

Knockdown of the E3 Ubiquitin ligase UBR5 and its role in skeletal muscle anabolism

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Abstract

UBR5 is an E3-ubiquitin-ligase positively associated with anabolism, hypertrophy and recovery from atrophy in skeletal muscle. The precise mechanisms underpinning UBR5's role in the regulation of skeletal muscle mass remains unknown. The present study aimed to elucidate these mechanisms by silencing the UBR5 gene *in-vivo*. To achieve this aim, we electroporated a UBR5-RNAi plasmid into mouse tibialis anterior muscle to investigate the impact of reduced UBR5 on mechano-transduction signalling MEK/ERK/p90RSK and Akt/GSK3 β /p70S6K/4E-BP1/rpS6 pathways. Seven days post UBR5 RNAi electroporation, while reductions in overall muscle mass were not detected, mean CSA of GFP-positive fibers was reduced (-9.5%) and the number of large fibers was lower versus the control. Importantly, UBR5-RNAi significantly reduced total RNA, muscle protein synthesis, ERK1/2, Akt and GSK3 β activity. Whilst p90RSK phosphorylation significantly increased, total p90RSK protein levels demonstrated a 45% reduction with UBR5-RNAi. Finally, these early events after 7 days of UBR5 knockdown culminated in significant reductions in muscle mass (-4.6%) and larger reductions in fiber CSA (-18.5%) after 30 days. This was associated with increased levels of the phosphatase PP2Ac, and inappropriate chronic elevation of p70S6K and rpS6 between 7 and 30 days, and corresponding reductions in eIF4e. This study demonstrates UBR5 plays an important role in anabolism/hypertrophy, whereby knockdown of UBR5 culminates in skeletal muscle atrophy.

Running title: UBR5 knockdown and skeletal muscle anabolism

Keywords UBR5, siRNA, RNAi, Skeletal Muscle, Hypertrophy, Electroporation, MAPK, ERK, p90RSK, Akt, P70S6K, rpS6, eIF4e, PP2Ac.

1 Introduction

2 The regulation of skeletal muscle (SkM) mass is orchestrated by the activity of key signalling
3 pathways that control protein breakdown and synthesis within myofibers. The breakdown or
4 atrophy of SkM mass is mediated, in-part, by the ubiquitin-proteasome system (3, 7), which is
5 composed of three key enzymes that activate and conjugate (E1 & E2 enzymes) small ubiquitin
6 molecules to target protein substrates (E3 ligases) for recognition and subsequent degradation in
7 the 26S proteasome. The most characterised E3 ubiquitin ligases associated with SkM atrophy
8 are the muscle specific RING finger protein 1 (MuRF1 or Trim63) and the F-box containing ubiquitin
9 protein ligase atrogin-1 (Atrogin-1; 16)), otherwise known as muscle atrophy F-box (MAFbx; 4).

10

11 Interestingly, recent work identified that a HECT domain E3 ligase named, ubiquitin protein ligase
12 E3 component n-recognin 5 (EDD1 or UBR5) was significantly altered at the DNA methylation and
13 gene expression level following resistance exercise (RE) in human SkM (32, 33). UBR5 DNA
14 methylation decreased (hypomethylated) and mRNA expression increased after 7 weeks of training-
15 induced SkM hypertrophy, with further enhanced changes reported following a later 7 weeks of
16 retraining (32, 33). The pattern observed in DNA methylation and gene expression, which
17 significantly correlated with changes in lean mass, suggested that there may be a role for UBR5
18 during muscle hypertrophy in contrast to E3 ligases, MuRF1 and MAFbx that are associated with
19 atrophy. Additional work has provided further support for this hypothesis, whereby UBR5
20 expression significantly increased after acute mechanical loading in bioengineered SkM *in-vitro*, in
21 response to synergistic ablation/functional overload (FO), and after programmed resistance training
22 in rodent muscle *in-vivo*, yet with no change in MuRF1 and MAFbx expression (31). UBR5 also
23 increased during recovery from hindlimb unloading (HU) and tetrodotoxin induced-disuse atrophy,
24 again with no increase in MuRF1 and MAFbx (31). Furthermore, increased gene expression of UBR5
25 in these models resulted in greater abundance of UBR5 protein content following FO-induced
26 hypertrophy of the mouse plantaris muscle *in-vivo*, and over the time-course of regeneration in
27 primary human muscle cells *in-vitro* (31). A recent study also supported the role of UBR5 as being
28 essential for muscle growth through RNAi screening in *Drosophila* larvae, where UBR5 inhibition led
29 to smaller sized larvae (22). Collectively, these data support the notion that UBR5 is involved in load-
30 induced anabolism and hypertrophy, in contrast with the well-known MuRF1 and MAFbx E3 ligases
31 that are associated with muscle atrophy.

32 The most characterised signalling pathway involved in anabolism, protein synthesis and
33 hypertrophy of SkM is the Akt/mTORC1/p70S6K pathway (Baar *et al.*, 2000; Bodine *et al.*, 2001;

Goodman *et al.*, 2011). Further work by the Esser laboratory also reported PI3K/Akt-independent activation of mTORC via mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) signalling which was critical for overload-induced hypertrophy (27). Earlier work also demonstrated increased ERK1/2 phosphorylation after acute resistance exercise (RE) in humans (12) and after mechanical loading in C₂C₁₂ myotubes (20). Downstream of ERK1/2, phosphorylation of the ribosomal S6 kinase (p90RSK) has been shown to increase with acute endurance and resistance exercise in rodent and human SkM (28, 38, 40). Interestingly, previous work also suggests that UBR5 is regulated via ERK/p90RSK signalling in non-muscle cells (9). Indeed, UBR5 has been shown to be a target substrate for ERK2 in the COS-1 kidney fibroblast cell line after treatment with epidermal growth factor (EGF), an established ligand that initiates downstream ERK signalling when bound to its receptor (EGFR) (14). Others have also shown that p90RSK, phosphorylates UBR5 in HeLa cancer cells at various sites, and may therefore be involved in growth of cancer cells (9). Further, UBR5 has been suggested to target protein phosphatase 2A subunit C (PP2Ac; catalytic subunit) for proteasomal degradation which is reported to negatively regulate Akt and ERK cascades (24–26, 34). Therefore, given that ERK/p90RSK signalling is associated with load-induced SkM hypertrophy (27) and UBR5 is associated with ERK/p90RSK signalling in non-muscle cells (9, 14), assessing ERK/p90RSK signalling as well as established load-induced Akt/ p70S6K signalling, together with negative regulator PP2Ac, after experimental manipulation of UBR5 is required to elucidate UBR5s mechanistic role in positively regulating muscle mass.

Therefore, in the present study, we knocked down UBR5 into murine tibialis anterior (TA) muscle using a miR-based RNAi via pcDNA TM 6.2-GW/EmGFP-miR to investigate the impact of reduced UBR5 on MEK/ERK/p90RSK/MSK1 and PP2Ac/Akt/GSK3 β /p70S6K/rpS6/eIF4e signalling *in-vivo*, protein synthesis, fibre size and muscle mass. Given that increased UBR5 is associated with anabolism and hypertrophy, we hypothesised that knockdown of UBR5 would perturb ERK and Akt signalling pathways, reduce protein synthesis and result in muscle atrophy.

Methods

Skeletal Muscle Tissue

C57Bl/6 male mice between twelve and sixteen weeks-old ($n = 10$ /time point) were obtained from Charles River Laboratories for electroporation experiments. Animals were housed in ventilated cages maintained in a room at 21 °C with 12-h light/ dark cycles and had ad libitum access to standard chow (Harlan-Teklad 190 formula 7913) and water throughout the study. All animal

67 procedures were approved by the Institutional Animal Care and Use Committee at the University of
68 Iowa. During tissue collection, animals were anaesthetized with 2–3% inhaled isoflurane. On
69 completion of tissue removal, mice were euthanised by exsanguination.

