-response experiments, a one-way repeated-measures analysis of variance (ANOVA) was performed with Dunnett's post hoc test compared with control (CON). Data are presented as means ± SD. All experiments were performed three times in duplication, and the average value was taken in each experiment ($n = 3$).

RESULTS

Evidence of Insulin/Akt/FoxO Signaling Pathway Modulating Atrogin-1 and MuRF1 Protein Content

We first confirmed whether the insulin/Akt/FoxO signaling pathway is sufficient to modulate both Atrogin-1 and MuRF1 protein content in C2C12 myotubes. Using an allosteric Akt inhibitor, MK-2206, we showed that Atrogin-1 protein content was significantly increased at 3 h, 6 h, and 9 h after the treatment of 10 μM MK-2206 (Fig. 1*B*). MuRF1 protein content was also significantly increased at 6 h and 9 h after the treatment of MK-2206 (Fig. 1*C*). In line with a previous study (21), Akt phosphorylation at Ser^{473} and Thr^{308} was completely abolished over the course of 9-h treatment with MK-2206 (Fig. 1*A*). We also confirmed that inhibition of Akt activity prevents FoxO1 and FoxO3a phosphorylation and reduces S6K1 and rpS6 phosphorylation (Fig. 1*A*).

Atrogin-1 protein content was significantly decreased at 3 h, 6 h, and 9 h after the treatment of 100 nM insulin stimulation (Fig. 1*B*). MuRF1 protein content was also significantly decreased at 6 h and 9 h after insulin treatment (Fig. 1*C*). As expected, insulin stimulated Akt phosphorylation at both Ser^{473} and Thr^{308} sites. The enhanced Akt activity was also confirmed by the increases in its downstream targets, such as FoxO1, FoxO3a, S6K1, and rpS6 phosphorylation (Fig. 1*A*).

Atrogin-1, but Not MuRF1, Protein Content Is Increased by Rapamycin-Sensitive mTOR Inhibition

Acute treatment with Rapamycin can specifically inhibit mTORC1 activity without directly affecting mTORC2 activity, but a long-term treatment (≥24 h) is known to inhibit mTORC2 activity (22). Therefore, we have limited the treatment time of small molecules to ≤9 h. Interestingly, Atrogin-1 protein content was increased at 3 h, 6 h, and 9 h after treatment with 100 nM rapamycin (Fig. 2*B*). Although Atrogin-1 protein content was increased, MuRF1 protein content was decreased at 9 h after rapamycin treatment (Fig. 2*C*). As anticipated, rapamycin treatment completely inhibited S6K1 and rpS6 phosphorylation (Fig. 2*A*) without inducing a significant change in Akt phosphorylation at Ser^{473} (Fig. 2*D*; $P = 0.56$) and Thr^{308} (Fig. 2*E*; $P = 0.55$). Despite unchanged activity of Akt, FoxO1 and FoxO3a phosphorylation (Fig. 2*F*) was increased at 9 h after rapamycin treatment, suggesting that Akt is not the sole kinase regulating FoxO phosphorylation, but an activity of phosphatases cannot also be ruled out.

Distinct Time-Dependent Changes of Atrogin-1 and MuRF1 Protein Content after AMPK Activation

AMPK activation is known to inhibit mTORC1 activity (26) via the phosphorylation of tuberous sclerosis complex

