

## **Ubiquitin E3 ligases Atrogin-1 and MuRF1 protein contents are differentially regulated in the rapamycin-sensitive mTOR-S6K1 signaling pathway in C2C12 myotubes**

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Running Title: mTORC1/S6K1 and ubiquitin E3 ligase protein content

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1    **Abstract**

2    Muscle-specific ubiquitin E3 ligases, Atrogin-1 and MuRF1, are highly expressed in multiple  
3    conditions of skeletal muscle atrophy. The PI3K/Akt/FoxO signaling pathway is well known  
4    to regulate Atrogin-1 and MuRF1 gene expressions. Evidence supporting this is largely based  
5    on stimuli by insulin and IGF-1, that activate anabolic signaling, including Akt and Akt-  
6    dependent transcription factors. However, Akt activation also activates the mammalian target  
7    of rapamycin complex 1 (mTORC1) which induces skeletal muscle hypertrophy. However,  
8    whether mTORC1-dependent signaling has a role in regulating Atrogin-1 and/or MuRF1  
9    gene and protein expression is currently unclear. In this study, we confirmed that activation  
10   of insulin-mediated Akt signaling suppresses both Atrogin-1 and MuRF1 protein content and  
11   that inhibition of Akt increases both Atrogin-1 and MuRF1 protein content in C2C12  
12   myotubes. Interestingly, inhibition of mTORC1 using a specific mTORC1 inhibitor,  
13   rapamycin, increased Atrogin-1, but not MuRF1, protein content. Furthermore, activation of  
14   AMP-activated protein kinase (AMPK), a negative regulator of the mTORC1 signaling  
15   pathway, also showed distinct time-dependent changes between Atrogin-1 and MuRF1  
16   protein content, suggesting differential regulatory mechanisms between Atrogin-1 and  
17   MuRF1 protein content. To further explore the downstream of mTORC1 signaling, we  
18   employed a specific S6K1 inhibitor, PF-4708671, and found that Atrogin-1 protein content  
19   was dose-dependently increased with PF-4708671 treatment, whereas MuRF1 protein content  
20   was not significantly altered. Overall, our results indicate that Atrogin-1 and MuRF1 protein  
21   contents are regulated by different mechanisms, the downstream of Akt, and that Atrogin-1  
22   protein content can be regulated by rapamycin-sensitive mTOR-S6K1 dependent signaling  
23   pathway.

24

25    **KEY WORDS:** The ubiquitin proteasome system, skeletal muscle

26 **Abbreviations:**

27 MuRF1, Muscle-specific RING finger protein 1  
28 MAFbx, Muscle atrophy F-box protein  
29 PI3K, phosphoinositide 3-kinase  
30 FoxO, forkhead box  
31 mTORC, mechanistic target of rapamycin complex  
32 TSC2, tuberous sclerosis complex 2  
33 S6K, p70 ribosomal S6 kinase  
34 AMPK, adenosine monophosphate activated protein kinase  
35 SDS-PAGE, sodium dodecyl sulfate– polyacrylamide gel electrophoresis  
36 PVDF, polyvinylidene fluoride  
37 TBS-T, Tris-buffered saline Tween-20  
38 ANOVA, analysis of variance  
39 CON, control  
40

41 **1. Introduction**

42 Atrogin-1 (also known as Muscle atrophy F-box protein: MAFbx or *FBXO32*) and Muscle-  
43 specific RING finger protein 1 (MuRF1 or *TRIM63*) are muscle specific E3 ligases and their  
44 expression is highly associated with various skeletal muscle atrophic models [1, 2]. In  
45 agreement with above, a plethora of studies have confirmed that Atrogin-1 and MuRF1  
46 mRNA expression are useful molecular biomarkers of skeletal muscle atrophy [3]. Although  
47 both Atrogin-1 and MuRF1 gene expressions increase in almost all atrophic models, various  
48 muscle atrophic conditions (e.g., fasting, immobilization, diabetes, insulin resistance) are  
49 likely to alter multiple signaling pathways to control Atrogin-1 and MuRF1 gene and protein  
50 expression [4]. While the PI3K-Akt signaling pathway is known to regulate Atrogin-1 and  
51 MuRF1 gene expression, the mechanisms that regulate protein content of these two E3  
52 ligases remain to be elucidated. Many studies have assumed that mRNA expressions  
53 implicitly reflect the corresponding changes of protein content, but in reality the expression  
54 levels of individual mRNA and its corresponding protein are indeed poorly correlated [5, 6].  
55 The poor correlation can be explained by multiple processes, including transcription and  
56 degradation of mRNAs, translation, folding, and degradation of proteins [7, 8]. As protein is  
57 the final product executing gene function, direct measurement of protein content should be  
58 more relevant to biological functions [8, 9]. However, in the cases of Atrogin-1 and MuRF1,  
59 poor quality of antibodies is often a major obstacle to reveal protein content in biological  
60 samples [3, 10, 11].

61

62 PI3K/Akt/forkhead box (FoxO) signaling is one of the most well studied pathways known to  
63 regulate Atrogin-1 and MuRF1 mRNA transcription expression [12-14]. Studies have shown  
64 that treatment of IGF-1 or an introduction of constitutively active Akt prevents both Atrogin-  
65 1 and MuRF1 mRNA transcription expression in C2C12 myotubes [12, 13]. In addition,

66 denervation-induced skeletal muscle atrophy was prevented by IGF-1 treatment [13]. IGF-1  
67 increases Akt phosphorylation and suppresses Atrogin-1 and MuRF1 mRNA transcription  
68 expression in mouse skeletal muscle [13], indicating a link between Akt and Atrogin-  
69 1/MuRF1 axis in skeletal muscle atrophy. Mechanistically, Akt phosphorylates the  
70 transcription factor FoxO to induce FoxO nuclear exclusion, which downregulates FoxO-  
71 dependent gene transcription [15]. A study has also confirmed that overexpression of FoxO3a  
72 in mouse skeletal muscle is able to induce Atrogin-1 mRNA expression and an atrophic  
73 phenotype [12]. In contrast, siRNA knockdown of FoxO1-3 inhibits Atrogin-1 promoter  
74 activity measured by Atrogin-1 luciferase reporter constructs during fasting-induced muscle  
75 atrophy [12]. All these findings have evidenced that Akt-FoxO axis is critical for regulating  
76 Atrogin-1 and MuRF1 mRNA transcriptional expression. However, some contradictory  
77 results have also been reported. For example, a study showed that deletion of Akt1 or Akt2  
78 did not alter Atrogin-1 mRNA and protein expressions in mouse skeletal muscle [16].  
79 Atrogin-1 and MuRF1 mRNA expression, including Atrogin-1 protein content, were shown  
80 to be unchanged in ageing-induced muscle atrophy, where Akt activity and FoxO3a  
81 phosphorylation were elevated, compared to young control skeletal muscles [17]. These  
82 contradictory findings raise the question of whether Akt-FoxO axis is the solely signaling  
83 pathway that regulate Atrogin-1 and MuRF1 expression.

84

85 mTORC1 plays an important role in regulating protein synthesis and autophagy-lysosome  
86 system [18], and its activation has been well associated with skeletal muscle hypertrophy [19,  
87 20]. Surprisingly, the involvement of mTORC1 in regulating muscle protein degradation has  
88 not been well investigated. A recent study led by Zhao et al. [21] suggested that mTOR  
89 (including mTORC1 and mTORC2) may be involved in the regulation of protein degradation  
90 in C2C12 myotubes. Their previous study has shown that treatment of rapamycin, a specific

91 mTORC1 inhibitor, can increase protein degradation in C2C12 myotubes [22], which lead  
92 the authors to suggest mTORC1 as a contributor for controlling protein degradation.  
93 Furthermore, there is also evidence suggesting that Atrogin-1 and MuRF1 mRNA  
94 expressions are regulated by distinct signaling mechanisms. Sacheck et al. [22] showed that  
95 rapamycin treatment increases Atrogin-1, but not MuRF1, mRNA expression. However,  
96 proof at protein level is currently lacking and such information is needed to better understand  
97 what the signaling mechanisms are controlling Atrogin-1 and MuRF1 protein content, which  
98 essentially execute the enzymatic ubiquitin E3 ligase activity.

99

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

109

## 110 **2. MATERIALS AND METHODS**

### 111 *2.1 C2C12 cell culture*

112 Mouse skeletal muscle C2C12 myoblast cells were obtained from the American Type Culture  
113 Collection (ATCC, Manassas, VA, USA). Cells were seeded and maintained in Dulbecco's  
114 Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Loughborough, UK, 31966021)  
115 containing GlutaMAX, 25 mM of glucose, and 1 mM of sodium pyruvate, supplemented with

116 10% (v/v) of Hyclone fetal bovine serum (FBS, Fisher Scientific, Loughborough, UK,  
117 SV30180.03), 1% (v/v) of Penicillin-Streptomycin (10 000 Units/mL-ug/mL, Thermo Fisher  
118 Scientific, Loughborough, UK, 15140122). Myoblasts were seeded onto six-well multidishes  
119 (greiner bio-one, 657 160) and when confluency reached at 90%, myoblasts were  
120 differentiated into myotubes in DMEM supplemented with 2% (v/v) of horse serum (Sigma-  
121 Aldrich, Cambridgeshire, UK, H1270), 1% (v/v) of Penicillin-Streptomycin. The media was  
122 changed every 48 h. Cultures were maintained in a humified incubator at 37 °C with an  
123 atmosphere of 5% of CO<sub>2</sub> and 95% of air.