70 71 *Plasmid Design and Electroporation of Skeletal Muscle Tissue In-Vivo*

72 An RNAi system which was designed and purchased from Invitrogen (Thermo Fisher Scientific, USA)
73 was implemented. Specifically, the negative control/empty vector (EV) RNAi plasmid was described
74 previously (13, 21, 31) and encodes emerald green fluorescent protein (EmGFP) and a non-targeting
75 pre-miRNA under bicistronic control of the CMV promoter in the pcDNA6.2GW/EmGFP-miR plasmid
76 (Invitrogen). The UBR5 RNAi plasmid also encodes EmGFP plus an artificial pre-miRNA targeting
77 mouse UBR5 under bicistronic control of the CMV promoter. It was generated by ligating the
78 Mmi571982 oligonucleotide duplex (Invitrogen) into the pcDNA6.2GW/EmGFP-miR plasmid. The
79 UBR5 RNAi plasmid is designed to target the nucleotide sequence in the HECT domain between
80 amino acids positions 2695-2754 (location/schematic of the RNAi on UBR5 protein structure is also
81 included in the results). The electroporation technique was performed as previously described (31).
82 Briefly, after a 2hr pre-treatment with 0.4 units/ul of hyaluronidase, 20 µg plasmid DNA was injected
83 into the tibialis anterior (TA) muscle and the hind limbs were placed between two-paddle electrodes
84 and subjected to 10 pulses (20 msec) of 175 V/cm using an ECM-830 electroporator (BTX Harvard
85 Apparatus). Using a within-animal experimental design, mice were injected with the UBR5 RNAi
86 plasmid and an empty vector (EV) control into the contralateral muscle. TA muscles were harvested
87 after 7 and 30 days.

88 89 *Skeletal Muscle Tissue Collection*

90 Following completion of the appropriate time period, mice were anesthetized with isoflurane, and
91 the TA muscles were excised, weighed, frozen in liquid nitrogen, and stored at –80°C for later
92 analyses. Muscles were collected for histology (n = 5/time point) and RNA/Protein isolation (n =
93 5/time point) and processed as described below. On completion of tissue removal, mice were
94 euthanized by exsanguination.

95 96 *Immunohistochemistry and Histology*

97 Harvested mouse TA muscles were immediately weighed and fixed in 4% (w/v) paraformaldehyde
98 for 16 h at 4°C and then placed in 30% sucrose for overnight incubation. The TA muscles were then
99 embedded in Tissue Freezing Medium (Triangle Biomedical Sciences), and a Thermo HM525 cryostat

100 was used to prepare 10 μ m sections from the muscle mid-belly. All sections were examined and
101 photographed using a Nikon Eclipse Ti automated inverted microscope equipped with NIS-Elements
102 BR digital imaging software.

103

104 *Laminin Staining*

105 TA muscle sections were permeabilized in PBS with 1% triton for 10 minutes at room temperature.
106 After washing with PBS, sections were blocked with 5% goat serum for 15 minutes at room
107 temperature. Sections were incubated with Anti-Laminin (1:500, Sigma-Aldrich Cat# L9393, RRID:
108 AB_477163) in 5% goat serum for 2 hours at room temperature, followed by two 5-minute washes
109 with PBS. Goat-anti-rabbit AlexaFluor® 555 secondary (1:333, Invitrogen Cat# A28180, RRID:
110 AB_2536164) in 5% goat serum was then added for 1 hour at room temperature. Slides were cover
111 slipped using ProLong Gold Antifade reagent (Life Technologies). Image analysis was performed
112 using Myovision software (36). Skeletal muscle fiber size was analyzed by measuring ≥ 250
113 transfected muscle fibers per muscle, per animal (10x magnification). Transfected muscle fibers
114 were identified as GFP-positive as shown in results figures for 7 days and 30 days. We also measured
115 the size of non-transfected fibers (GFP-negative) from the transfected muscles at all time points.
116 Comparison of the CSA distributions of GFP-negative fibers in EV and UBR5 RNAi transfected
117 muscles revealed no difference between groups. Therefore, fiber size comparisons were made
118 between the GFP-positive fibers in the EV controls and UBR5 RNAi transfected muscles.

119

120 *RNA Isolation and Total RNA Quantification*

121 Prior to RNA isolation, aliquots of frozen muscle powder were weighed in order to calculate RNA
122 per milligram of wet muscle tissue. Muscle powder was homogenized using RNeasy lysis reagent
123 (Sigma-Aldrich, St Louis, MO) in accordance with the manufacturer's instructions. Total RNA
124 quantity and quality was assessed for 260/280 ratios using a SpectraMax M2 Microplate reader
125 (Molecular Devices, CA, USA).

126

127 *Muscle Protein Synthesis (MPS)*

128 Changes in MPS were assessed in TA muscles transfected for 7 days by measuring the incorporation
129 of exogenous puromycin into nascent peptides as described previously (18, 37). Puromycin (EMD
130 Millipore, Billerica, MA, USA; cat. no. 540222) was dissolved in sterile saline and delivered
131 (0.02 μ mol g⁻¹ body weight by i.p. injection) 30 min prior to muscle collection. Protein synthesis was
132 measured under fed conditions and studied in the light cycle.

133 Immunoblotting

134 Frozen TA muscles were homogenized in sucrose lysis buffer (50 mM Tris pH 7.5, 250 mM sucrose,
135 1 mM EDTA, 1 mM EGTA, 1% Triton X 100, 50 mM NaF). The supernatant was collected following
136 centrifugation at 8,000 *g* for 10 minutes and protein concentrations were determined using the 660-
137 protein assay (Thermo Fisher Scientific, Waltham, MA). Twelve micrograms of protein were
138 subjected to SDS-PAGE on 4-20% Criterion TGX stain-free gels (Bio-Rad, Hercules, CA) and
139 transferred to polyvinylidene difluoride membranes (PVDF, Millipore, Burlington, MA). Membranes
140 were blocked in 3% nonfat milk in Tris-buffered saline with 0.1% Tween-20 added for one hour and
141 then probed with primary antibody (concentrations detailed below) overnight at 4°C. Membranes
142 were washed and incubated with HRP-conjugated secondary antibodies at 1:10,000 (Mouse, Cell
143 Signaling technology, RRID AB_330924; Rabbit, Cell Signaling technology, RRID AB_2099233) for one
144 hour at room temperature. Immobilon Western Chemiluminescent HRP substrate was then applied
145 to the membranes prior to image acquisition. Image acquisition and band quantification were
146 performed using the Azure C400 System (Azure Biosystems, Dublin, CA, USA) and Image Lab, version
147 6.0.1 (Bio-Rad), respectively. Total protein loading of the membranes captured from images using
148 stain-free gel technology was used as the normalization control for each phosphorylated and total
149 protein analyte. For phosphorylated to total protein ratios, normalized phosphorylation values were
150 divided by total normalized values. The following primary antibodies were used all used at a
151 concentration of 1:1000: Cell Signaling Technologies (Danvers, MA, USA) – phospho-ERK1/2
152 Thr202/Tyr204 (RRID:AB_2315112), ERK1/2 (RRID:AB_390779), phospho-p90RSK Ser380
153 (RRID:AB_2687613), p90RSK (RRID:AB_659900), phospho-MSK1 Thr581 (RRID:AB_2181783),
154 phospho-MEK1/2 Ser217/221 (RRID:AB_2138017), phospho-Akt Ser473 (RRID:AB_2315049), Akt
155 (RRID:AB_329827), phospho-GSK3β Ser9 (RRID:AB_331405), GSK3β (RRID:AB_2335664), phospho-
156 p70S6K Thr389 (RRID:AB_330944), p70S6K (RRID:AB_331676), phospho-rpS6 Ser240/244
157 (RRID:AB_10694233), rpS6 (RRID:AB_331355), phospho-4EBP1 Thr37/46 (RRID: AB_560835), 4EBP1
158 (RRID:AB_2097841) UBR5 (RRID:AB_2799679), PP2Ac (RRID:AB_561239), eIF4e (RRID:AB_823488)
159 and EMD Millipore – puromycin (RRID:AB_2566826). Knockdown of UBR5 protein was confirmed
160 previously with a reduction of 65% (± 11.8%) (Seaborne *et al.*, 2019) and all signaling antibodies in
161 the present study were processed on the same samples as this UBR5 protein data.