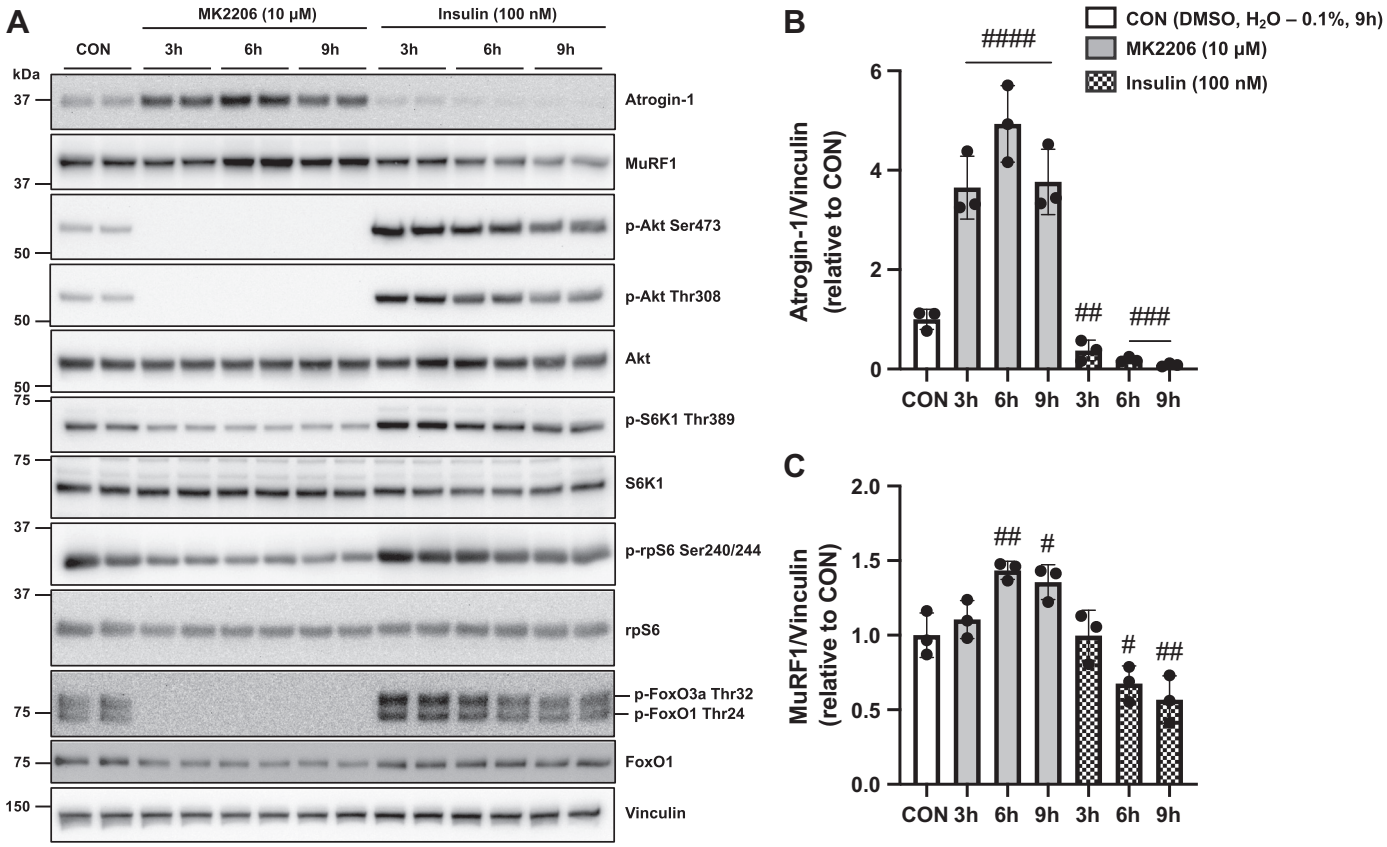

Figure 1. Insulin/Akt signaling pathway is sufficient to modulate Atrogin-1 and Muscle-specific RING finger protein 1 (MuRF1) protein contents in C2C12 myotubes. C2C12 myotubes were treated with DMSO (0.1%, 9 h) as a vehicle control (CON), MK2206 (10 μM), or insulin (100 nM) for 3, 6, or 9 h. Lysates were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. *A*: representative images from 1 of 3 independent experiments in duplication. *B* and *C*: quantification of Atrogin-1 (*B*) and MuRF1 (*C*). Data are expressed as mean ± SD (n = 3) fold changes relative to CON. One-way repeated-measures ANOVA with Dunnett's post hoc test, #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, ####$P < 0.0001$ compared with CON.

2 (TSC2) (27) and Raptor (28). To further investigate the role of mTORC1 on the regulation of Atrogin-1 and MuRF1 protein content, we used a direct AMPK activator, 991, to increase AMPK activity in C2C12 myotubes (26, 29). Interestingly, Atrogin-1 protein content was increased at 3 h (Fig. 3*B*). In contrast, MuRF1 protein content had obviously delayed increase at 9 h after 991 treatment (Fig. 3*C*). These results again suggest that Atrogin-1 and MuRF1 protein contents are regulated by distinct signaling mechanisms. As expected, ULK1 phosphorylation at Ser^{555} was increased by the treatment with 991 (Fig. 3*A*) (30), and the inhibition of mTORC1 activity was confirmed by showing a decrease in S6K1 and rpS6 phosphorylation (Fig. 3*A*). Whereas Akt phosphorylation at Ser^{473} (Fig. 3*D*) was decreased at 9 h, Akt phosphorylation at Thr^{308} (Fig. 3*E*; ANOVA P = 0.02 with no statistical significance in Dunnett's post hoc test) and FoxO1 and FoxO3a phosphorylation (Fig. 3*F*; P = 0.22) were not altered. These data highlight that Atrogin-1 protein content is not fully dependent on Akt-FoxO signaling axis.

Atrogin-1 Protein Content Is Increased by S6K1 Inhibition

To further explore the distinct mechanisms that regulate Atrogin-1 and MuRF1 protein content, we asked whether mTORC1 downstream, such as S6K1, is involved in regulating Atrogin-1 or MuRF1 protein content. Using a specific S6K1 inhibitor (24), we showed that Atrogin-1 (Fig. 4*B*) protein content was increased in a dose-response manner, where significant increases were seen with treatment from 20 to 50 μM PF-4708671. Instead of increasing, MuRF1 protein content was indeed decreased at 50 μM (Fig. 4*C*). Inhibition of S6K1 was confirmed by the observation of reduced rpS6 phosphorylation (Fig. 4*A*). As expected, the phosphorylation of S6K1 was increased by the treatment with PF-4708671 (24) (Fig. 4*A*). Whereas Akt phosphorylation at Thr^{308} (Fig. 4*E*) was increased at 40 μM and 50 μM, Akt phosphorylation at Ser^{473} (Fig. 4*D*, P = 0.12) and FoxO1 and FoxO3a phosphorylation (Fig. 4*F*; P = 0.4) were not altered. Next, we performed Pearson's correlation coefficient to identify the relationship between p-rpS6$^{Ser240/244}$/rpS6 and Atrogin-1 or MuRF1 by plotting the dose-response data (Fig. 4*G*). Interestingly, a negative correlation was observed between p-rpS6$^{Ser240/244}$/rpS6 and Atrogin-1 (r = −0.83, $P < 0.0001$), whereas no significant association was observed between p-rpS6$^{Ser240/244}$/rpS6 and MuRF1 (r = 0.31, P = 0.2). The 95% confidence interval for a correlation coefficient is −0.9343 to −0.5908 and −0.1794 to 0.6809 for Atrogin-1 and MuRF1, respectively. Simple linear regression shows that the equation is $Y = -1.013 \times X + 2.148$ and $Y = 0.2655 \times X + 0.7792$ for Atrogin-