124

125 *2.2 Drug reconstitution and cell treatment*

126 Akt1/2/3 inhibitor MK-2206 dihydrochloride (ApexBio, A3010), Rapamycin (Sigma-Aldrich,  
127 553211), adenosine monophosphate (AMP)-activated protein kinase (AMPK) activator 991  
128 (AOBIOUS, MA, USA, AOB8150), S6K1 Inhibitor, PF-4708671 (Sigma-Aldrich, Dorset,  
129 UK, 559278) were prepared in DMSO and treatment conditions were described in the figure  
130 legend. Insulin solution human was obtained from Sigma (Sigma Aldrich, Dorset, UK,  
131 I9278).

132

133 *2.3 Cell lysis*

134 Cells were lysed in ice-cold sucrose lysis buffer containing: 250 mM of sucrose, 50 mM of  
135 Tris-base (pH 7.5), 50 mM of sodium fluoride, 10 mM of sodium β-glycerophosphate, 5 mM  
136 of sodium pyrophosphate, 1 mM of EDTA, 1 mM of EGTA, 1 mM of benzamidine, 1 mM of  
137 sodium orthovanadate, 1 x complete Mini EDTA-free protease inhibitor cocktail (Roche), 1%  
138 of Triton X-100, and 100 mM of 2-chloroacetamide. Cell lysates were centrifuged for 15  
139 minutes at 13 000 g at 4°C and the supernatant was stored at -80°C before analysis for total  
140 protein concentrations using the Bradford protein assay (Thermo Fisher Scientific,

141 Leicestershire, UK, 23200). Protein in each sample was quantified from a standard curve  
142 using BSA standards (Thermo Fisher Scientific, Leicestershire, UK, 23209).

143

144 *2.4 Western blot*

145 Cell lysates were prepared in 1x NuPAGE LDS sample buffer (Invitrogen, NP0008)  
146 containing 2-mercaptoethanol (final concentration 1.5%) and left to denature overnight at  
147 room temperature. Prepared cell lysates (10-15 µg of total protein) were loaded into 8% or  
148 10% Bis/Tris gels prior to sodium dodecyl sulfate– polyacrylamide gel electrophoresis (SDS-  
149 PAGE). Gels were run in 1x MOPS buffer for approximately 60 minutes at 140V. Proteins  
150 were transferred onto 0.2 µm polyvinylidene fluoride (PVDF) membranes (Millipore,  
151 Hertfordshire, UK) for 1 hour at 100V. Membranes were blocked in 5% of milk diluted in  
152 Tris-buffered saline Tween-20 (TBS-T): 137 mM of sodium chloride, 20 mM of Tris-base  
153 7.5 pH, 0.1% of Tween-20 for 1 hour. After blocking, membranes were washed 3 times for 5  
154 min in TBS-T before being incubated overnight at 4°C with the appropriate primary  
155 antibodies (**Table 1**). Membranes were washed 3 times for 5 min in TBS-T prior to  
156 incubation in horse radish peroxidase-conjugated secondary antibodies (see Supplementary  
157 Table 1) at room temperature for 1 h. Membranes were washed a further three times in TBS-  
158 T prior to antibody detection using enhanced chemiluminescence horseradish peroxidase  
159 substrate detection kit (Millipore, Hertfordshire, UK). Imaging was undertaken using a  
160 G:BOX Chemi-XR5 (Syngene, Cambridgeshire, UK). Band intensities were quantified using  
161 ImageJ/Fiji (NIH, Bethesda, MD, USA). Phosphorylation levels were determined by the  
162 expression of phosphorylated protein divided by expression of non-phosphorylated total  
163 protein. Vinculin was used as the loading control.

164

165 *2.5 Statistical analysis*

166 The statistical analyses were performed using Prism version 8.1.2 (GraphPad Software, San  
167 Diego, California USA, [www.graphpad.com](http://www.graphpad.com)). Values of  $P < 0.05$  (\*) were considered  
168 statistically significant. For time course and dose-response experiments, a one-way analysis  
169 of variance (ANOVA) was performed with Dunnett's post-hoc test compared to control  
170 (CON). Data are presented as mean  $\pm$  SD. All experiments were performed in duplication and  
171 repeated at least twice.

172 **3. Results**

173 *3.1 Evidence of Insulin/Akt/FoxO signaling pathway modulating Atrogin-1 and MuRF1*  
174 *protein content*

175 We first confirmed if insulin/Akt/FoxO signaling pathway is sufficient to modulate both  
176 Atrogin-1 and MuRF1 protein content in C2C12 myotubes. Using an allosteric Akt inhibitor  
177 (MK-2206), we showed that Atrogin-1 protein content was significantly increased at 3 h, 6 h,  
178 and 9 h after the treatment of 10  $\mu$ M MK-2206 (**Fig. 1 B**). MuRF1 protein content was also  
179 significantly increased at 6 h after the treatment of MK-2206 (**Fig. 1 C**). In line with a  
180 previous study [23], Akt phosphorylation at Ser<sup>473</sup> and Thr<sup>308</sup> was completely abolished over  
181 the course of 9 h treatment with MK-2206 (**Fig. 1 A**). We also confirmed that inhibition of  
182 Akt activity prevents FoxO1 and FoxO3a phosphorylation and reduces S6K1 and rpS6  
183 phosphorylation (**Fig. 1 A**).

184

185 Atrogin-1 protein content was significantly decreased at 3 h, 6 h, and 9 h following the  
186 treatment of 100 nM insulin stimulation (**Fig. 1 B**). MuRF1 protein content was also  
187 significantly decreased at 6 h and 9 h after insulin treatment (**Fig. 1 C**). As expected, insulin  
188 stimulated Akt phosphorylation at both Ser<sup>473</sup> and Thr<sup>308</sup> sites. The enhanced Akt activity was

189 also confirmed by the increases of its downstream, such as FoxO1, FoxO3a, S6K1, and rpS6  
190 phosphorylation (**Fig. 1 A**).

191

192 *3.2 Atrogin-1, but not MuRF1, protein content is increased by the rapamycin sensitive*  
193 *mTORC1 inhibition*

194 Treatment with Rapamycin can specifically inhibit mTORC1 activity without directly  
195 affecting mTORC2 activity, but a long-term treatment ( $\geq 24$  h) is known to inhibit mTORC2  
196 activity [24]. Therefore, we have limited the treatment time of small molecules to not more  
197 than 9 h. Interestingly, Atrogin-1 protein content was increased at 3 h, 6 h, and 9 h following  
198 the treatment of 100 nM rapamycin (**Fig. 2 B**). Despite that Atrogin-1 protein content was  
199 increased, MuRF1 protein content remained unchanged (**Fig. 2 C**). As anticipated, rapamycin  
200 treatment completely inhibited S6K1 and rpS6 phosphorylation (**Fig. 2 A**) without inducing a  
201 significant change in Akt phosphorylation ( $P = 0.38$ ).

202

203 *3.3 Distinct time-dependent changes of Atrogin-1 and MuRF1 protein content following*  
204 *AMPK activation*

205 AMPK activation is known to inhibit mTORC1 activity [25] via the phosphorylation of  
206 tuberous sclerosis complex 2 (TSC2) [26] and Raptor [27]. To further investigate the role of  
207 mTORC1 on the regulation of Atrogin-1 and MuRF1 protein content, we used a direct  
208 AMPK activator, 991, to increase AMPK activity in C2C12 myotubes [25, 28]. Interestingly,  
209 Atrogin-1 protein content was increased rapidly at 3 h and 6 h, despite returning to the basal  
210 level after 9 h of treatment (**Fig. 3 B**). In contrast, MuRF1 protein content had obviously  
211 delayed increases at 6 h and 9 h after 991 treatment (**Fig. 3 C**). These results again suggest  
212 that Atrogin-1 and MuRF1 protein content are regulated by distinct signaling mechanisms.  
213 As expected, ULK1 phosphorylation at Ser<sup>555</sup> was increased by the treatment of 991 (**Fig. 3**

214 A) [29] and the inhibition of mTORC1 activity was confirmed by showing a decrease in  
215 S6K1 and rpS6 phosphorylation (**Fig. 3 A**).

216

217 *3.4 Atrogin-1 protein content is increased by S6K1 inhibition*

218 To further explore the distinct mechanisms that regulate Atrogin-1 and MuRF1 protein  
219 content, we asked whether mTORC1 downstream, such as S6K1, is involved in regulating  
220 Atrogin-1 or MuRF1 protein content. Using a specific S6K1 inhibitor [30], we showed that  
221 Atrogin-1 (**Fig. 4 B**) protein content was increased in a dose-response manner, where  
222 significant increases was seen with the treatment of 40  $\mu$ M and 50  $\mu$ M PF-4708671. Instead  
223 of increasing, MuRF1 protein content was indeed decreased at 50  $\mu$ M (**Fig. 4 C**). Inhibition  
224 of S6K1 was confirmed by the observation of reduced rpS6 phosphorylation (**Fig. 4 A**). As  
225 expected, the phosphorylation of S6K1 was increased by the treatment of PF-4708671 [30]  
226 (**Fig. 4 A**). Next, we performed Pearson's correlation coefficient to identify the relationship  
227 between p-rpS6<sup>Ser240/244</sup>/rpS6 and Atrogin-1 or MuRF1 by plotting the dose-response data  
228 (**Fig. 4 D**). Interestingly, a strong negative correlation was observed between p-  
229 rpS6<sup>Ser240/244</sup>/rpS6 and Atrogin-1 ( $r = -0.90, P < 0.0001$ ), whereas no significant association  
230 was observed between p-rpS6<sup>Ser240/244</sup>/rpS6 and MuRF1 ( $r = 0.17, P = 0.44$ ).