162

163 Statistical Analysis

164 Paired *t*-tests were carried out when comparing empty control (EV) vs. UBR5 RNAi transfected
165 mouse TA. All statistical analysis was performed using GraphPad Software (Prism, Version 7.0a, San

166 Diego, CA). Data is presented as means \pm standard error of the mean (SEM). $P \leq 0.05$ represents
167 statistical significance.

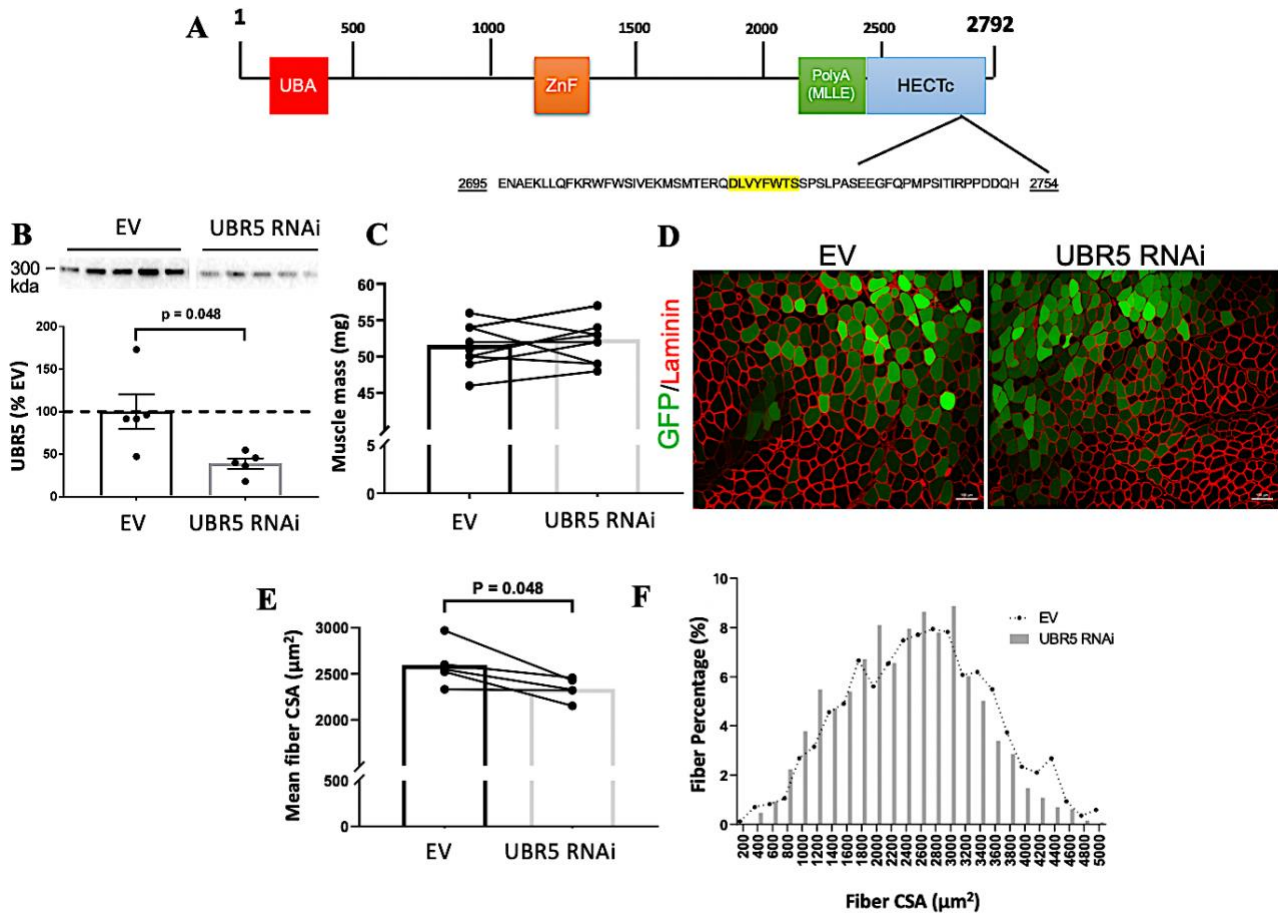
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169 **Results**

170 *Fiber CSA was significantly reduced at 7 days post UBR5 RNAi electroporation in mice*

171 To examine the effect of reducing UBR5 expression *in-vivo*, mouse TA muscles were transfected
172 with either UBR5 RNAi plasmid targeting UBR5s HECT domain (Figure 1A) or an EV control. UBR5
173 protein was significantly reduced by 65% ($\pm 11.8\%$) in UBR5 RNAi conditions (Figure 1B), as
174 previously demonstrated (31). The TA mass (mg) did not significantly change at 7 days post
175 electroporation in UBR5 RNAi vs. EV transfected TA muscle (Figure 1C). However, measurement of
176 the CSA of GFP-positive fibers revealed that the mean fiber CSA of GFP-positive fibers was
177 significantly reduced ($-9.5 \pm 3.2\%$) in RNAi transfected TA muscle ($P = 0.048$; Figure 1D & E) with
178 fewer larger fibers ($\geq 3400 \mu\text{m}^2$) present versus the EV (Figure 1D & F).

179



180

181 **Figure 1. Characteristics of tibialis anterior muscle mass and fiber CSA in UBR5 RNAi transfected TA muscle after 7**
182 **days. (A)** Schematic/ putative domain structure of UBR5 protein consisting of ubiquitin-associated (UBA) domain-like
183 superfamily, Putative zinc finger in N-recogin (ZnF), Poly-adenylate binding protein (PolyA), MLE protein/protein