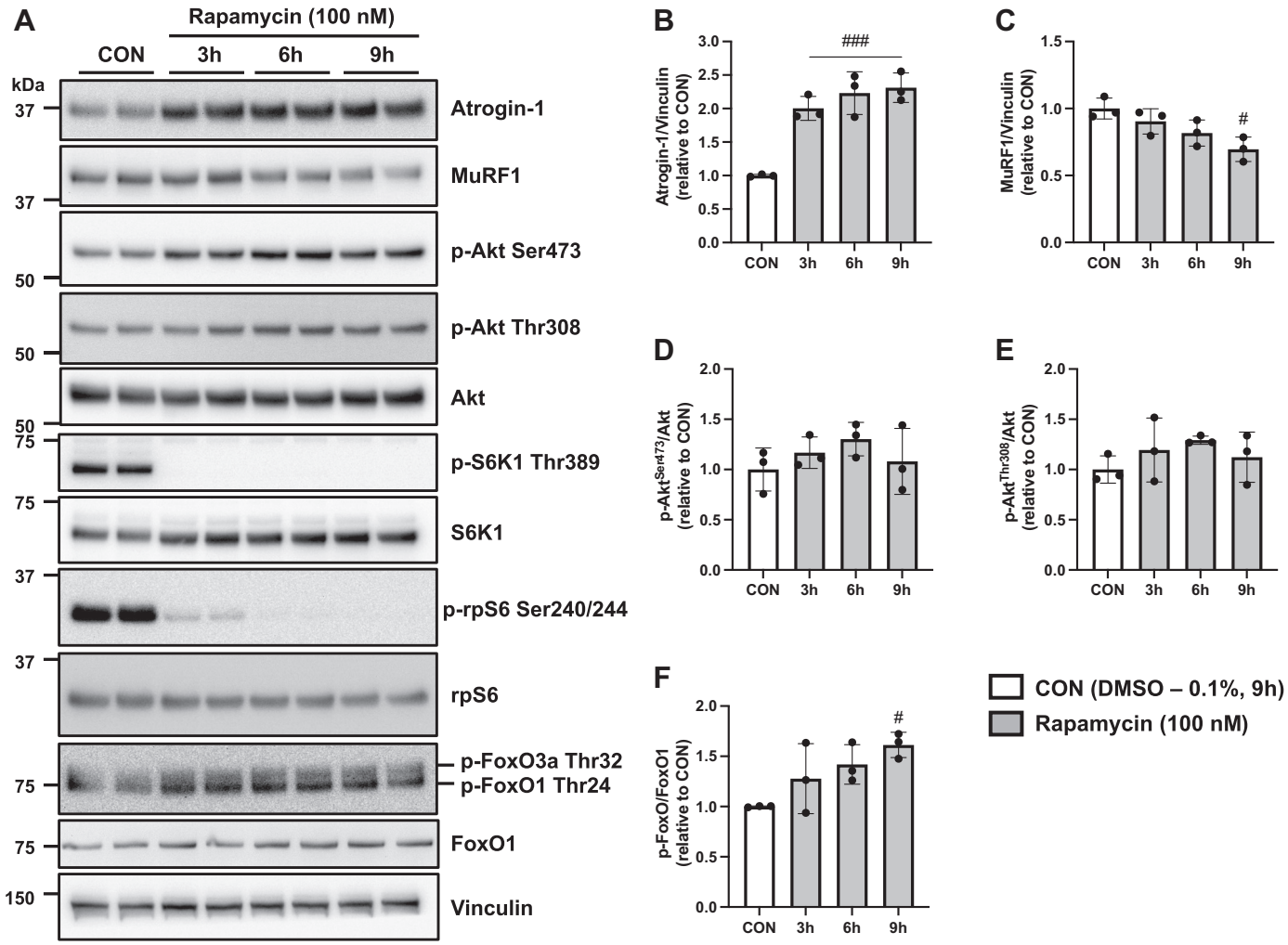

Figure 2. Rapamycin-sensitive mechanistic target of rapamycin (mTOR) inhibition increases Atrogin-1, but not Muscle-specific RING finger protein 1 (MuRF1), protein content in C2C12 myotubes. C2C12 myotubes were treated with DMSO (0.1%, 9 h) as a vehicle control (CON) or Rapamycin (100 nM) for 3, 6, or 9 h. Lysates were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. *A*: representative images from 1 of 3 independent experiments in duplication. *B–F*: quantification of Atrogin-1 (*B*), MuRF1 (*C*), p-Akt Ser473 (*D*), p-Akt Thr308 (*E*), and p-FoxO (*F*). Data are expressed as mean ± SD (n = 3) fold changes relative to CON. One-way repeated-measures ANOVA with Dunnett's post hoc test, #$P < 0.05$, ###$P < 0.001$ compared with CON.

1 and MuRF1, respectively. This suggests that the slope is different between Atrogin-1 and MuRF1.

To confirm that S6K1 inhibition increases Atrogin-1, but not MuRF1, protein content, we performed a time course experiment using 30 μM PF-4708671 for up to 24 h (Fig. 5*A*). In line with Fig. 4*B*, the protein content of Atrogin-1 was increased 3 h after PF-4708671 treatment (Fig. 5*B*). Based on PF-4708671 dose-response data in Fig. 4*C*, increases in MuRF1 protein content at 6 h and 9 h after PF-4708671 treatment were unexpected (Fig. 5*C*). Phosphorylation at Akt^{473} ($P = 0.17$) and Thr^{308} ($P = 0.29$) and FoxO1 and FoxO3a phosphorylation ($P = 0.22$) remained unchanged over the course of 24-h treatment with PF-4708671.

