



LJMU Research Online

Hughes, D, Turner, DC, Baehr, LM, Seaborne, RA, Viggars, M, Jarvis, JC, Gorski, P, Stewart, CE, Owens, DJ, Bodine, S and Sharples, AP

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

<http://researchonline.ljmu.ac.uk/id/eprint/13814/>

Article

Citation (please note it is advisable to refer to the publisher's version if you intend to cite from this work)

Hughes, D, Turner, DC, Baehr, LM, Seaborne, RA, Viggars, M, Jarvis, JC, Gorski, P, Stewart, CE, Owens, DJ, Bodine, S and Sharples, AP (2021) Knockdown of the E3 Ubiquitin ligase UBR5 and its role in skeletal muscle anabolism. American Journal of Physiology: Cell Physiology. 320 (1). C45-

LJMU has developed [LJMU Research Online](#) for users to access the research output of the University more effectively. Copyright © and Moral Rights for the papers on this site are retained by the individual authors and/or other copyright owners. Users may download and/or print one copy of any article(s) in LJMU Research Online to facilitate their private study or for non-commercial research. You may not engage in further distribution of the material or use it for any profit-making activities or any commercial gain.

The version presented here may differ from the published version or from the version of the record. Please see the repository URL above for details on accessing the published version and note that access may require a subscription.

For more information please contact researchonline@ljmu.ac.uk

<http://researchonline.ljmu.ac.uk/>

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

David C. Hughes^{*4}, Daniel C. Turner^{1,2,3*}, Leslie M. Baehr^{4*}, Robert A. Seaborne^{3,5}, Mark Viggars³,
Jonathan C. Jarvis³, Piotr P. Gorski^{1,2}, Claire E. Stewart³, Daniel J. Owens³,
Sue C. Bodine^{4#} and Adam P. Sharples^{1,2,3#}

¹*Institute for Physical Performance, Norwegian School of Sport Sciences (NiH), Oslo, Norway*

²*Inst. for Science & Technology in Medicine (ISTM), School of Pharmacy and Bioengineering, Keele University, Staffordshire, UK*

³*Stem Cells, Ageing and Molecular Physiology Unit (SCAMP), Research Institute for Sport & Exercise Sciences (RISES), Liverpool John Moores University, Liverpool, UK*

⁴*Department of Internal Medicine, Division of Endocrinology and Metabolism, Carver College of Medicine, University of Iowa, Iowa City, IA, USA*

⁵*Centre for Genomics and Child Health, Blizard Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, UK*

* Primary Authors. These authors contributed equally to this work.

Corresponding authors

Corresponding Authors Details:

Sue C. Bodine & Adam P. Sharples

Email: sue-bodine@uiowa.edu & a.p.sharples@googlemail.com

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;

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

53

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

60

61 **Methods**

62 *Skeletal Muscle Tissue*

63 C57Bl/6 male mice between twelve and sixteen weeks-old ($n = 10$ /time point) were obtained from
64 Charles River Laboratories for electroporation experiments. Animals were housed in ventilated
65 cages maintained in a room at 21 °C with 12-h light/ dark cycles and had ad libitum access to
66 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 μm 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 ($0.02 \mu\text{mol g}^{-1}$ 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-GSK3 β ^{Ser9} (RRID:AB_331405), GSK3 β (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% (\pm 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.