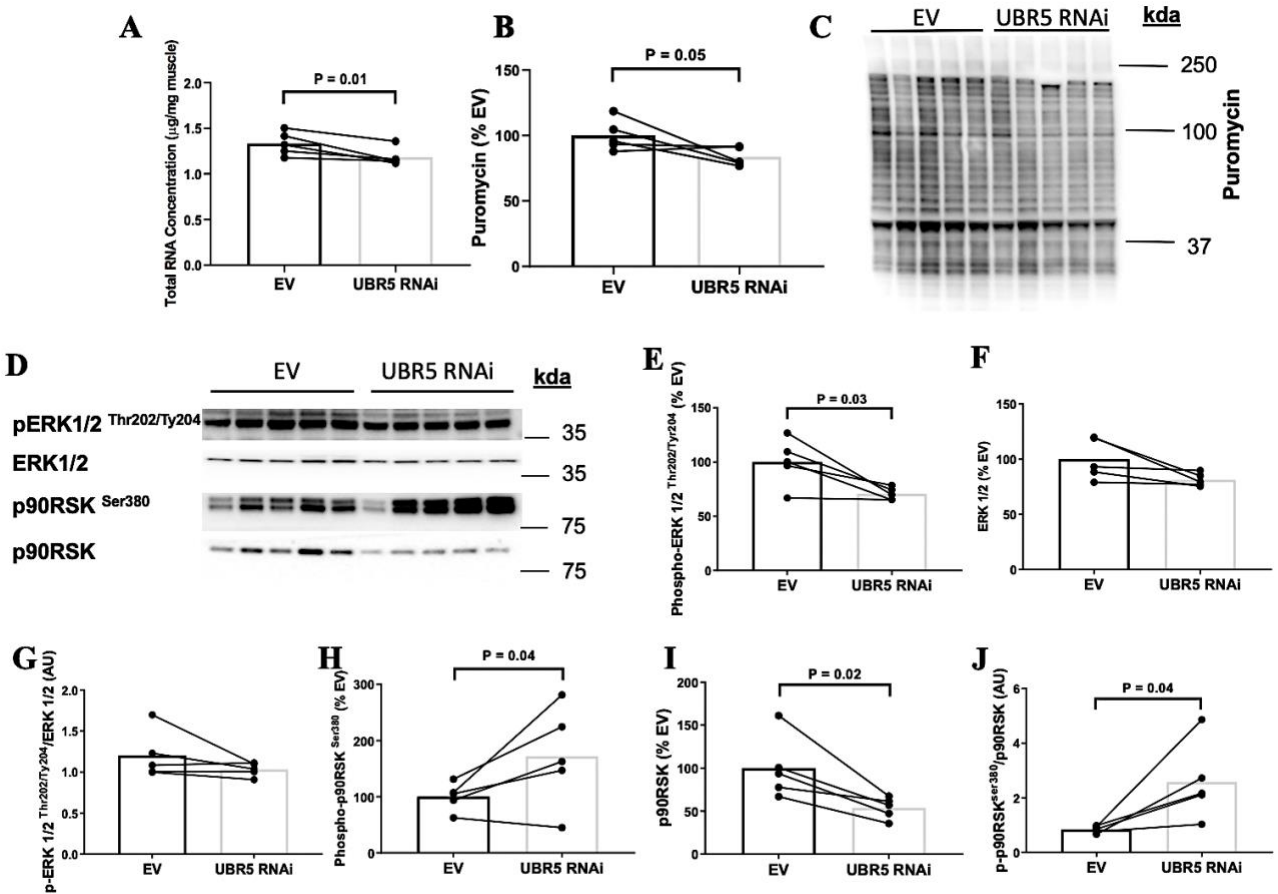
184 interaction domain and a HECT domain. The UBR5 RNAi plasmid was designed to target the nucleotide sequence in the
 185 HECT domain between amino acids position 2695-2754 (highlighted in yellow in A). **(B)** Reduction of UBR5 protein levels
 186 by 65% (Seaborne et al., 2019) in UBR5 RNAi conditions at 7 days. We have previously confirmed significant reductions
 187 in UBR5 protein levels at 7 days with this UBR5 RNAi plasmid (Seaborne et al., 2019), and all 7 day data in the present
 188 manuscript (muscle mass (C), histology (D), fiber CSA (E) RNA and signaling data – figures below) are analyzed from the
 189 same animals/samples as this UBR5 protein data. Figure 1B is therefore reused with permissions from; R. A. Seaborne
 190 et al., *Journal of Physiology* (Wiley), 597.14 (2019) pp 3727–3749, Copyright-2019 The Authors. *The Journal of*
 191 *Physiology*. Copyright-2019 The Physiological Society). **(C)** Muscle mass between empty vector control (EV) and UBR5
 192 RNAi transfected TA muscles ($n = 10$ per group). **(D)** Representative images (10x magnification; scale bar = 100 μm) for
 193 GFP transfected fiber identification and CSA quantification through laminin staining. **(E)** Mean transfected fiber CSA size
 194 in the RNAi transfected vs. EV muscles ($n = 5$ per group). **(F)** Quantification of muscle CSA revealed changes in the
 195 percentage of large fibers ($\geq 3400 \mu\text{m}^2$) with RNAi transfected muscles verses the EV group ($n = 5$ per group). Statistical
 196 significance is depicted where present ($P \leq 0.05$).

197
 198

199 *Total RNA concentrations, muscle protein synthesis, ERK1/2 and Akt activity and total p90RSK are*
 200 *reduced in UBR5 RNAi TA muscle after 7 days*

201 Along with a reduction in fiber CSA at 7 days post UBR5 RNAi, a significant reduction in total RNA
 202 concentrations ($P = 0.01$, Figure 2A) and protein synthesis ($-17 \pm 3.2\%$, $P = 0.05$, Figure 2B & 3C) was
 203 evident. Wishing to determine potential regulators of this process in the ERK signalling pathway
 204 (Figure 2D), ERK1/2 (p44/42 MAPK) was examined and its phosphorylation found to be reduced ($-$
 205 $43 \pm 2.3\%$, $P = 0.03$, Figure 2E). When calculating ERK1/2 total levels there was also a reduction,
 206 albeit non-significant (Figure 2F) making the phosphorylation to total activity ratio of ERK1/2
 207 unchanged (Figure 2G). Interestingly, the reduction in RNA, protein synthesis and ERK1/2
 208 phosphorylation coincided with a significant increase in phospho-p90RSK^{Ser380} ($170 \pm 39.8\%$, $P =$
 209 0.04 ; Figure 2H) at 7 days. This was on the background of a significant 45% decrease in total p90RSK
 210 ($-45 \pm 5.6\%$, $P = 0.02$; Figure 2I), resulting overall in an increased phosphorylation / total activity
 211 ratio for p90RSK (0.83 ± 0.1 EV vs. 2.57 ± 0.6 UBR5 RNAi, $P = 0.04$, Figure 2J). We also observed no
 212 differences in the phosphorylation of MEK 1/2^{Ser 217/221} or MSK-1^{Thr581} between RNAi and EV
 213 transfected TA muscles after 7 days (data not depicted). We next assessed protein signalling
 214 associated with the Akt pathway (Figure 3A). As with ERK1/2, there were significant reductions in
 215 Akt^{Ser473} phosphorylation ($-26 \pm 9\%$, $P = 0.03$; Figure 3B), with no changes in total levels of Akt
 216 (Figure 3C) and therefore significant reductions in Akt phosphorylation activity ratio (1.37 ± 0.1 EV
 217 vs. 1.15 ± 0.2 UBR5 RNAi, $P = 0.04$, Figure 3D). There were also significant reductions in GSK-
 218 3β ^{Ser9} phosphorylation to total ratio after 7 days post-electroporation (1.60 ± 0.1 EV vs. 1.32 ± 0.2

219 UBR5 RNAi, $P = 0.03$, Figure 3E, F, G). Interestingly, despite reductions in ERK1/2 phosphorylation,
 220 Akt and protein synthesis, we observed significant elevations in phosphorylation for p70S6K
 221 ^{Thr389} ($176 \pm 57\%$, $P = 0.02$; Figure 3H), with no changes in its total levels (Figure 3I) and therefore
 222 significantly increased phosphorylation to total ratios (2.62 ± 0.9 EV vs. 5.4 ± 1.7 UBR5 RNAi, $P =$
 223 0.02 , Figure 3J). The same trend was observed for rpS6 ^{Ser240/244} with increases in its phosphorylation
 224 ($178 \pm 39.4\%$, $P = 0.01$; Figure 4K), no change in its total (Figure 3L) and thus an increase in its activity
 225 ratio (1.62 ± 0.3 EV vs. 2.82 ± 0.3 UBR5 RNAi, $P = 0.01$, Figure 3M) in UBR5 RNAi transfected muscles
 226 at 7 days. Furthermore, no reduction in 4E-BP1 ^{Thr37/46} phosphorylation levels (Figure 3N), yet a
 227 significant reduction in its total levels (Figure 3O), resulted in a significant reduction in its activity
 228 ratio (0.68 ± 0.01 EV vs. 0.84 ± 0.05 UBR5 RNAi, $P = 0.01$, Figure 3P). Finally, there were no changes
 229 to total protein levels of eIF4e or PP2Ac at 7 days in UBR5 RNAi transfected muscles (Figure 3Q &
 230 4R respectively).



231
 232 **Figure 2. Alteration in total RNA, protein synthesis and MAPK (ERK/p90RSK) signaling in UBR5 RNAi transfected TA**
 233 **muscle after 7 days. (A)** Total RNA concentration, **(B & C)** muscle protein synthesis **(B)** assessed via western blots for
 234 puromycin incorporation **(C)**. **(D)** Western blot images for the ERK signaling pathway. **(E)** Phosphorylation levels of
 235 ERK1/2 (p44/42 MAPK), **(F)** total levels of ERK1/2, **(G)** phosphorylation to total activity ratio of ERK1/2. **(H)**
 236 Phosphorylation levels of p90RSK, **(I)** total levels of p90RSK ($P = 0.02$), and **(J)** phosphorylation to total p90RSK activity

ratio.. Total protein loading of the membranes captured from images using stain-free gel technology was used as the normalization control. N = 5 per group. Statistical significance is depicted where present ($P \leq 0.05$).