DISCUSSION

Here, we have made use of small molecules to evaluate some key signaling pathways that modulate Atrogin-1 and MuRF1 protein contents in C2C12 myotubes. In accordance with previous studies, we confirmed that the insulin/Akt/FoxO pathway is sufficient to modulate both Atrogin-1 and MuRF1 protein contents, which agrees with the previous studies measuring mRNA transcription (10, 11, 20, 31). Further investigation revealed that Atrogin-1, but not MuRF1, protein content is predominantly increased when the rapamycin-sensitive mTOR signaling pathway is inhibited. More interestingly, our study is the first to reveal that Atrogin-1 protein content can be regulated by a S6K1-dependent signaling pathway. These findings indicate that Atrogin-1 protein content can be regulated via Akt-FoxO-independent mechanisms.

Inactivation of the PI3K/Akt/FoxO signaling pathway is well known as an "atrophic signal" that increases both MuRF1 and Atrogin-1 mRNA expression (32). However, few studies have investigated whether MuRF1 and Atrogin-1 protein contents are regulated in accordance with their gene/mRNA expressions. The present study confirmed that protein contents of both Atrogin-1 and MuRF1 were suppressed

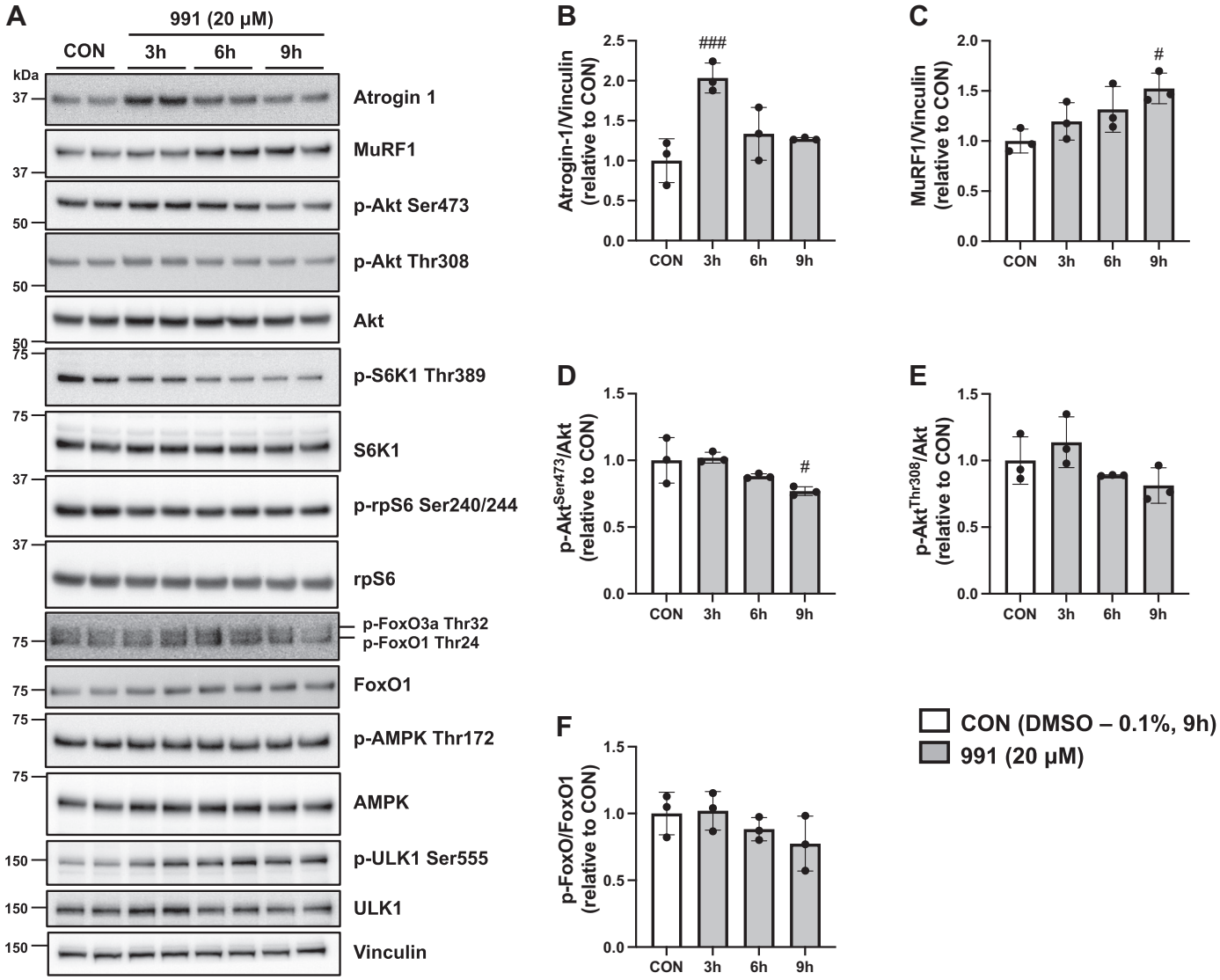

Figure 3. Inhibition of the mechanistic target of rapamycin complex 1 (mTORC1) pathway by adenosine monophosphate-activated protein kinase (AMPK) activator 991 on Atrogin-1 and Muscle-specific RING finger protein 1 (MuRF1) protein contents in C2C12 myotubes. C2C12 myotubes were treated with DMSO (0.1%, 9 h) as a vehicle control (CON) or 991 (20 μM) for 3, 6, or 9 h. Lysates were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. *A*: representative images from 1 of 3 independent experiments in duplication. *B–F*: quantification of Atrogin-1 (*B*), MuRF1 (*C*), p-Akt Ser473 (*D*), p-Akt Thr308 (*E*), and p-FoxO (*F*). Data are expressed as mean ± SD (n = 3) fold changes relative to CON. One-way repeated-measures ANOVA with Dunnett's post hoc test, $\#P < 0.05$, $\#\#\#P < 0.001$ compared with CON.

by insulin, whereas Atrogin-1 and MuRF1 protein contents were upregulated by the treatment of MK-2206 (Akt1/2/3 inhibitor). These findings are consistent with the mRNA expressions investigated by previous studies (10, 11, 20, 31).