231

232 To confirm that S6K1 inhibition increases Atrogin-1, but not MuRF1, protein content, we  
233 performed a time course experiment using 30  $\mu$ M of PF-4708671 for up to 24 h (**Fig. 5 A**).  
234 As anticipated, the protein content of Atrogin-1 was increased over the course of PF-4708671  
235 treatment at 3 h, 6 h, and 24 h (**Fig. 5 B**). Although MuRF1 protein content (**Fig. 5 C**)  
236 remained unchanged over majority of the time points, there was still an unexpected increase  
237 occurred at 6 h after PF-4708671 treatment.

238

239 **4. Discussion**

240 The gene expression of Atrogin-1 and MuRF1 are highly associated with almost all kinds of  
241 skeletal muscle atrophy [1-3]. Genetic studies have also shown that knockout of Atrogin-1 or  
242 MuRF1 partially rescue denervation-induced skeletal muscle atrophy [1]. However, the  
243 molecular mechanisms of how Atrogin-1 and MuRF1 contribute to skeletal muscle atrophy  
244 are still unclear. The most recent study has indicated that the enzymatic activity of these  
245 ubiquitin E3 ligases is particularly important in controlling skeletal muscle mass [31].  
246 Therefore, obtaining information relevant to the regulation of Atrogin-1 and MuRF1 protein  
247 content will provide an alternative opportunity to manipulate their functional E3 ligase  
248 activity. This information will also help identify new therapeutic targets to treat and/or  
249 prevent skeletal muscle atrophy. Here, we have made use of small molecules to evaluate  
250 some key signaling pathways that modulate Atrogin-1 and MuRF1 protein contents in C2C12  
251 myotubes. In accordance with previous studies, we confirmed that insulin/Akt/FoxO pathway  
252 is sufficient to modulate both Atrogin-1 and MuRF1 protein contents, which is in agreement  
253 with the tendency of measuring mRNA transcriptional expression [12-14, 22]. Further  
254 investigation revealed that Atrogin-1, but not MuRF1, protein content is predominantly  
255 increased when rapamycin-sensitive signaling pathways is inhibited. These findings show  
256 that Atrogin-1 and MuRF1 protein contents are regulated through different mechanisms  
257 downstream of Akt. More interestingly, our studies also revealed that Atrogin-1 protein  
258 content can be regulated by S6K1 dependent signaling pathway.

259

260 Inactivation of PI3K/Akt/FoxO signaling pathway is well known as an “atrophic signal” that  
261 increases both MuRF1 and Atrogin-1 mRNA expression [32]. However, few studies have  
262 investigated whether MuRF1 and Atrogin-1 protein contents are regulated in accordance with  
263 their gene/mRNA expressions. The current study confirmed that protein content of both

264 Atrogin-1 and MuRF1 were suppressed by insulin, whereas Atrogin-1 and MuRF1 protein  
265 contents were upregulated by the treatment of MK-2206. These findings are consistent with  
266 the mRNA expressions investigated by previous studies [12-14, 22].

267

268 In the present study, we showed that Atrogin-1 protein content was increased after 3 h  
269 treatment of rapamycin, whereas MuRF1 protein content was not changed throughout the  
270 time course (**Fig. 2**). This data indicates that inhibition of mTORC1 signaling can enhance  
271 Atrogin-1, but not MuRF1, protein content. This is indeed consistent with a previous study  
272 reported that inhibition of rapamycin-sensitive signaling pathway increases Atrogin-1, but not  
273 MuRF1, mRNA expression [22]. Our findings strengthened the previous evidence of mRNA  
274 data [22] by showing that inhibition of rapamycin-sensitive mTOR-S6K1 signaling pathway  
275 also induces an increase in Atrogin-1 protein content.

276

277 The most interesting findings in the present study are that Atrogin-1 and MuRF1 protein  
278 contents can be regulated differently, and that Atrogin-1 protein content is regulated by  
279 rapamycin-sensitive and S6K1 dependent signaling pathways. In the present study, the  
280 phosphorylation of FoxO3a at Thr<sup>32</sup> and FoxO1 at Thr<sup>24</sup> was not altered after rapamycin or  
281 PF-4708671 treatment, suggesting that FoxOs are not the most critical factor regulating  
282 Atrogin-1 (as well as MuRF1) protein content. Multiple transcription factors, including the  
283 NF-κB transcription factors CCAAT/enhancer-binding protein-β (C/EBPβ) and Smad3, can  
284 work cooperatively to regulate Atrogin-1 mRNA transcription Atrogin-1 [3, 33]. Thus,  
285 complex cooperative mechanisms of transcription factors might have been involved in the  
286 distinct protein expression patterns between Atrogin-1 and MuRF1 protein content. In  
287 supporting our finding that Atrogin-1 protein content is regulated by S6K1 dependent  
288 signaling, previous studies have also shown that the absence of S6K1 causes skeletal muscle

289 atrophy in mice [34]. In addition, Marabita et al. [35] reported that S6K1 is required for the  
290 prevention of protein aggregation during skeletal muscle hypertrophy in mice. These  
291 observations led us to hypothesize that protein quality control, mainly protein degradation, is  
292 the mechanism inducing the increased Atrogin-1 protein content, when rapamycin-sensitive  
293 mTOR-S6K1 signaling is inhibited. However, future studies should confirm this hypothesis  
294 by investigating the process of Atrogin-1 protein turnover rate, and subsequent protein  
295 content.

296

297 The mTORC1 signaling pathway has been shown as a positive regulator of skeletal muscle  
298 mass in several models of hypertrophy [19, 20, 36]. In support of age-related muscle loss,  
299 studies have demonstrated that muscle contraction-induced activation of mTORC1 signaling  
300 is impaired with ageing [37, 38]. In contrast, constant activation of mTORC1 is known to  
301 cause myopathy, but not hypertrophy [39]. Moreover, a most recent study led by Joseph et al.  
302 [40] showed that mTORC1 signaling pathway is indeed hyperactivated in age-related muscle  
303 loss with a concomitant increase in both Atrogin-1 and MuRF1 mRNA expression in basal  
304 rat skeletal muscle. More interestingly, a partial inhibition of mTORC1 via RAD001 restored  
305 age-related skeletal muscle loss [40]. RAD001 treatment also decreased MuRF1 mRNA  
306 expression while Atrogin-1 mRNA was not altered in ageing muscle. We cannot directly  
307 compare our findings to their results as they did not report Akt activity and information of  
308 MuRF1 and Atrogin-1 protein contents was not available. Nonetheless, these findings  
309 indicate the importance of fine tuning the mTORC1 activity in maintaining skeletal muscle  
310 mass and Atrogin-1 and/or MuRF1 may be responsible for this.

311

312 Protein content is determined by protein turnover, which is a continuous process of protein  
313 synthesis and protein degradation [41]. In this study, Atrogin-1 and MuRF1 protein contents

314 were investigated following time-course and/or dose-dependent small molecule treatments,  
315 which is a snapshot in time of the impact of the protein turnover kinetics on protein balance.  
316 mTORC1 is a well-known signaling pathway to control protein synthesis. Thus, after the  
317 treatment of rapamycin or PF-4708671, a decrease in protein synthesis would be expected  
318 and a greater decrease in protein degradation would, in theory, contribute to the observed  
319 increase in Atrogin-1 protein content. Post-translational modifications and the subsequent  
320 degradation make it more complicated to understand how protein content is regulated. For  
321 example, many ubiquitin E3 ligases have been implicated to regulate their own protein  
322 abundance [42] because most of E3 ligases have the ability to ubiquitylate itself (known as  
323 autoubiquitylation) and trigger self-degradation processes (either via proteasome or  
324 autophagic lysosome). For example, the greater autoubiquitylation usually demonstrates  
325 greater E3 ligase activity [11], which was observed in MuRF1 via in vitro reaction [1].  
326 However, the degree of autoubiquitylation on MuRF1 and Atrogin-1 is currently not clear in  
327 any of muscle atrophy conditions. Although it is not clear from the present study,  
328 autoubiquitylation might have been involved in the regulation of Atrogin-1 and MuRF1  
329 protein contents.

330  
331 While our findings suggest that Atrogin-1 and MuRF1 protein contents are regulated by  
332 different signaling mechanisms, future studies should determine which molecules in the  
333 rapamycin-sensitive mTOR-S6K1 signaling cascade are responsible for regulating Atrogin-1  
334 protein content. With the use of our protein content data, other studies should also investigate  
335 whether E3 ligase activity of MuRF1 and/or Atrogin-1 is associated with their protein content,  
336 and thus a measurement of protein content can be used as a biomarker for E3 ligase activity  
337 or vice versa. Additionally, protein degradation contributes half of the equation to determine  
338 protein content (i.e., protein synthesis – protein degradation = protein content). Thus,

339 determining degradation mechanisms of Atrogin-1 and MuRF1 protein contents is also  
340 important to modulate protein half-life. Thus, understanding of the degradation mechanisms  
341 of Atrogin-1 and MuRF1 is required as an important step towards understanding the  
342 underlying mechanisms of skeletal muscle atrophy and manipulating their functional E3  
343 ligase activity.