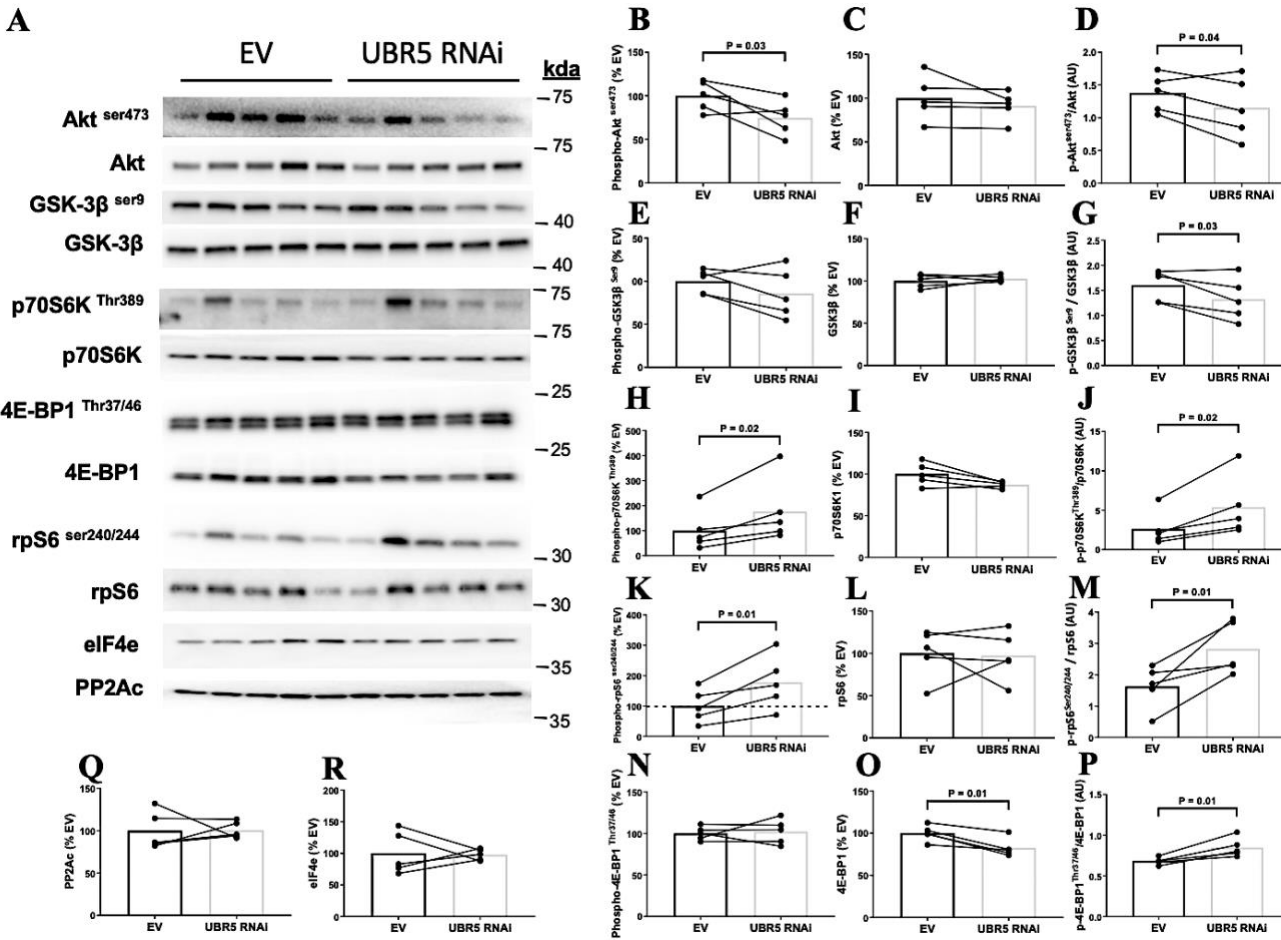


Figure 3. (A) Western blot analysis for the Akt signaling pathway (Akt, GSK-3β, p70S6K, 4E-BP1, rpS6, eIF4e, PP2Ac) in UBR5 RNAi transfected TA muscle after 7 days. (B-P) Phosphorylation, total levels and phosphorylation / total ratios respectively for Akt (B-D), GSK-3β (E-G), p70S6K (H-I), rpS6 (K-M), 4E-BP1 (N-P). (Q) PP2Ac protein levels, and (R) eIF4e levels. All n = 5 per group – EV control and UBR5 RNAi. Total protein loading of the membranes captured from images using stain-free gel technology was used as the normalization control. Statistical significance is depicted where present ($P \leq 0.05$).

Prolonged UBR5 RNAi transfection leads to significant loss of muscle mass and fiber CSA atrophy in mouse skeletal muscle

Given the data over 7 days, the next question to interrogate was the impact of UBR5 RNAi transfection in TA muscle after a prolonged period (30 days), with the hypothesis that this would perhaps lead to greater muscle atrophy vs. 7 days of UBR5 suppression. In line with this hypothesis, while UBR5 protein reductions were not maintained out to this 30-day timepoint (Figure 4A), the earlier reductions in UBR5 at 7 days led to a significant reduction in muscle mass by 30 days ($-4.6 \pm 1.5\%$) in UBR5 RNAi transfected vs. EV muscles ($P = 0.01$; Figure 4B). Alongside the reduction in muscle mass, a significant larger reduction in GFP-positive fiber CSA ($-18.2 \pm 2.3\%$ at 30 d vs. $-9.5 \pm$

3.2% at 7 d) was evident after UBR5 RNAi transfection ($P = 0.01$; Figure 4C & D) vs. EV control, with the RNAi transfected muscles displaying a shift in the distribution of fiber CSA primarily towards smaller fibers ($\geq 2600 \mu\text{m}^2$) compared to the EV control muscle (Figure 4E). These observations provide further support towards UBR5 being a positive modulator of muscle mass, with its knockdown evoking considerable atrophy.