It was unexpected to see that Atrogin-1 protein content was increased after 3-h treatment with rapamycin, whereas MuRF1 protein content was decreased after 9-h treatment with rapamycin (Fig. 2). These data indicate that inhibition of the rapamycin-sensitive mTOR signaling pathway can enhance Atrogin-1, but not MuRF1, protein content. However, this is indeed consistent with a previous study that reported that inhibition of the rapamycin-sensitive mTOR signaling pathway increases Atrogin-1, but not MuRF1, mRNA expression (20). Our findings strengthened the previous evidence of mRNA data (20) by showing that inhibition of the rapamycin-sensitive mTOR-S6K1 signaling pathway also induces an increase in Atrogin-1 protein content. We also used AMPK activator 991 to reduce mTORC1 activity, as AMPK activation is known to inhibit mTORC1 activity (26) via the phosphorylation of tuberous sclerosis complex 2 (TSC2) (27) and Raptor (28). However, AMPK possibly modulated Atrogin-1 and/or MuRF1 protein content via mTORC1-independent mechanisms. For example, AMPK is known to phosphorylate FoxO3 at different amino acid residues and regulate FoxO3 transcriptional activity without affecting cellular localization (33). Thus, the AMPK-FoxO axis can also participate in modulating Atrogin-1 and MuRF1 protein content. The distinct time course changes of Atrogin-1 and MuRF1 protein content (Figs. 2 and 3) support that these proteins are controlled by distinct signaling mechanisms.