344 **5. Conclusions**

345 Based on the findings from the preset study and the existing literature, we propose potential  
346 signaling mechanisms that may be involved in the regulation of Atrogin-1 and MuRF1  
347 protein contents in skeletal muscle (**Fig. 6**). The anabolic Akt signaling, which can be  
348 activated by Insulin/IGF-1, is a critical upstream signal to modulate MuRF1 and Atrogin-1 at  
349 both gene and protein expression levels. However, Atrogin-1, but not MuRF1, protein  
350 content is increased when the rapamycin-sensitive and S6K1 dependent signaling pathways  
351 are inhibited. Thus, the regulatory mechanisms of protein content are distinct between  
352 Atrogin-1 and MuRF1. Our study provides evidence that Atrogin-1 protein content can be  
353 regulated by the rapamycin-sensitive mTOR-S6K dependent signaling pathway. Future  
354 studies should determine the underlying mechanisms by which the rapamycin-sensitive  
355 mTOR-S6K1 signaling regulates Atrogin-1 protein content.

356

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365 **CRediT authorship contribution statement**

366 Yusuke Nishimura: Conceptualization, Investigation, Visualization, Writing - original draft,  
367 Writing - review & editing. Ibrahim Musa: Investigation, Writing - review & editing. Peter  
368 Dawson: Writing - review & editing. Lars Holm: Writing - review & editing. Yu-Chiang Lai:  
369 Conceptualization, Writing - review & editing.

370 **CONFLICT OF INTEREST**

371 The authors have no conflicts of interest to declare.

372

373 **References**

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505

506 **Table 1. Antibodies for western blot**

Antibodies	Dilution	Source	Identifier
phospho-Ser473 Akt	1:1000	Cell Signaling Technology	Cat# 4060
phospho-Thr308 Akt	1:1000	Cell Signaling Technology	Cat# 2965
Akt	1:1000	Cell Signaling Technology	Cat# 4691
phospho-Thr389 p70 S6 Kinase	1:1000	Cell Signaling Technology	Cat# 9234
p70 S6 Kinase	1:1000	Cell Signaling Technology	Cat# 2708
phospho-Ser240/244 S6 Ribosomal Protein	1:8000	Cell Signaling Technology	Cat# 5364
S6 Ribosomal Protein	1:8000	Cell Signaling Technology	Cat# 2217
phospho-Thr172 AMPK $\alpha$	1:1000	Cell Signaling Technology	Cat# 2535
AMPK $\alpha$	1:1000	Cell Signaling Technology	Cat# 2532
Atrogin-1	1:1000	ECM Biosciences	Cat# AM3141
MuRF1	1:1000	Santa Cruz	Cat# SC-398608
phospho-Ser555 ULK1	1:1000	Cell Signaling Technology	Cat# 5869
ULK1	1:1000	Cell Signaling Technology	Cat# 4773
Phospho-FoxO1 (Thr24)/FoxO3a (Thr32)	1:750	Cell Signaling Technology	Cat# 9464
Vinculin	1:2000	Abcam	Cat# Ab129002
Anti-mouse IgG, HRP-linked Antibody	1:10000	Cell Signaling Technology	Cat# 7076
Anti-rabbit IgG, HRP-linked Antibody	1:10000	Cell Signaling Technology	Cat# 7074
Anti-Rat IgG, HRP-linked Antibody	1:10000	Cell Signaling Technology	Cat# 7077

507

508 **Figure 1. Insulin/Akt signaling pathway is sufficient to modulate Atrogin-1 and MuRF1**  
509 **protein contents in C2C12 myotubes.**

510 C2C12 myotubes were treated with DMSO (0.1%, 9 h) as a vehicle control (CON), MK2206  
511 (10  $\mu$ M), or insulin (100 nM) for 3, 6, or 9 h. Lysates were analyzed by SDS-PAGE and  
512 western blotting with the indicated antibodies. (A) Representative images from one of two  
513 independent experiments. (B) Quantification of Atrogin-1. (C) Quantification of MuRF1.  
514 Data are expressed as means  $\pm$  SD (n = 4) fold changes relative to CON. One-way ANOVA  
515 with Dunnett's post-hoc test, \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001  
516 compared to CON.

517 **Figure 2. Rapamycin-sensitive mTOR inhibition increases Atrogin-1, but not MuRF1**  
518 **protein contents in C2C12 myotubes.**

519 C2C12 myotubes were treated with DMSO (0.1%, 9 h) as a vehicle control (CON) or  
520 Rapamycin (100 nM) for 3, 6, or 9 h. Lysates were analyzed by SDS-PAGE and western  
521 blotting with the indicated antibodies. (A) Representative images from one of two  
522 independent experiments. (B) Quantification of Atrogin-1. (C) Quantification of MuRF1.  
523 Data are expressed as means  $\pm$  SD (n = 4) fold changes relative to CON. One-way ANOVA  
524 with Dunnett's post-hoc test, \*\*\*P < 0.001 compared to CON.

525 **Figure 3. Inhibition of the mTORC1 pathway by AMPK activator 991 on Atrogin-1 and**  
526 **MuRF1 protein contents in C2C12 myotubes.**

527 C2C12 myotubes were treated with DMSO (0.1%, 9 h) as a vehicle control (CON) or 991  
528 (20  $\mu$ M) for 3, 6, or 9 h. Lysates were analyzed by SDS-PAGE and western blotting with the  
529 indicated antibodies. (A) Representative images from one of two independent experiments.  
530 (B) Quantification of Atrogin-1. (C) Quantification of MuRF1. Data are expressed as means

531  $\pm$  SD (n = 4) fold changes relative to CON. One-way ANOVA with Dunnett's post-hoc test,  
532 \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.0001 compared to CON.

533 **Figure 4. A dose-response effect of S6K1 inhibitor on Atrogin-1 and MuRF1 protein**  
534 **contents in C2C12 myotubes.**

535 C2C12 myotubes were treated with DMSO (0.1%, 3 h) as a vehicle control (CON) or PF-  
536 4708671 at the indicated doses for 3 h. Lysates were analyzed by SDS-PAGE and western  
537 blotting with the indicated antibodies. (A) Representative images of 2 independent  
538 experiments. (B) Quantification of Atrogin-1. (C) Quantification of MuRF1. Data are  
539 expressed as means  $\pm$  SD (n = 4) fold changes relative to CON. One-way ANOVA with  
540 Dunnett's post-hoc test, \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001 compared to  
541 CON. (D) Pearson's correlation coefficient to identify the association between p-  
542 rpS6<sup>Ser240/244</sup>/rpS6 and Atrogin-1 or MuRF1.