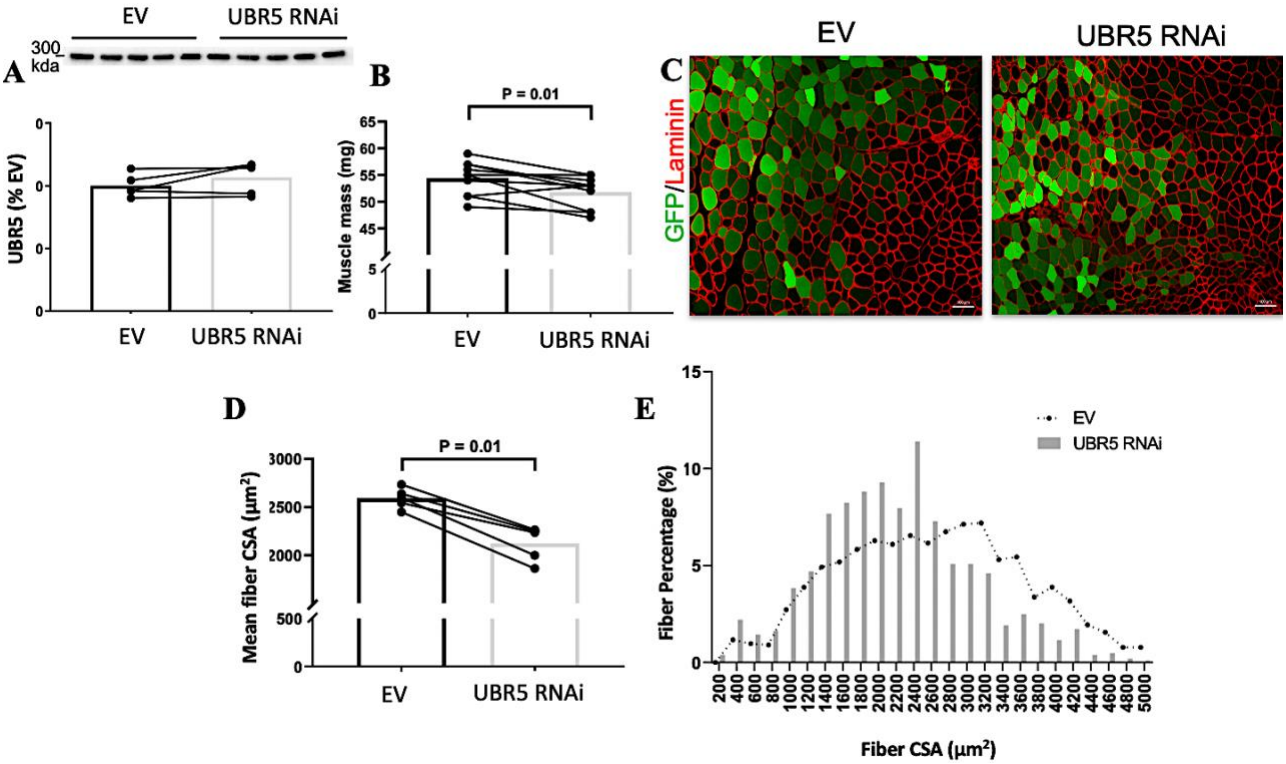
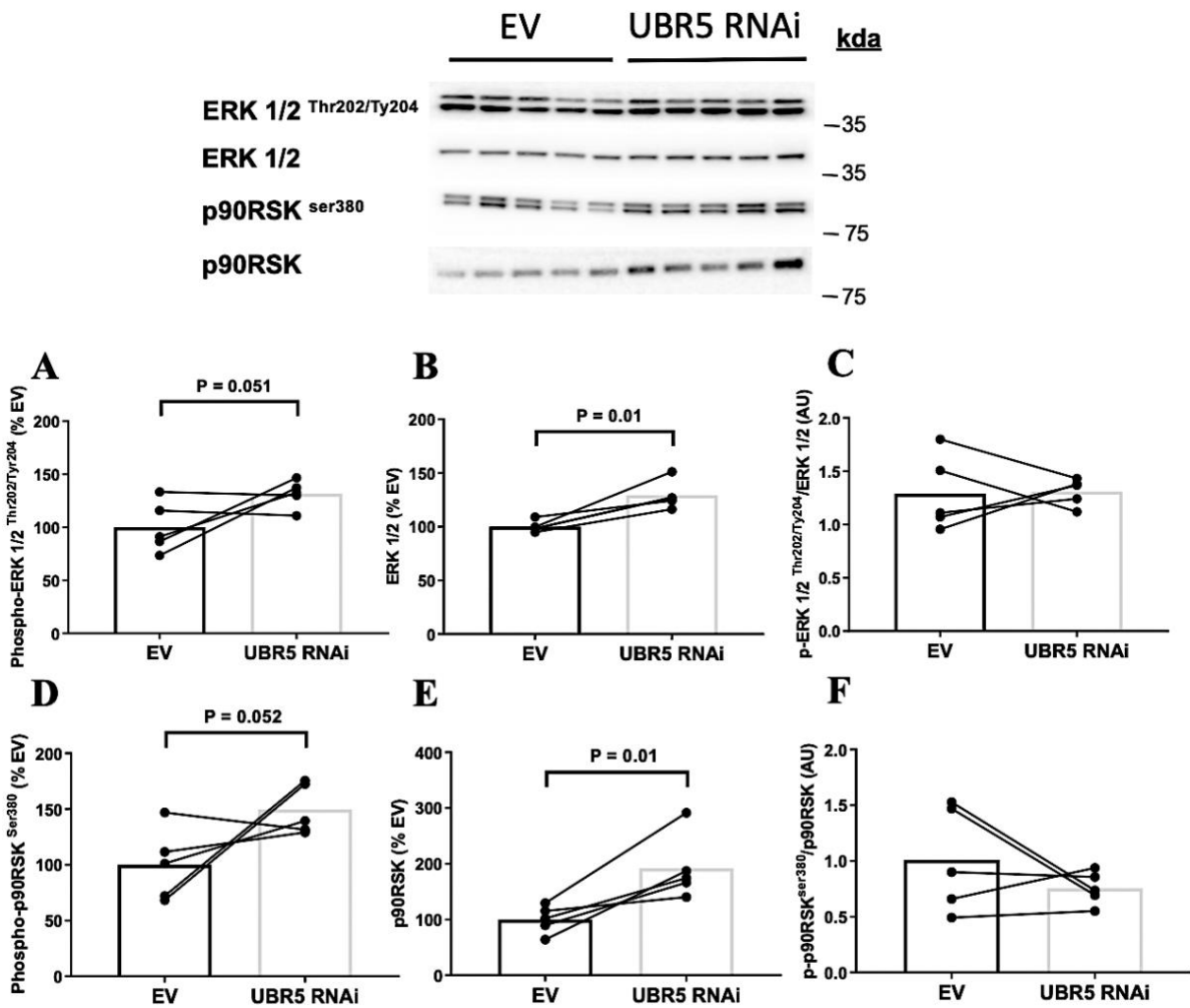


Figure 4. Loss of muscle mass and fiber CSA in UBR5 RNAi transfected TA muscle after 30 days. (A) UBR5 protein after 30 d UBR5 RNAi. (B) After 30 days, muscle mass was significantly reduced in UBR5 RNAi transfected vs. empty vector control (EV) TA muscles ($P = 0.01$; $n = 10$ per group). (C) Representative images (10x magnification; scale bar = 100 μm) for GFP transfected fiber identification and CSA quantification through Laminin staining. (D) A significant reduction ($P = 0.01$) in mean transfected fiber CSA size was observed in the RNAi transfected vs. EV muscles ($n = 5$ per group). (E) Quantification of transfected muscle CSA revealed a leftward shift from large to smaller fiber sizes ($\geq 3400 \mu\text{m}^2$) with RNAi transfected muscles versus EV group (D). Statistical significance is depicted where present ($P \leq 0.05$).

Prolonged UBR5 RNAi at 30 days and reductions in muscle mass and fiber size are associated with increased PP2Ac, reduced eIF4e and inappropriate chronic elevation of p70S6K and rpS6

At 30 days post RNAi transfection phosphorylated ERK1/2 was significantly increased (Figure 5A), as were its total levels (Figure 5B) resulting in no significant change in its activity ratio (Figure 5C). A similar trend was observed for p90RSK (Figures 5D, E, F). As with 7 days, we observed no differences in the phosphorylation of MEK 1/2^{Ser 217/221} or MSK-1^{Thr581} between RNAi and EV transfected TA muscles after 30 days (data not depicted). For the Akt signalling pathway (Figure 6A), by 30 days there was no reduction of phosphorylated Akt (Figure 6B, C, D) as observed at the 7-day timepoint.

278 However, there was a continued reduction in GSK-3 β phosphorylation with RNAi transfection
 279 (Figure 6E, F, G). Interestingly, elevation of p70S6K and rpS6 phosphorylation from the 7-day time
 280 point continued over the more chronic period of 30 days, demonstrating a 4- and 2-fold increase
 281 respectively in the RNAi transfected muscles verses the empty vector control group (Figure 6H, I, J
 282 and 6K, L, M respectively). There were no changes in activity of 4E-BP1^{Thr37/46} in UBR5 RNAi
 283 transfected muscles at 30 days (Figure 6N, O, P). Compared to the 7d time point, we also observed
 284 an 80% reduction in eIF4e protein levels in the RNAi group (Figure 6Q). Furthermore, the levels of
 285 PP2Ac were significantly increased at the 30 day time point (Figure 6R). Signalling events at 7 and
 286 30 days are summarised in the cell signalling diagram (Figure 7).