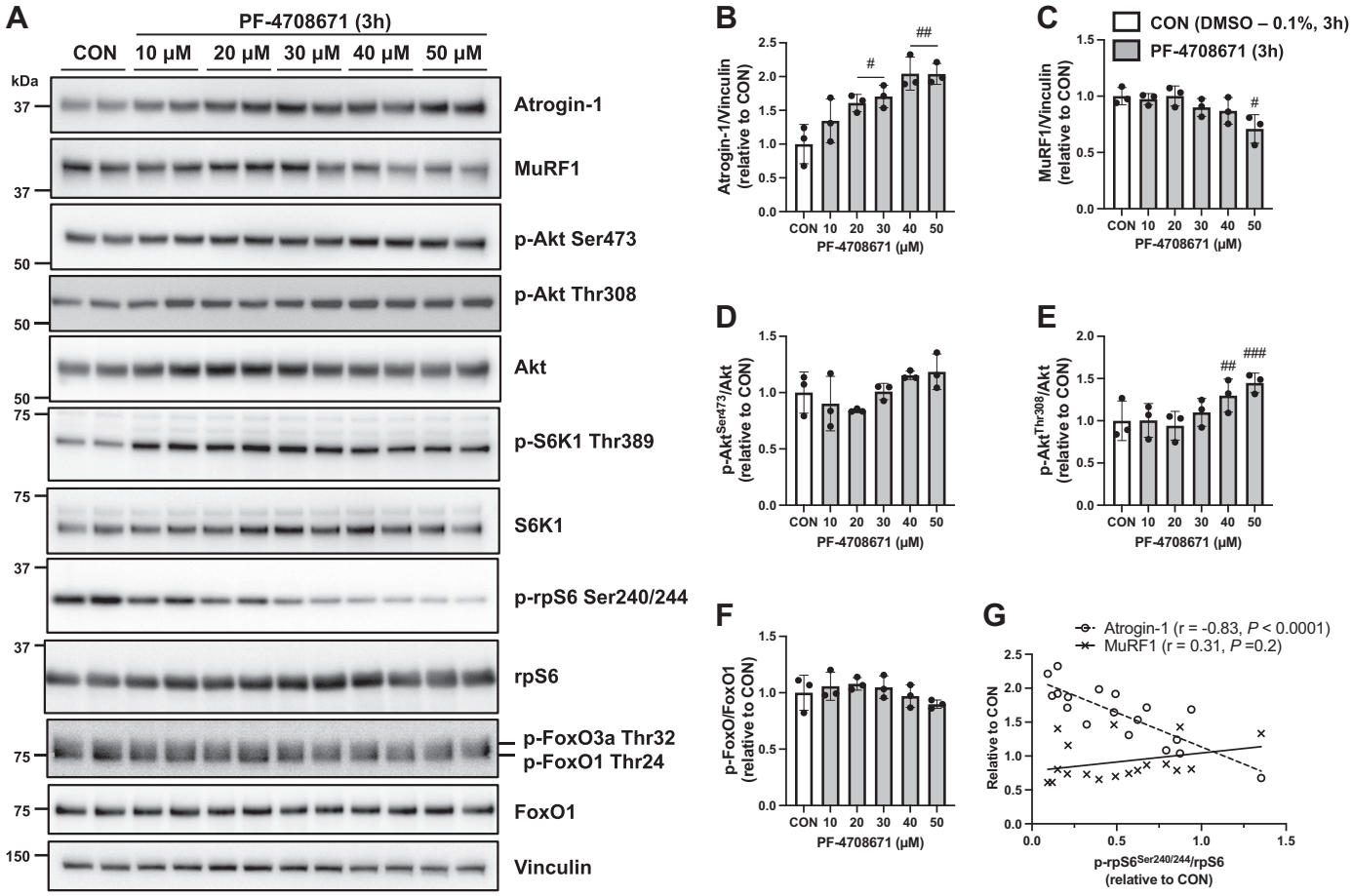

Figure 4. A dose-response effect of S6K1 inhibitor on Atrogin-1 and Muscle-specific RING finger protein 1 (MuRF1) protein contents in C2C12 myotubes. C2C12 myotubes were treated with DMSO (0.1%, 3 h) as a vehicle control (CON) or PF-4708671 at the indicated doses for 3 h. Lysates were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. *A*: representative images of 3 independent experiments in duplication. *B–F*: quantification of Atrogin-1 (*B*), MuRF1 (*C*), p-Akt Ser473 (*D*), p-Akt Thr308 (*E*), and p-FoxO (*F*). Data are expressed as mean ± SD (n = 3) fold changes relative to CON. One-way repeated-measures ANOVA with Dunnett's post hoc test, #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ compared with CON. *G*: Pearson's correlation coefficient to identify the association between p-rpS6 Ser240/244/rpS6 and Atrogin-1 or MuRF1. The 95% confidence interval for a correlation coefficient is −0.9343 to −0.5908 and −0.1794 to 0.6809 for Atrogin-1 and MuRF1, respectively. Simple linear regression shows that the equation is Y = −1.013 × X + 2.148 and Y = 0.2655 × X + 0.7792 for Atrogin-1 and MuRF1, respectively.

The most interesting discoveries in the present study are that Atrogin-1 and MuRF1 protein contents can be regulated by different mechanisms and that Atrogin-1 protein content is regulated by the rapamycin-sensitive mTOR-S6K1-dependent signaling pathway. In the present study, the phosphorylation of FoxO3a at Thr^{32} and FoxO1 at Thr^{24} was indeed increased after rapamycin treatment. In our study, Atrogin-1 was increased after rapamycin or PF-4708671 treatment, whereas phosphorylation of Akt at Thr^{308} was increased with PF-4708671 dose-response treatment and FoxO3a at Thr^{32} and FoxO1 at Thr^{24} were increased after rapamycin treatment. This suggests that the rapamycin-sensitive mTOR-S6K1 signaling pathway might be dominant over Akt in regulating Atrogin-1 protein content. This also suggests that FoxOs are not the only factor regulating Atrogin-1 protein content. In contrast, the changes of MuRF1 protein content (Figs. 1*C*–4*C*) seems to follow the changes of Akt-FoxO signaling (Fig. 1*A*, Fig. 2, *D–F*, Fig. 3, *D–F*, and Fig. 4, *D–F*). We reason that an increase in Akt phosphorylation after PF-4708671 treatment might be due to the suppression of a negative feedback loop to insulin receptor substrate 1 (IRS-1). S6K1 has been known to control a feedback loop that inhibits the PI3K/Akt/mTORC1 pathway (Fig. 6). It was reported that when PI3K/Akt/mTORC1 pathway is hyperactivated, S6K1 phosphorylates IRS-1 on multiple serine residues, which induces its degradation (34, 35). Furthermore, multiple transcription factors, including the NF-κB transcription factors CCAAT/enhancer-binding protein-β (C/EBPβ) and Smad3, can work cooperatively to regulate Atrogin-1 mRNA transcription (3, 15). Thus, complex cooperative mechanisms of transcription factors might have been involved in the distinct protein expression patterns between Atrogin-1 and MuRF1 protein content.

In supporting our finding that Atrogin-1 protein content is regulated by S6K1-dependent signaling, a previous study has also shown that the absence of S6K1 causes skeletal muscle atrophy in mice (36). In addition, Marabita et al. (37) reported that S6K1 is required for the prevention of protein aggregation during skeletal muscle hypertrophy in mice. These observations led us to hypothesize that increasing Atrogin-1 protein content is responsible for protein quality control when the rapamycin-sensitive mTOR-S6K1 signaling is