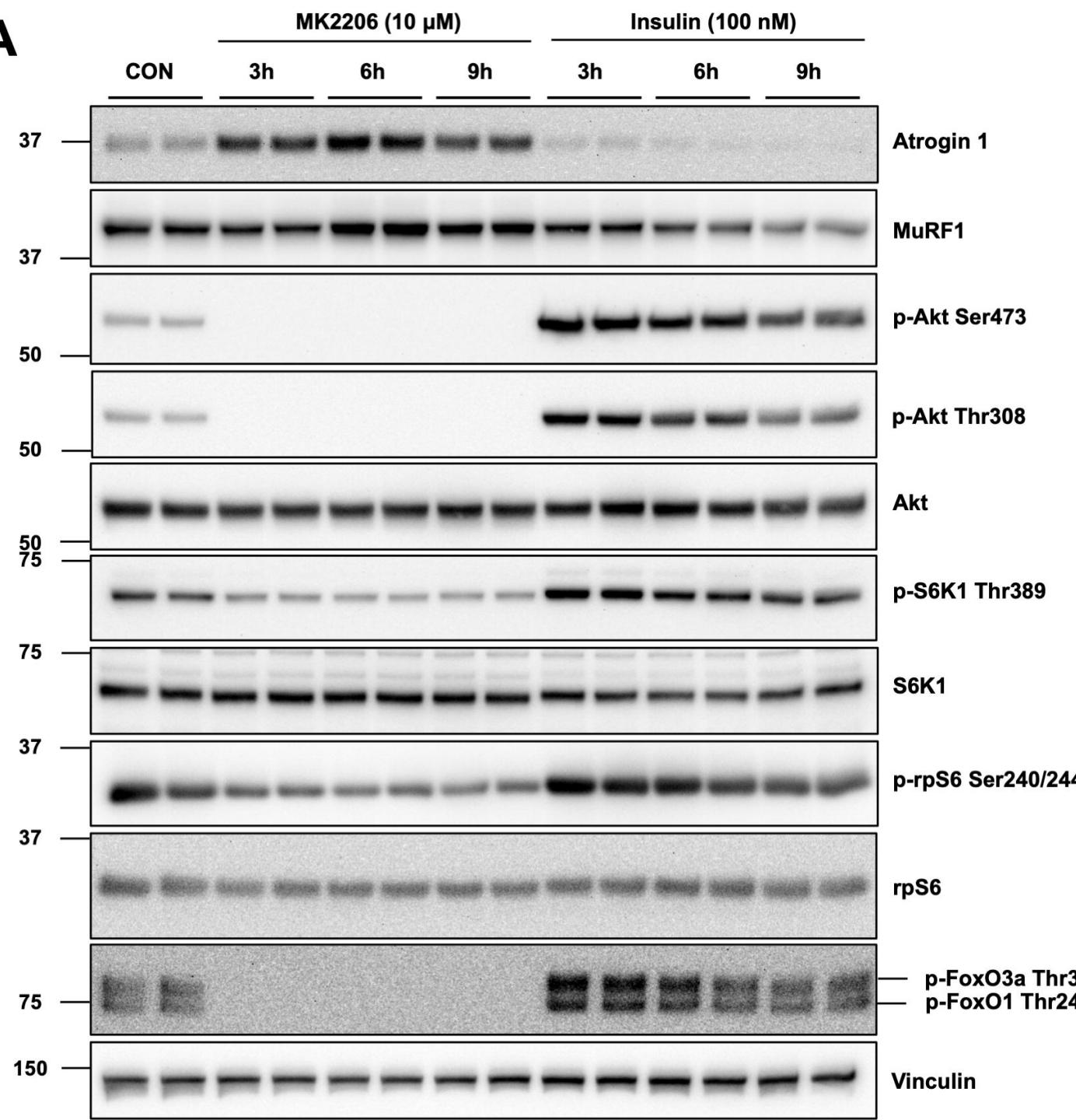
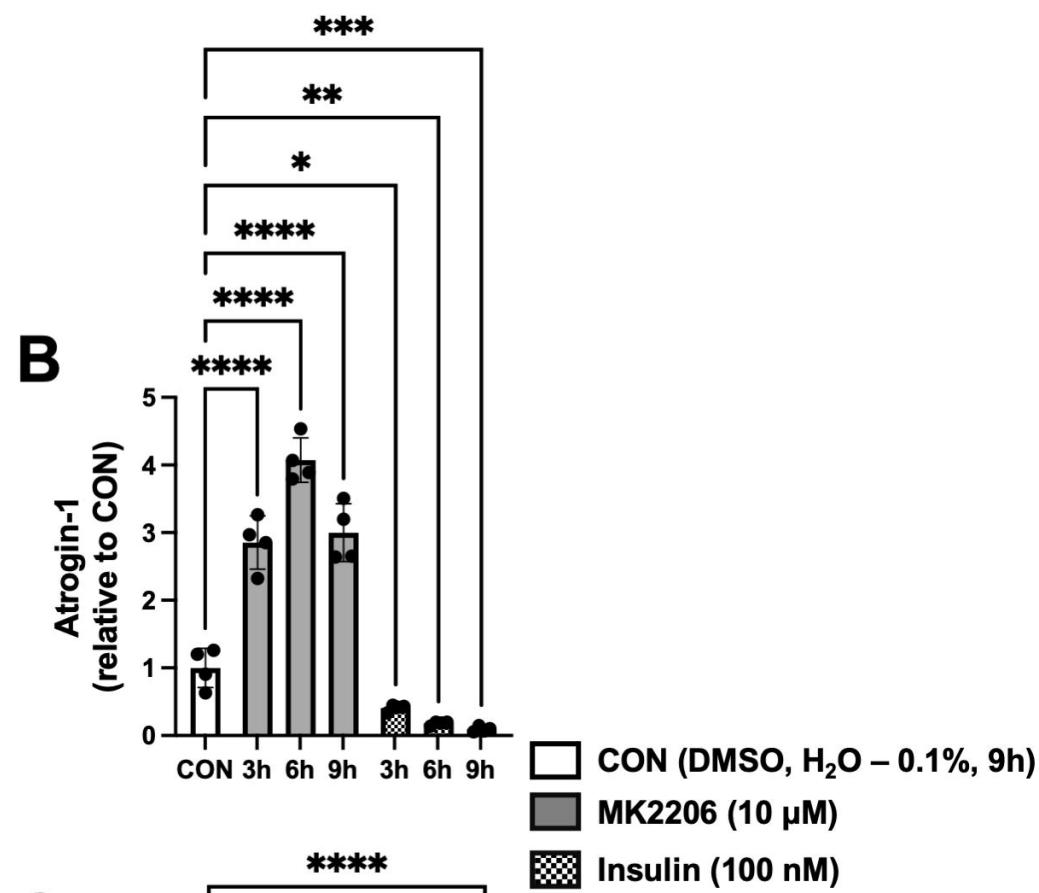
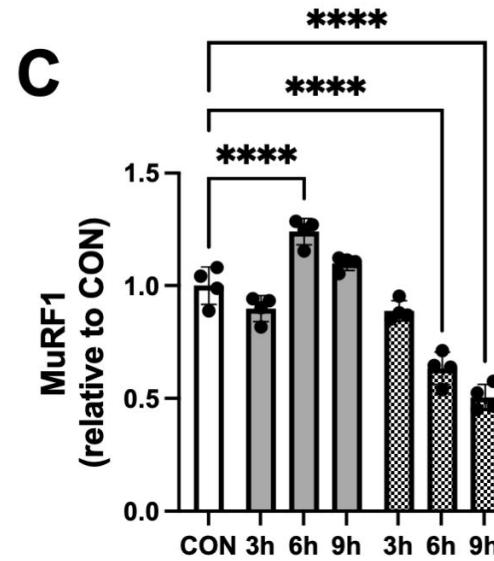
543 **Figure 5. A time course effect of S6K1 inhibitor on Atrogin-1 and MuRF1 protein**  
544 **contents in C2C12 myotubes.**