287
 288 **Figure 5. ERK pathways signaling in UBR5 RNAi transfected TA muscle after 30 days.** (A) Phosphorylated ERK1/2, (B)
 289 total ERK1/2, (C) ratio of phosphorylated ERK1/2 to total ERK1/2. (D) Phosphorylated p90RSK, (E) total p90RSK, (F) ratio
 290 of phosphorylated p90RSK to total p90RSK. All $n = 5$ per group – EV control and UBR5 RNAi. Total protein loading of the
 291 membranes captured from images using stain-free gel technology was used as the normalization control. Statistical
 292 significance is depicted on figures where present ($P \leq 0.05$).
 293

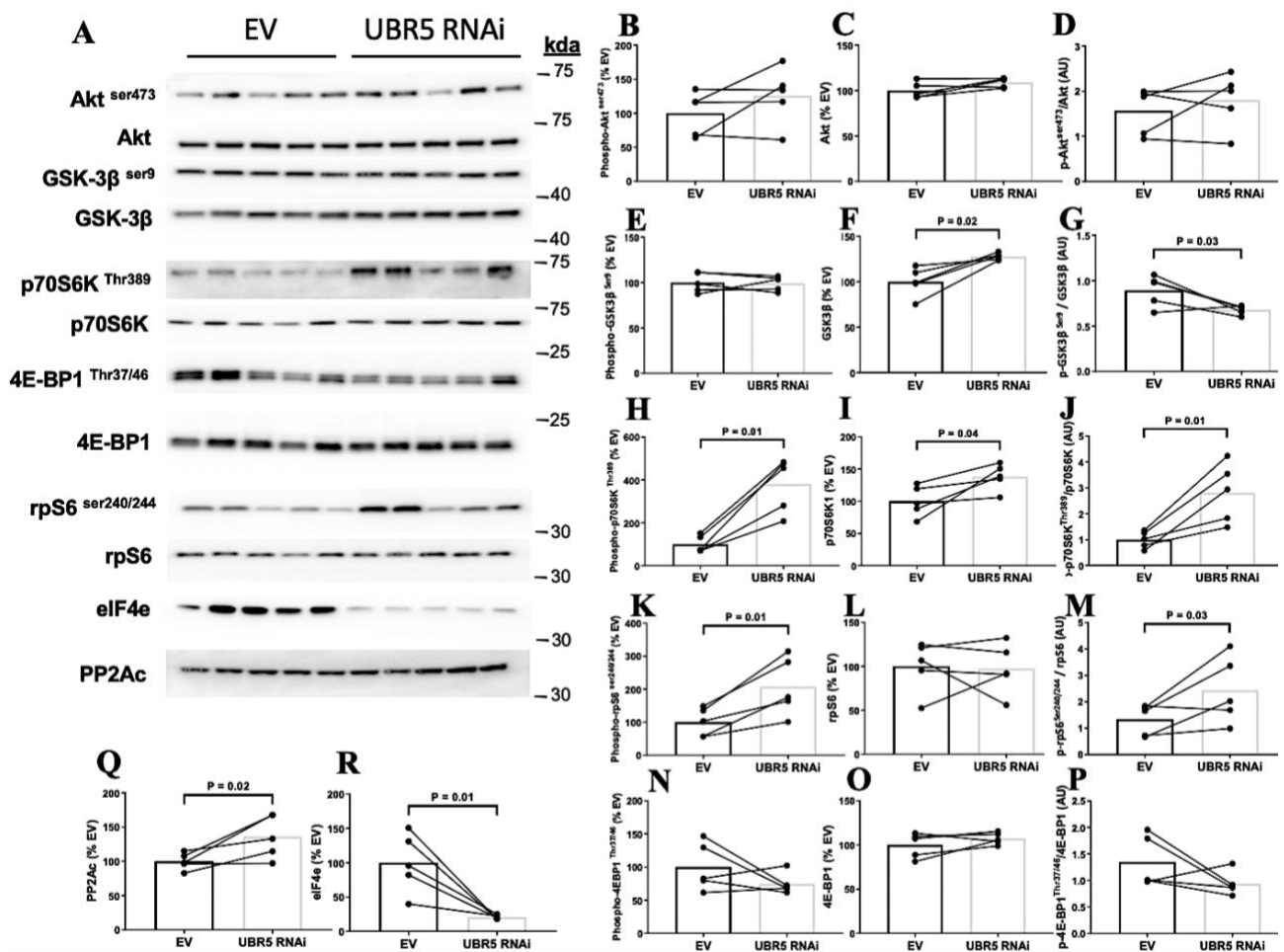


Figure 6. (A) Western blot analysis for the Akt signaling pathway (Akt, GSK-3β, p70S6K, 4E-BP1, rpS6, eIF4e, PP2Ac) in UBR5 RNAi transfected TA muscle after 30 days. (B-P) Phosphorylation, total levels and phosphorylation / total ratios respectively for Akt (B-D), GSK-3β (E-G), p70S6K (H-I), rpS6 (K-M), 4E-BP1 (N-P). (Q) PP2Ac protein levels, and (R) eIF4e levels. All $n = 5$ per group – EV control and UBR5 RNAi. Total protein loading of the membranes captured from images using stain-free gel technology was used as the normalization control. Statistical significance is depicted where present ($P \leq 0.05$).

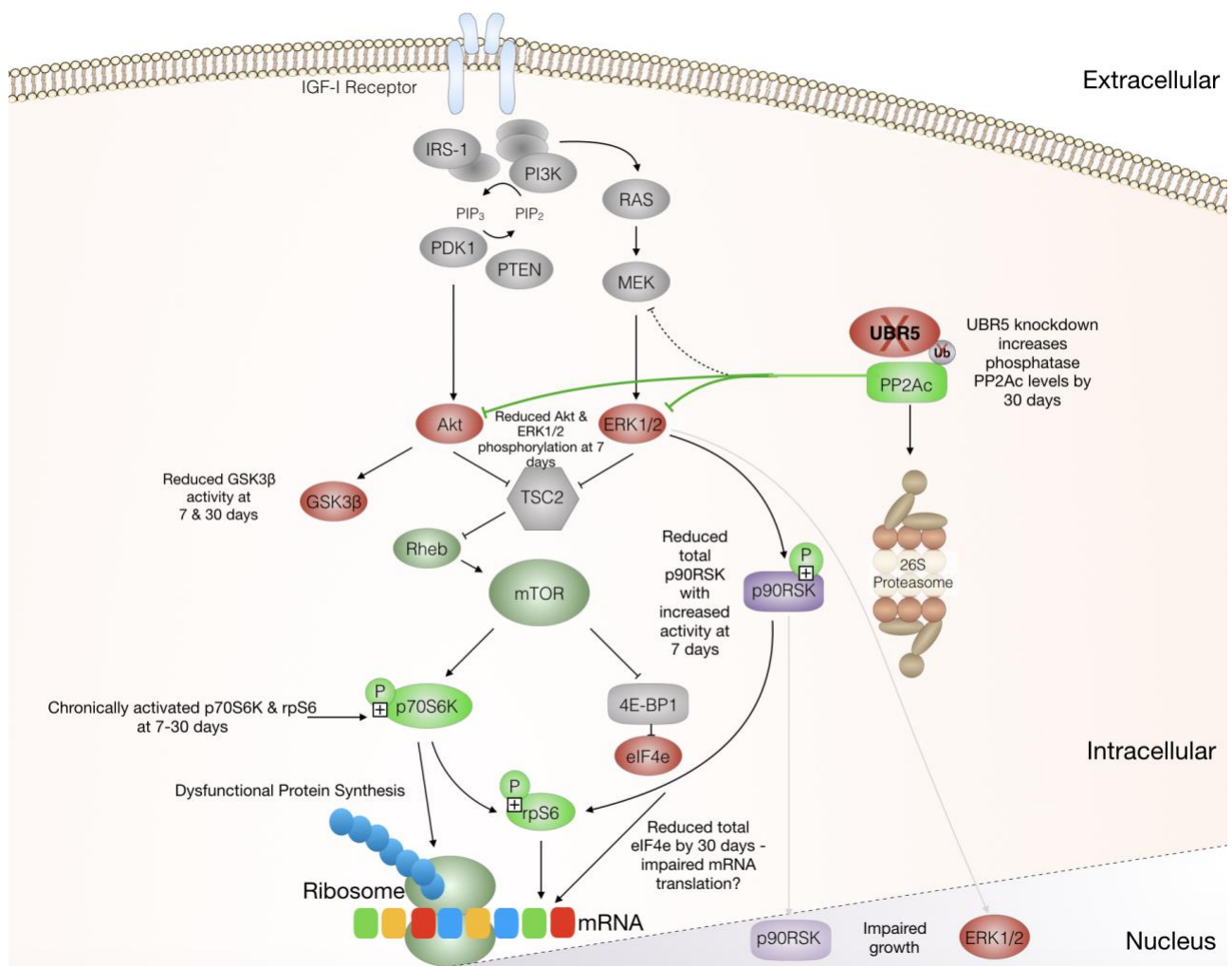


Figure 7. Schematic of cell signaling events after UBR5 knockdown in skeletal muscle in-vivo. UBR5 knockdown (X) increases phosphatase PP2Ac by 30 days; reduces ERK1/2, Akt phosphorylation at 7 days and GSK-3 β activity at 7 and 30 days (RED); reduces total p90RSK at 7 days and eIF4e at 30 days (PURPLE); chronically increases the phosphorylation of p70S6K and rpS6 (GREEN).