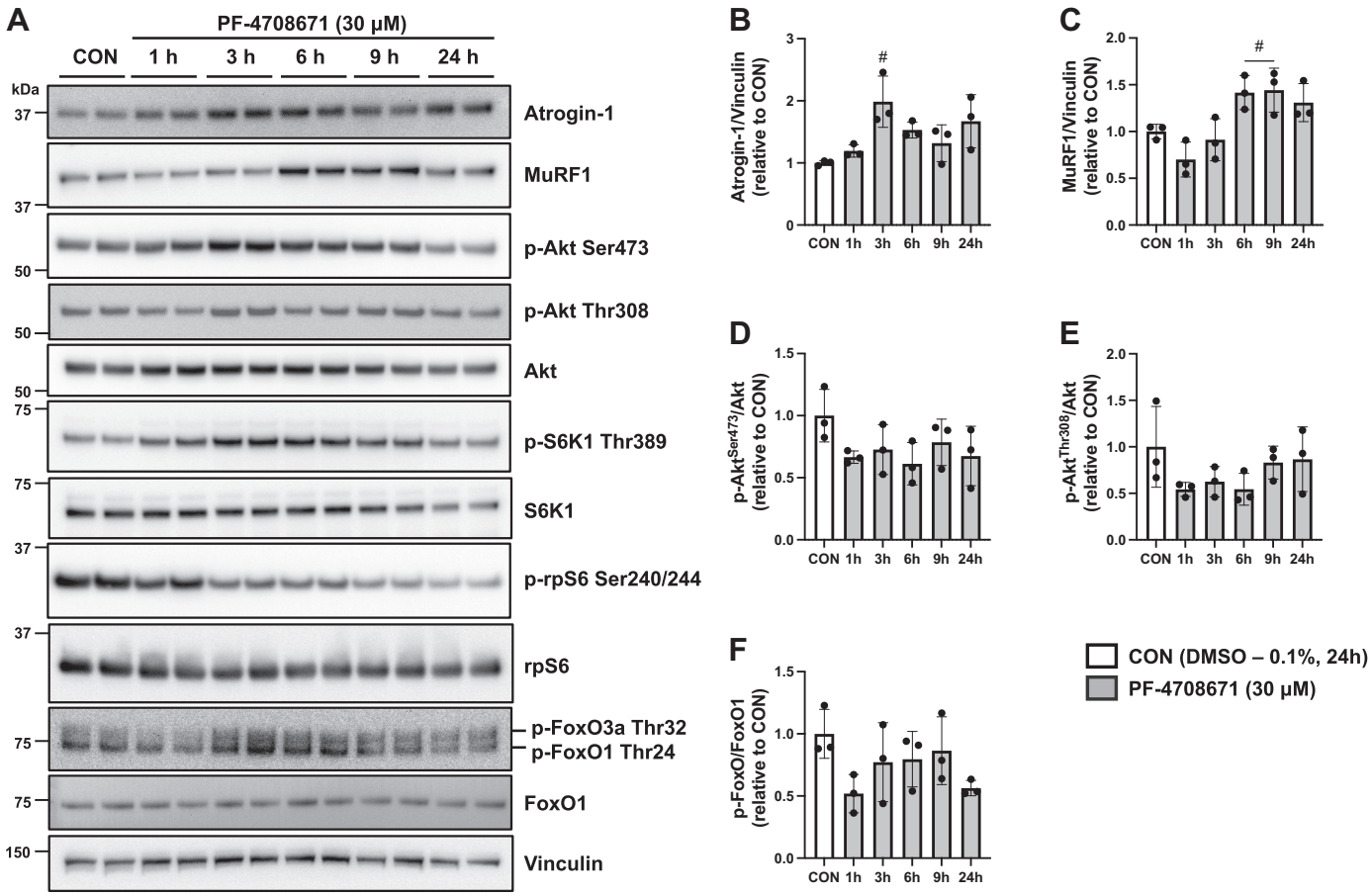

Figure 5. A time course effect of S6K1 inhibitor on Atrogin-1 and Muscle-specific RING finger protein 1 (MuRF1) protein contents in C2C12 myotubes. C2C12 myotubes were treated with DMSO (0.1%, 24 h) as a vehicle control (CON) or PF-4708671 (30 μM) for up to 24 h. Lysates were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. *A*: representative images from 1 of 3 experiments in duplication. *B*–*F*: quantification of Atrogin-1 (*B*), MuRF1 (*C*), p-Akt Ser473 (*D*), p-Akt Thr308 (*E*), and p-FoxO (*F*). Data are expressed as mean ± SD (n = 3) fold changes relative to CON. One-way repeated-measures ANOVA with Dunnett's post hoc test, #$P < 0.05$ compared with CON.

inhibited. However, future studies should confirm this hypothesis by investigating Atrogin-1 substrates and degradation mechanisms.

The mTORC1 signaling pathway has been shown as a positive regulator of skeletal muscle mass in several models of hypertrophy (17, 18, 38). In support of age-related muscle loss, studies have demonstrated that muscle contraction-induced activation of mTORC1 signaling is impaired with aging (39, 40). In contrast, constant activation of mTORC1 is known to cause myopathy but not hypertrophy (41). Moreover, a most recent study led by Joseph et al. (42) showed that the mTORC1 signaling pathway is indeed hyperactivated in age-related muscle loss, with a concomitant increase in both Atrogin-1 and MuRF1 mRNA expression in basal rat skeletal muscle. More interestingly, a partial inhibition of mTORC1 via RAD001 restored age-related skeletal muscle loss (42). RAD001 treatment also decreased MuRF1 mRNA expression, whereas Atrogin-1 mRNA was not altered in aging muscle. We cannot directly compare our findings to their results as they did not report Akt activity and information on MuRF1 and Atrogin-1 protein contents was not available. Nonetheless, these findings indicate the importance of fine-tuning the mTORC1 activity in maintaining skeletal muscle mass, and Atrogin-1 and/or MuRF1 may be responsible for this.

Although our findings suggest that Atrogin-1 and MuRF1 protein contents are regulated by different signaling mechanisms, future studies should determine what signaling molecules in the rapamycin-sensitive mTOR-S6K1 signaling cascade are responsible for regulating Atrogin-1 protein content (Fig. 6). With the use of our protein content data, other studies should also investigate whether E3 ligase activity of MuRF1 and/or Atrogin-1 is associated with their protein content, and thus a measurement of protein content can be used as a biomarker for E3 ligase activity or vice versa. The most recent study has indicated that the enzymatic activity of ubiquitin E3 ligases is particularly important in controlling skeletal muscle mass (43). The gene expressions of Atrogin-1 and MuRF1 are highly associated with almost all kinds of skeletal muscle atrophy (1–3). Genetic studies have also shown that knockout of Atrogin-1 or MuRF1 partially rescues denervation-induced skeletal muscle atrophy (1). However, the molecular mechanisms of how Atrogin-1 and MuRF1 contribute to skeletal muscle atrophy are still unclear (44). Thus, further understanding of the signaling mechanisms that control Atrogin-1 and MuRF1 protein content is required as an important step toward understanding the underlying mechanisms of skeletal