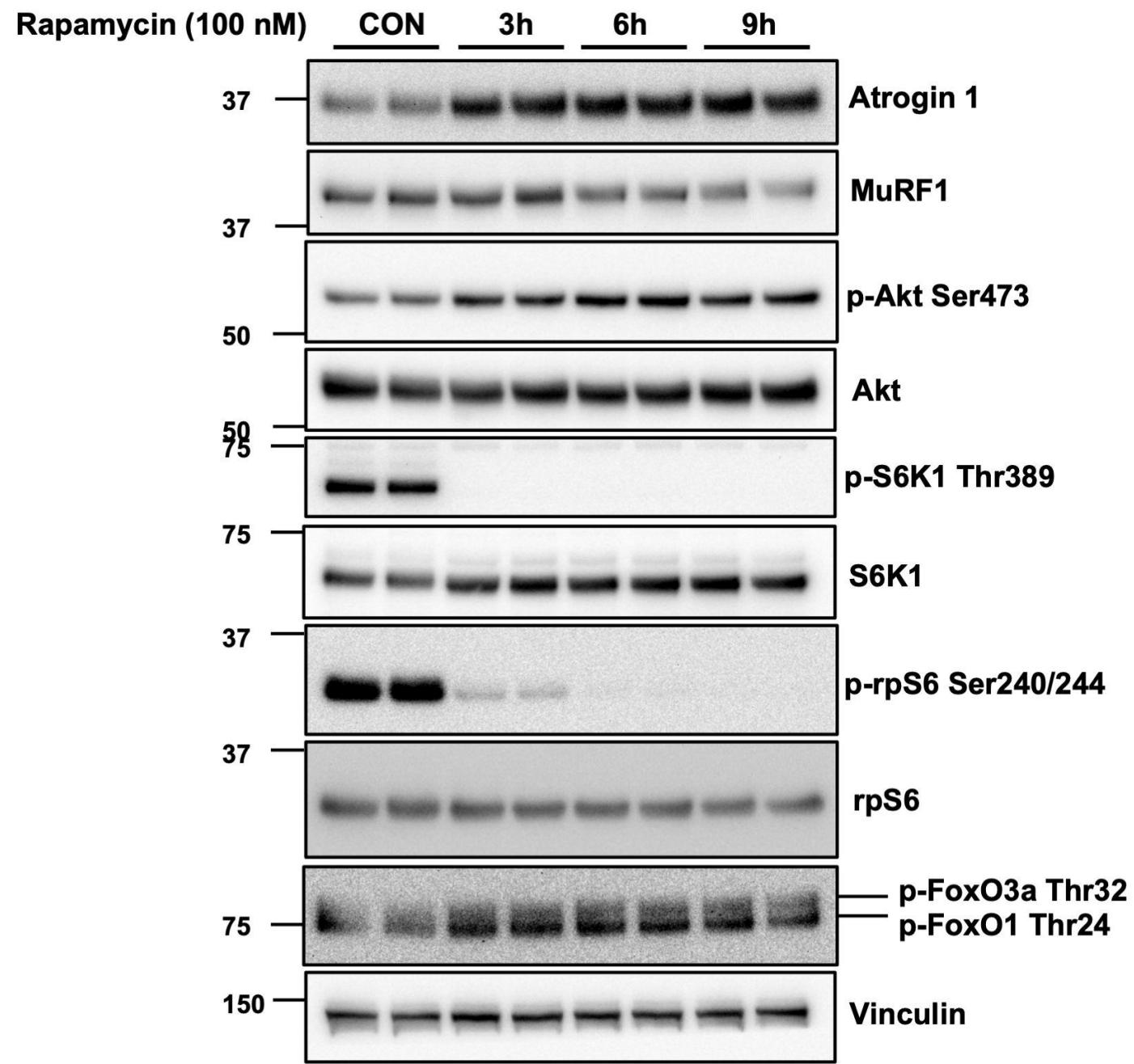
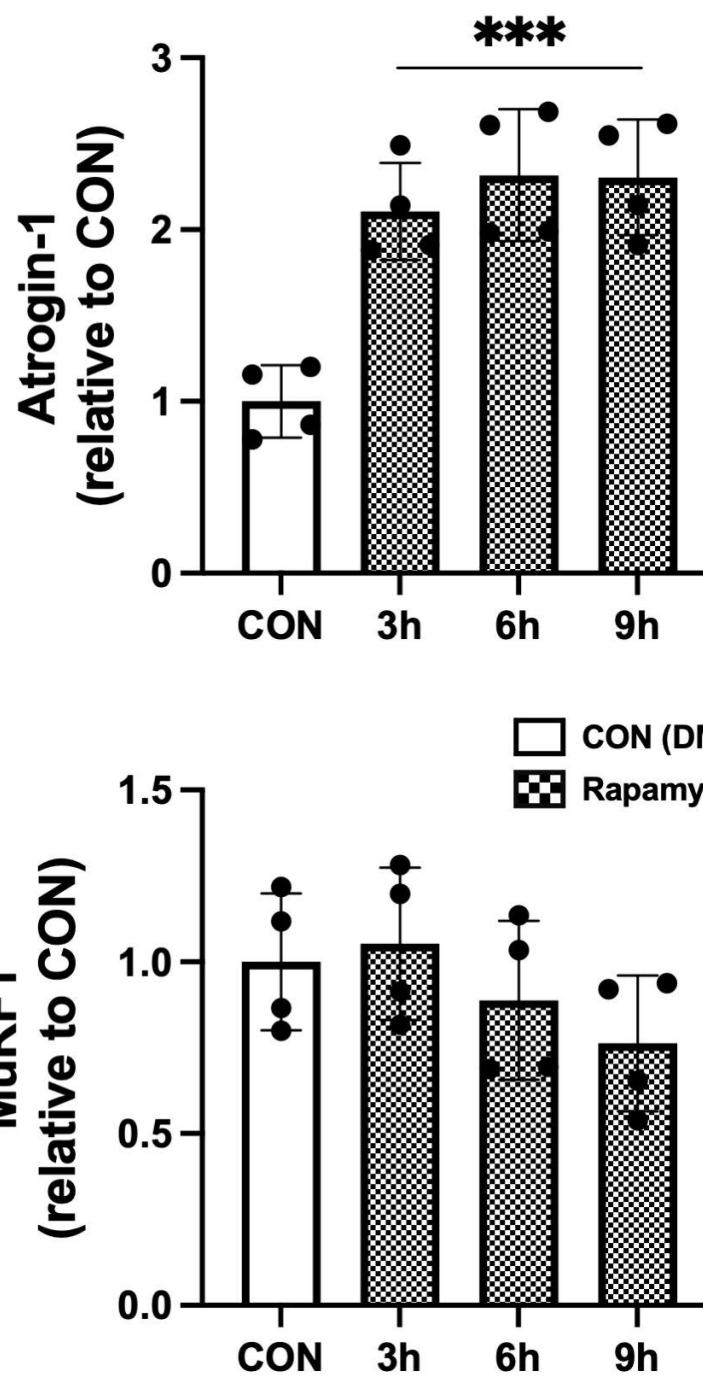
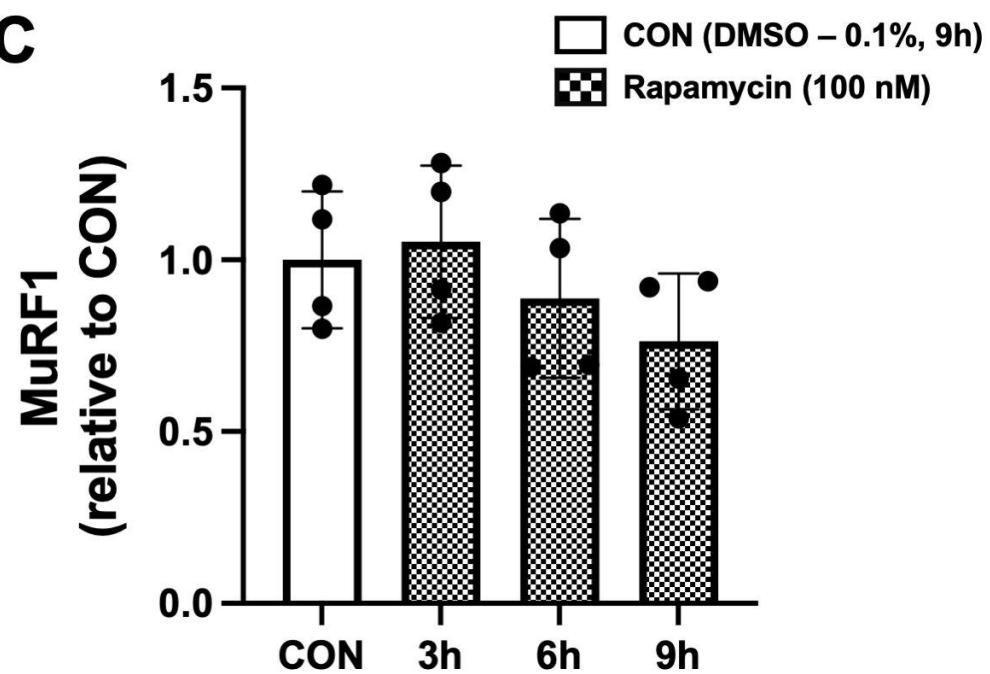
545 C2C12 myotubes were treated with DMSO (0.1%, 24 h) as a vehicle control (CON) or PF-  
546 4708671 (30  $\mu$ M) for up to 24 h. Lysates were analyzed by SDS-PAGE and western blotting  
547 with the indicated antibodies. (A) Representative images from one of two experiments. (B)  
548 Quantification of Atrogin-1. (C) Quantification of MuRF1. Data are expressed as means  $\pm$   
549 SD (n = 4) fold changes relative to CON. One-way ANOVA with Dunnett's post-hoc test, \*P  
550 < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 compared to CON.