Discussion

The present study aimed to investigate the impact of reduced UBR5 on ERK and Akt signalling pathways in SkM tissue using our miR-based RNAi for UBR5 electroporated into the tibialis anterior (TA) of mice. Indeed, transfection of UBR5 RNAi into the TA muscle of mice after 7 days caused a significant reduction in fiber CSA, total RNA, global protein synthesis and ERK1/2, Akt phosphorylation and GSK3 β activity. However, p90RSK, P70S6K and rps6 phosphorylation significantly increased, perhaps suggestive of a potential compensatory mechanism triggered by the reduction in UBR5 or inappropriate chronic activation of these pathways, like that observed in aged muscle (discussed below). Furthermore, the increases in p90RSK phosphorylation were on a background of considerably reduced total p90RSK levels. Finally, the changes observed above at 7 days culminated in further reductions in muscle mass and fiber CSA after prolonged (30 days) UBR5

320 RNAi. This was associated with increased PP2Ac and, as hypothesised above, continued chronic
321 elevation of P70S6K and rpS6 from 7 to 30 days, as well as reductions in elongation initiation factor
322 4e (eIF4e). Overall, the present study further supports the notion that UBR5 plays an important role
323 in muscle anabolism and hypertrophy, and its reduction culminates in atrophy.

324
325 Electroporation of UBR5 RNAi for 7 days also induced a significant reduction in average myofiber
326 CSA, frequency of larger myofibers ($\geq 3400 \mu\text{m}^2$), total RNA and muscle protein synthesis (~17%).
327 The reduction in total RNA and muscle protein synthesis is interesting given the classification of
328 UBR5 as an E3 ubiquitin ligase and thus its role in the ubiquitin-proteasome system. Indeed, UBR5
329 may have unidentified functions within skeletal muscle that are unrelated to proteasomal
330 degradation. An example of an E3 ubiquitin ligase having other functions in skeletal muscle is
331 TRIM72 (known as MG53), with its role being studied in cell membrane repair (6, 10, 11). UBR5 has
332 been suggested to have a role in the regulation of mRNA translation and gene regulation through
333 the MLLE domain on the UBR5 protein structure (29, 39). Recent studies have identified the
334 translation capacity and activity of skeletal muscle to be important for growth and hypertrophy in
335 rodent and human models (19, 30, 35, 37). Therefore, while we saw reductions in ERK/ Akt
336 phosphorylation in the present study, there were opposite increases in p90RSK, p70S6K and rps6
337 suggesting that the observed reductions in protein synthesis may not be regulated by p90RSK,
338 p70S6K and rps6 activity. However, there were significant reductions in total p90RSK as a
339 consequence of UBR5 knockdown at 7d, that may contribute to the reductions in protein synthesis
340 observed. However, given the observed reduction in total RNA, there could be a role for UBR5 in
341 the regulation of mRNA translational capacity that would in-turn lead to reductions in total protein
342 synthesis and skeletal muscle mass. Indeed, supporting this notion, we also observed 80%
343 reductions of eIF4e at 30 days, an elongation initiation factor important for enhanced RNA
344 translational efficiency, splicing and stability.

345
346 Other explanations for increased p90RSK, p70S6K and rpS6 signalling activity significantly increasing
347 following UBR5 knockdown, are perhaps due to a compensatory or protective mechanism in an
348 attempt to prevent the reductions in protein synthesis. Further, in a non-classically envisaged
349 pathway, the increase in p90RSK activity may be explained due to its suggested role as an upstream
350 regulator of UBR5 and its activation could therefore be altered due to the suppression of UBR5
351 protein levels (9). This could suggest that loss of UBR5 may indirectly lead to activation of p90RSK
352 because its normal target for action is no longer present. Alongside the alterations in p90RSK

phosphorylation, as suggested above, we did observe a significant loss in total protein levels for p90RSK at 7 days which may contribute towards the alterations in p90RSK activity and the overall reductions in protein synthesis. There are currently no commercial antibodies to assess UBR5 activity in relation to p90RSK, something that warrants investigation when antibodies are developed in the future. In addition to alterations in ERK and Akt phosphorylation and reduced total p90RSK and protein synthesis at 7 days, a prolonged time period after transfection of the UBR5 RNAi at 30 days resulted in greater reductions in both muscle mass and fiber CSA. The loss of muscle mass and fiber size at 30 days was surprisingly accompanied by increased p70S6K and rpS6 phosphorylation. The chronic activation of mTORC/p70S6K signalling has been observed to be detrimental in skeletal muscle homeostasis, such as that with increasing age (2, 8, 23, 34). Therefore, subsequent future studies will seek to address the interplay between UBR5 and Akt/mTORC/p70S6K signalling with aging.

Finally, in the present study we observed significant increases in PP2Ac levels at 30 d RNAi. PP2Ac is a phosphatase demonstrated to inhibit Akt and ERK signalling, and importantly, UBR5 in its capacity as an ubiquitin ligase has been demonstrated to target PP2A for degradation in non-muscle studies (24–26, 34). While observing an increase in PP2Ac at 30 days, the reductions in phosphorylation of Akt and ERK were observed at 7 days and not at 30 days. However, the data does suggest for the first time in skeletal muscle, that PP2A is perhaps a target of UBR5 and that knockdown of UBR5 leads to an increase in the levels of PP2A due to a potential decrease in its degradation. However, UBR5 and its protein targets remain to be elucidated in skeletal muscle and require further study given these interesting findings.

Despite the exciting and novel findings surrounding UBR5's mechanistic role in skeletal muscle, it is worth acknowledging the limitations of the study, where UBR5 protein levels were not reduced at 30 d to the same degree as the 7d time point and this may be reflective of the effectiveness of the RNAi construct being reduced and also the influence of non-transfected muscle fibers in the biochemical analyses. However, the earlier reductions in UBR5 at 7 days still evoked altered signalling and greatest loss of muscle mass by this 30-day timepoint. Suggesting the earlier reductions in UBR5 at 7 days still culminated in later atrophy by 30 days. Lastly, it would also be prudent in future experiments to also evoke hypertrophy (e.g. via synergistic ablation) in rodents in the presence or absence of the UBR5 RNAi plasmid.

386 **Conclusion**

387 The present study supports the notion that UBR5 plays an important role in muscle
388 anabolism/hypertrophy, and presents novel findings demonstrating knockdown of UBR5 is
389 associated with early reductions in ERK1/2 and Akt phosphorylation and GSK3 β activity, total
390 p90RSK and protein synthesis that culminates in later atrophy that is associated with increased
391 PP2Ac, reduced eIF4e levels and inappropriately chronically elevated activity of p70S6K and rpS6.

392

393 **Author contributions**

394 DCT, DCH, LMB, SCB, APS conceived and designed the research. All authors were involved in
395 acquisition or analysis or interpretation of data for the work. DCT, DCH, LMB, SCB, APS drafted the
396 work. All Authors were involved in revising the work critically for important intellectual content. All
397 authors approved the final version of the manuscript.

398

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407

408 **Declarations & Competing Interests**

409 SCB is on the scientific advisory board for Emmyon Inc. The authors declare that they have no other
410 competing interests.

411

412 **Data Availability Statement**

413 The data that support the findings of this study are available from the corresponding author upon
414 reasonable request.

415

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