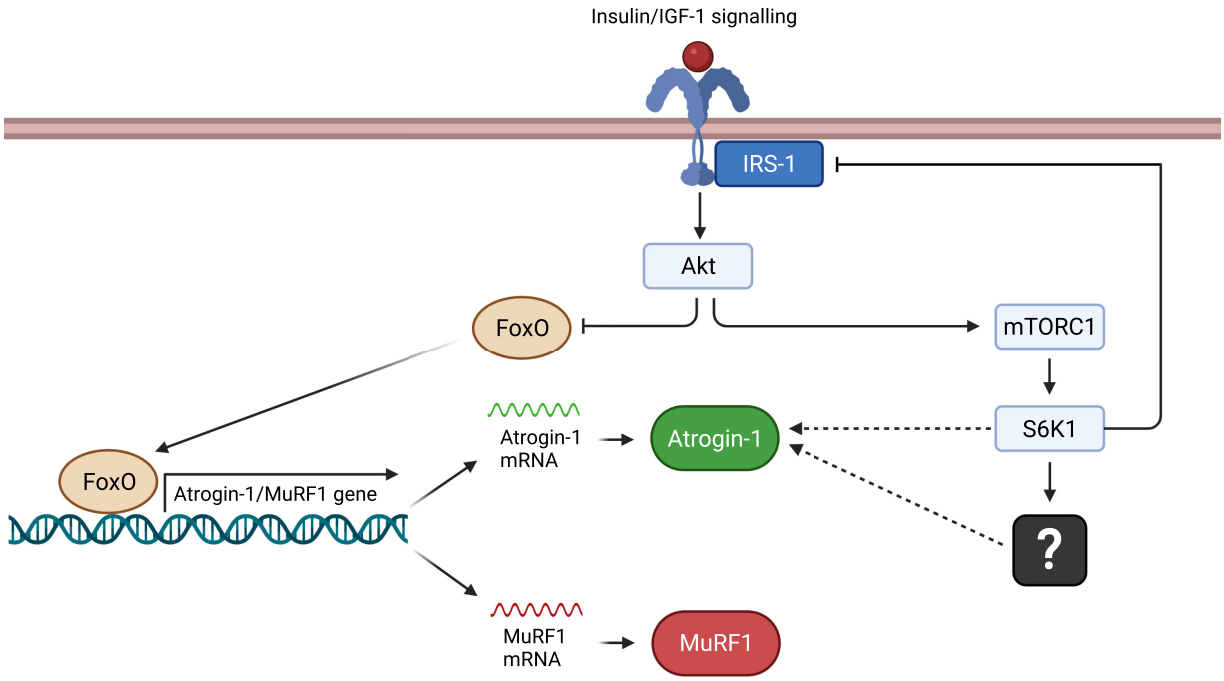

Figure 6. Atrogin-1 and Muscle-specific RING finger protein 1 (MuRF1) protein contents are differentially regulated in Akt and the rapamycin-sensitive mechanistic target of rapamycin (mTOR)-S6K1 signaling pathway. Insulin/IGF-1/Akt/FoxO signaling pathway is a predominant mechanism regulating Atrogin-1 and MuRF1 expression at both mRNA transcription and protein levels in skeletal muscle. Upon insulin or IGF-1 stimulation, the binding of their respective receptors triggers a signaling cascade to activate Akt. Akt phosphorylates and inhibits FoxO by preventing their localization to the nuclei, and thus FoxO remains in the cytoplasm. In catabolic conditions, FoxO is less phosphorylated and remains in the nuclei to promote Atrogin-1 and MuRF1 mRNA transcription, thereby increasing their protein content. When the rapamycin-sensitive mTOR or S6K1 signaling pathway is inhibited, one of the Akt downstream signalings can promote Atrogin-1, but not MuRF1, protein content via Akt-FoxO-independent mechanisms. The evidence indicates that Atrogin-1 and MuRF1 protein content are regulated by at least two different mechanisms. How rapamycin-sensitive mTOR and S6K-dependent signaling pathways regulate Atrogin-1 protein content remains undetermined. S6K1 has been known to control a feedback loop that inhibits the phosphoinositide 3-kinase (PI3K)/Akt/mTORC1 pathway when its signaling pathway is hyperactivated. Insulin receptor substrate-1 (IRS-1) on multiple serine residues is phosphorylated, which promotes its degradation. Image created with BioRender.com.

muscle atrophy and manipulating their functional E3 ligase activity. This information will also help identify new therapeutic targets to treat and/or prevent skeletal muscle atrophy. Moreover, identification of Atrogin-1 and MuRF1 substrates, their ubiquitylation profiles, and the fate of ubiquitylated substrates is required.

Conclusions

On the basis of the findings from the present study and the existing literature, we propose potential signaling mechanisms that may be involved in the regulation of Atrogin-1 and MuRF1 protein contents in skeletal muscle (Fig. 6). The anabolic Akt signaling, which can be activated by Insulin/IGF-1, is a critical upstream signal to modulate MuRF1 and Atrogin-1 at both gene and protein expression levels. However, Atrogin-1, but not MuRF1, protein content is increased when the rapamycin-sensitive mTOR-S6K1-dependent signaling pathway is inhibited. Thus, the regulatory mechanisms of controlling protein content are distinct between Atrogin-1 and MuRF1. Our study provides evidence for the first time that Atrogin-1 protein content can be regulated by the rapamycin-sensitive mTOR-S6K-dependent signaling pathway. Future studies should determine the underlying mechanisms by which rapamycin-sensitive mTOR-S6K1 signaling regulates Atrogin-1 protein content.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Y.N. and Y.-C.L. conceived and designed research; Y.N., J.C.-O., S.L., I.M., and P.D. performed experiments; Y.N., J.C.-O., S.L., I.M., and P.D. analyzed data; Y.N., L.H., and Y.-C.L. interpreted results of experiments; Y.N. prepared figures; Y.N. drafted manuscript; Y.N., I.M., L.H., and Y.-C.L. edited and revised manuscript; Y.N., J.C.-O.,

S.L., I.M., P.D., L.H., and Y.-C.L. approved final version of manuscript.

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