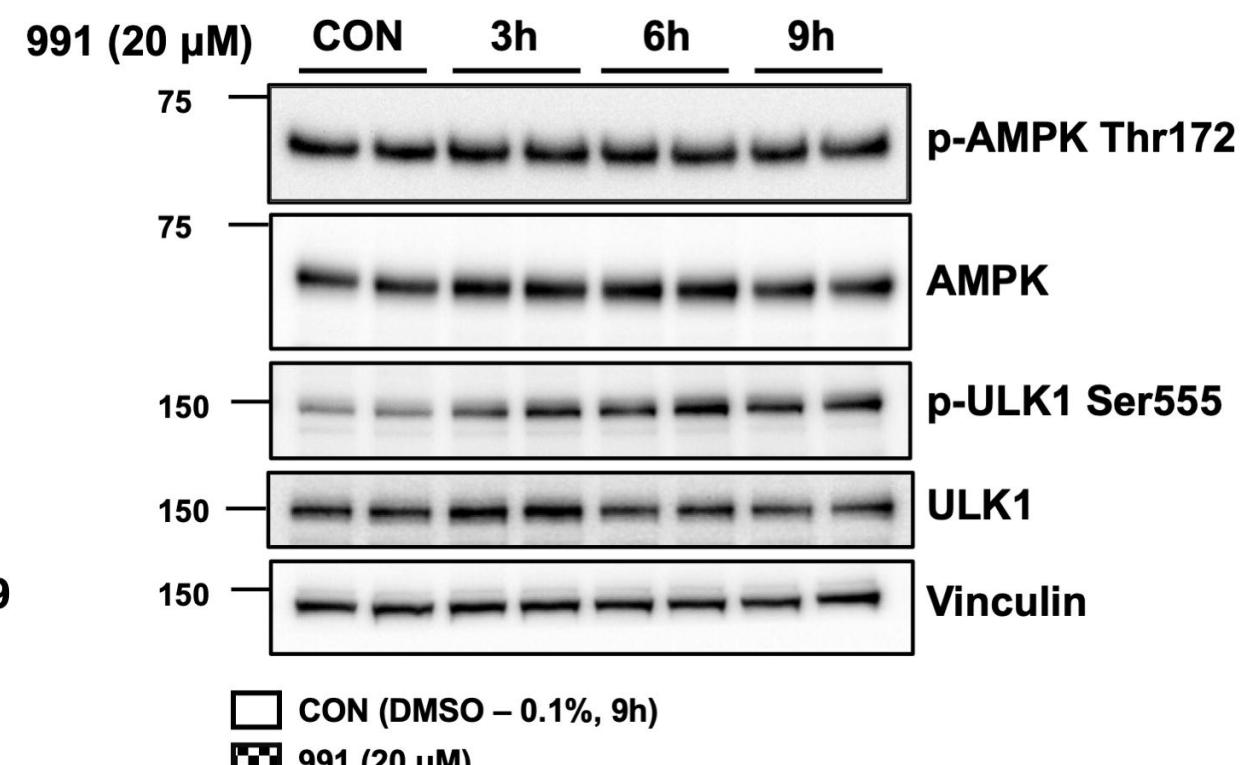
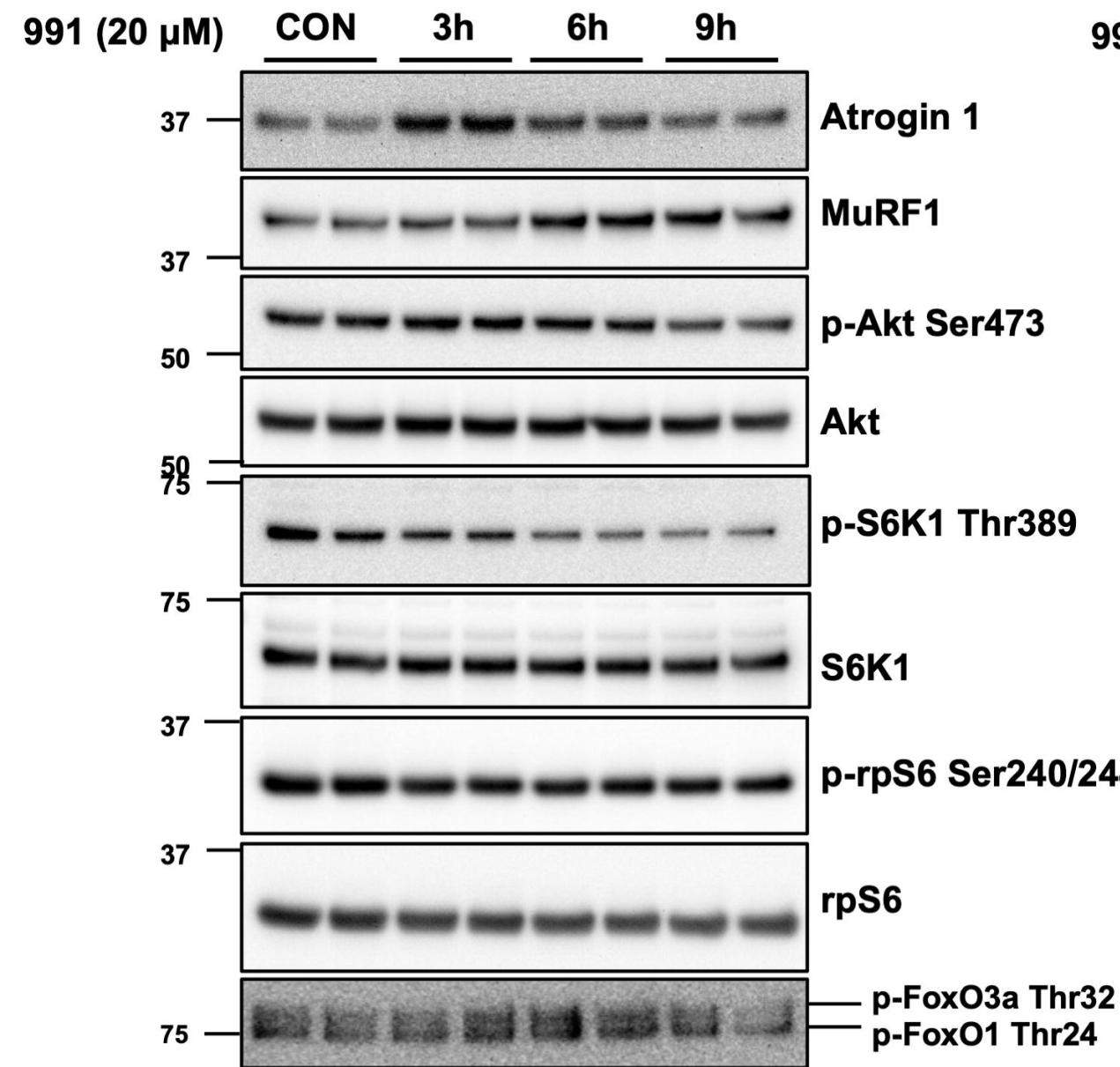
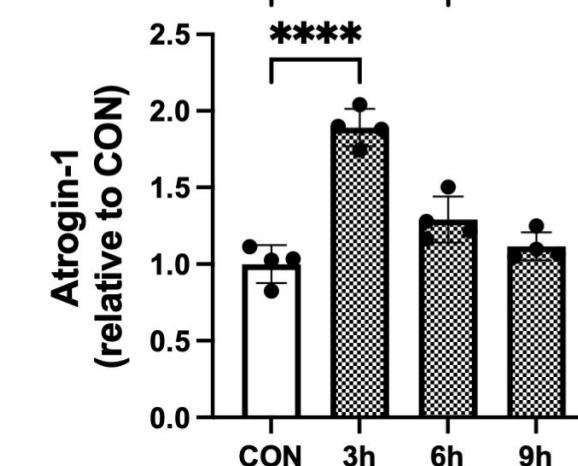
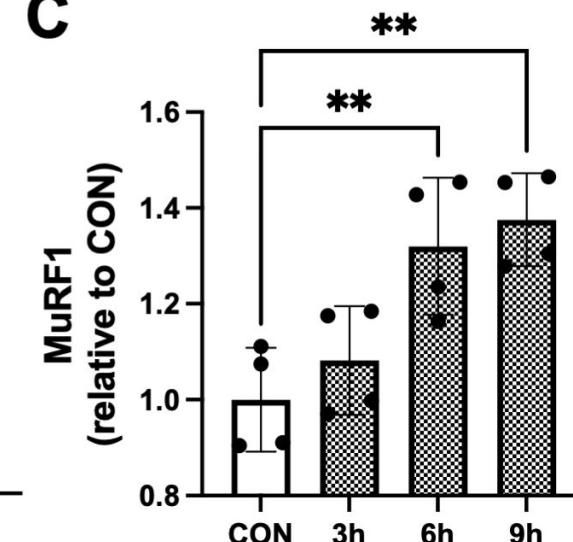
551 **Figure 6. Atrogin-1 and MuRF1 protein contents are differentially regulated in Akt and**  
552 **the rapamycin-sensitive mTOR-S6K1 signaling pathway.**

553 Insulin/IGF-1/Akt/FoxO signaling pathway is a predominant mechanism regulating Atrogin-  
554 1 and MuRF1 expression at both mRNA transcription and protein levels in skeletal muscle.

555 Upon insulin or IGF-1 stimulation, the binding of their respective receptors triggers a  
556 signaling cascade to activate Akt. Akt phosphorylates and inhibits FoxO by preventing their  
557 localization to the nuclei, and thus FoxO remains in the cytoplasm. In catabolic conditions,  
558 FoxO is less phosphorylated and remains in the nuclei to promote Atrogin-1 and MuRF1  
559 mRNA transcription and thereby increasing their protein content. Inhibition of mTORC1 or  
560 S6K1, one of the Akt downstream signaling, can promote Atrogin-1, but not MuRF1, protein  
561 content without altering Akt and FoxO phosphorylation. The evidence indicates that Atrogin-  
562 1 and MuRF1 protein content are regulated by at least two different mechanisms. How  
563 rapamycin-sensitive mTOR and S6K dependent signaling pathway regulate Atrogin-1 protein  
564 content remains undetermined.

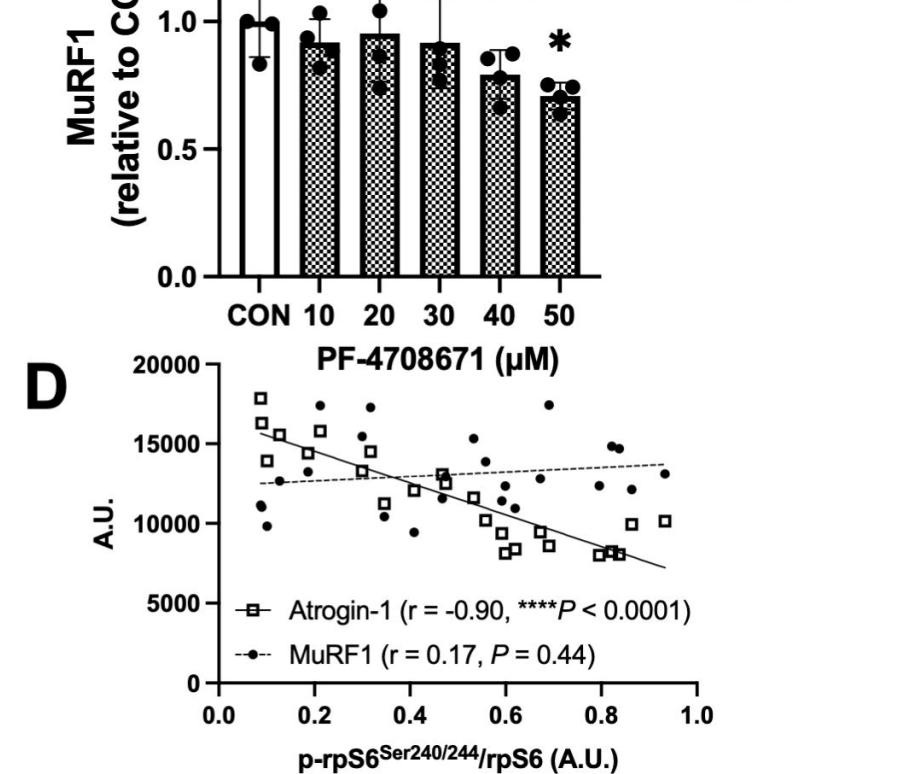
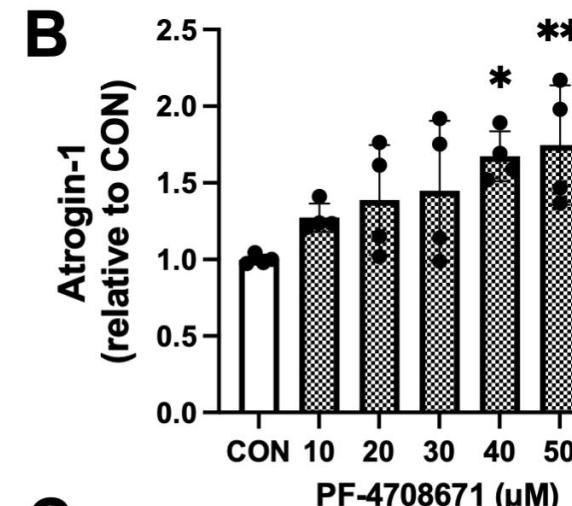
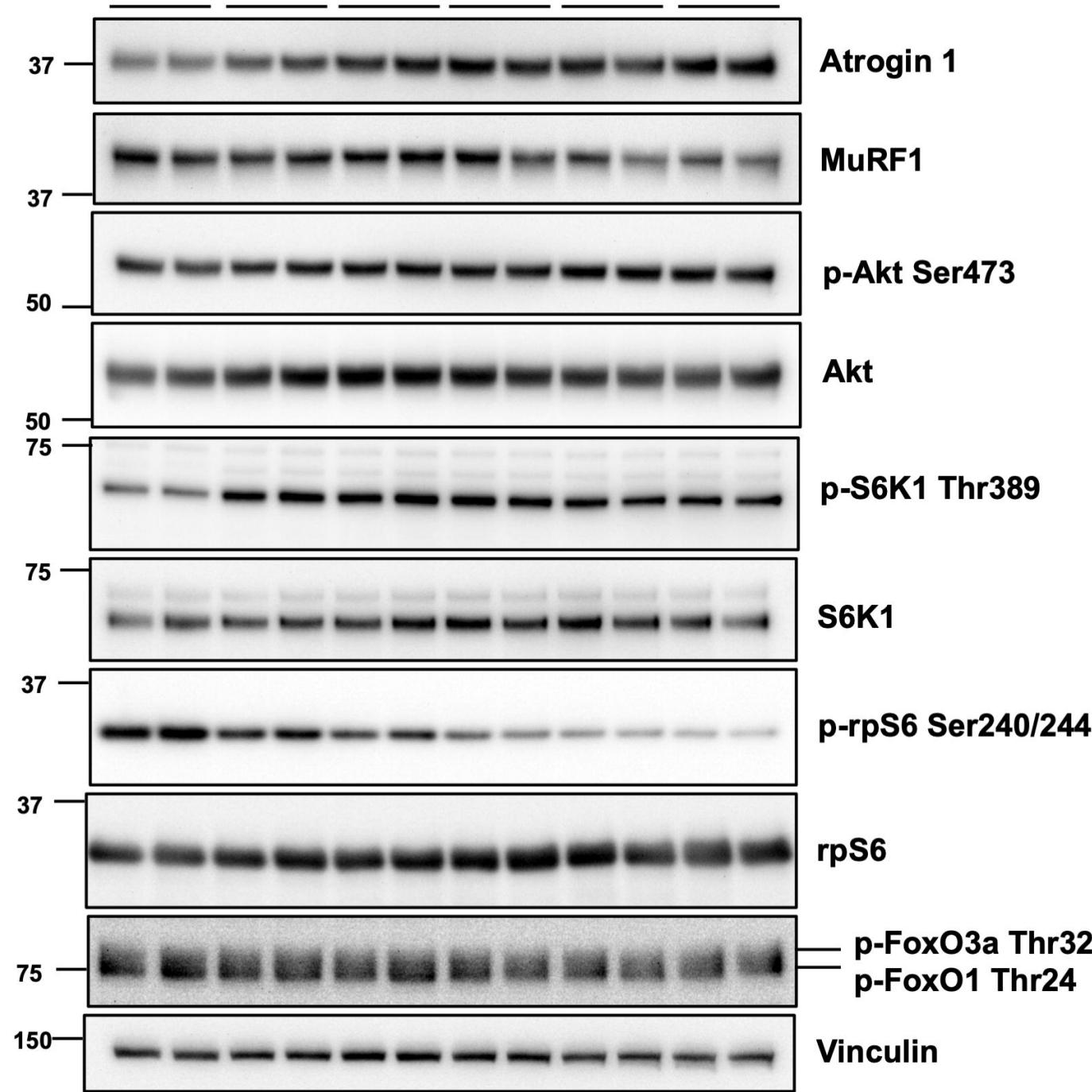
**A****B****C**

**A****B****C**

**A****B****C**

PF-4708671 (3h) CON 10  $\mu$ M 20  $\mu$ M 30  $\mu$ M 40  $\mu$ M 50  $\mu$ M

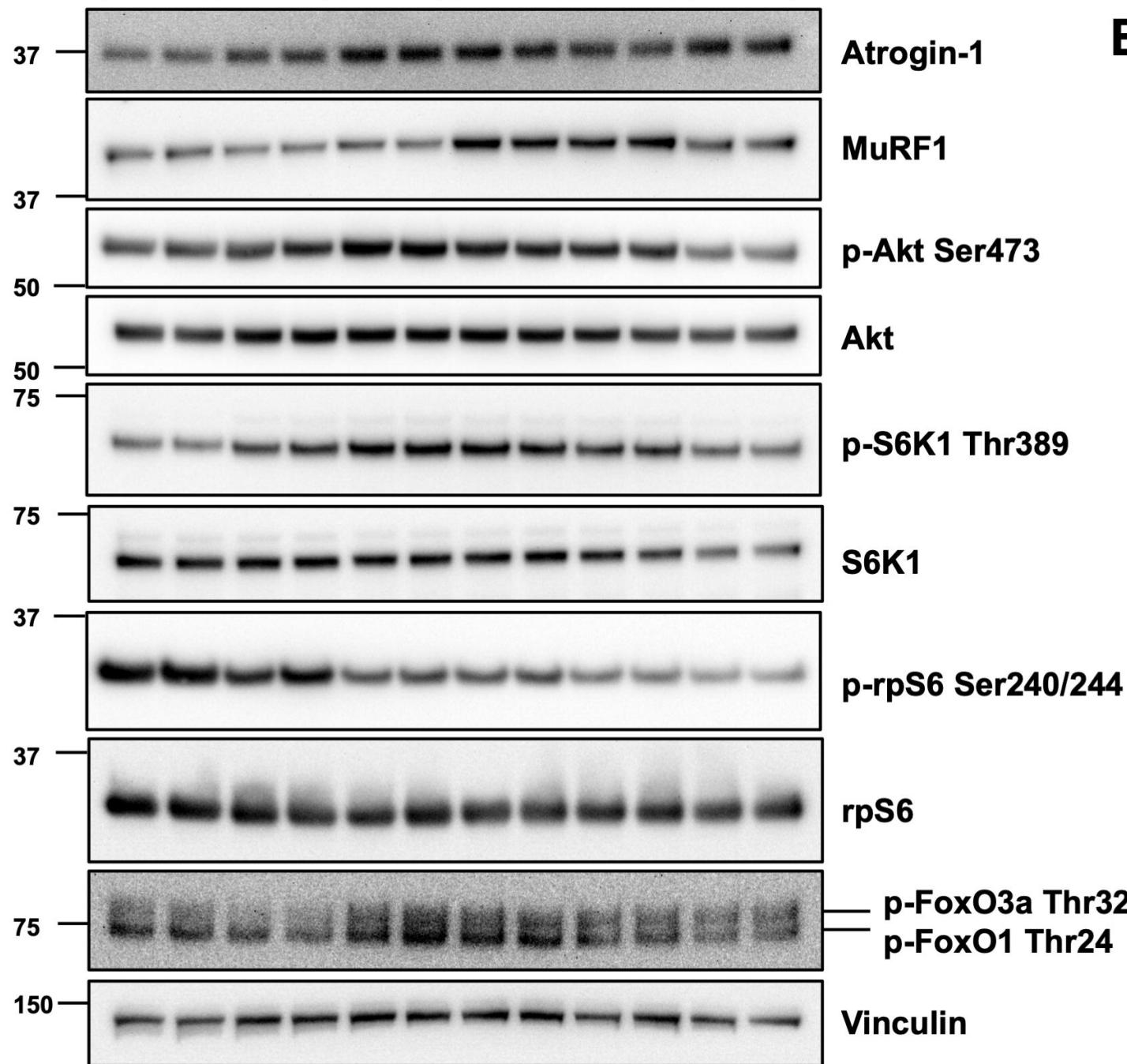
**A**



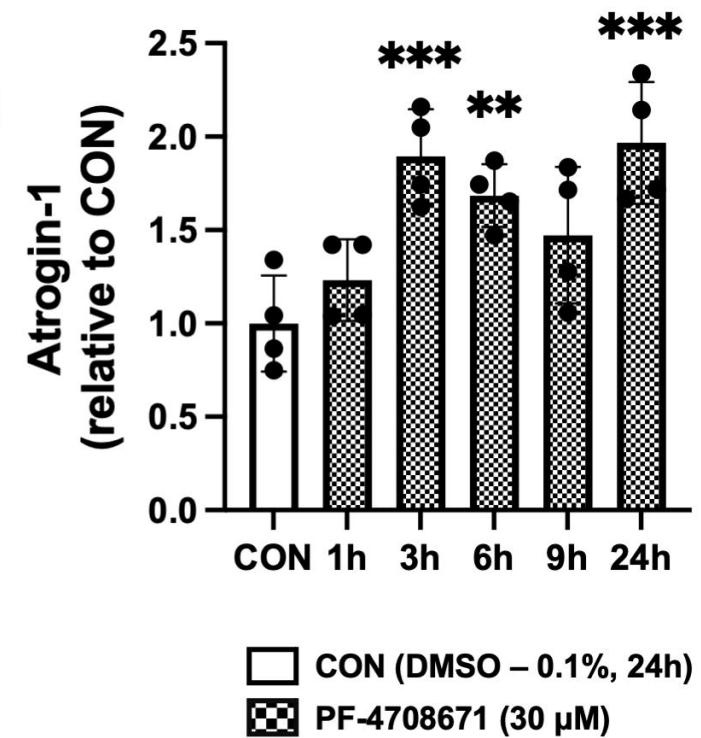
PF-4708671 (30  $\mu$ M)

CON 1 h 3 h 6 h 9 h 24 h

**A**



**B**



**C**