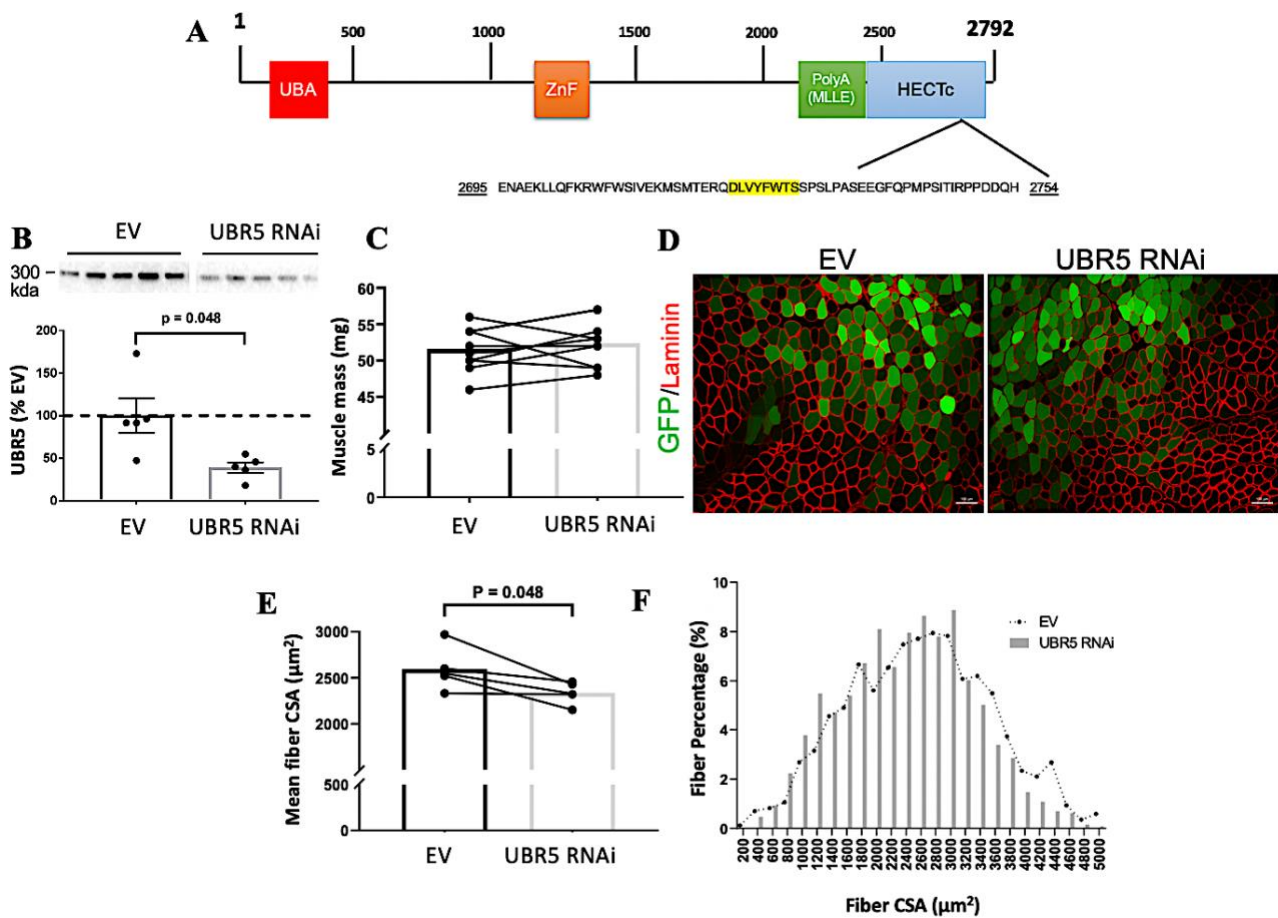
168

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), MLLE protein/protein

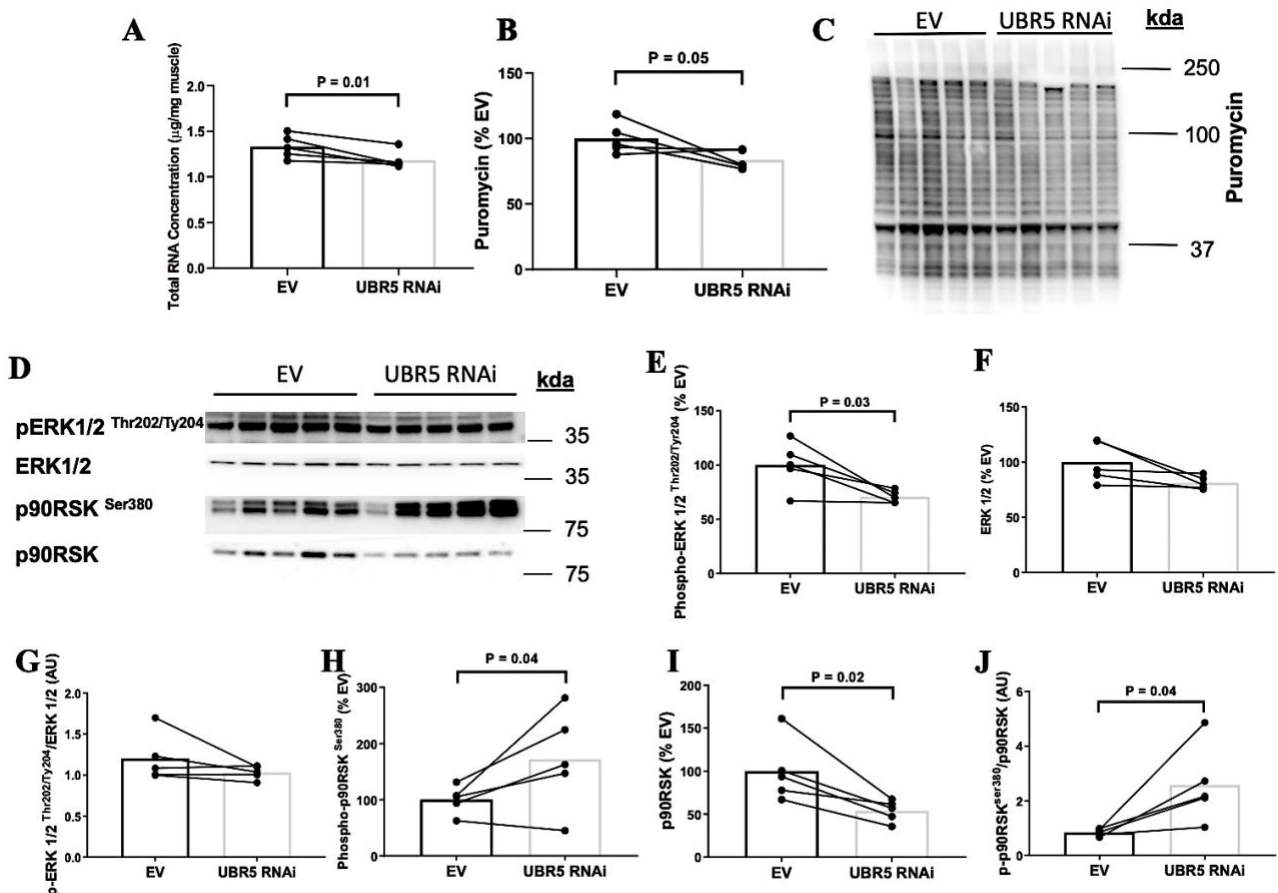
184 interaction domain and a HECT domain. The UBR5 RNAi plasmid was designed to target the nucleotide sequence in the
185 HECT domain between amino acids position 2695-2754 (highlighted in yellow in A). **(B)** Reduction of UBR5 protein levels
186 by 65% (Seaborne et al., 2019) in UBR5 RNAi conditions 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 versus 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*

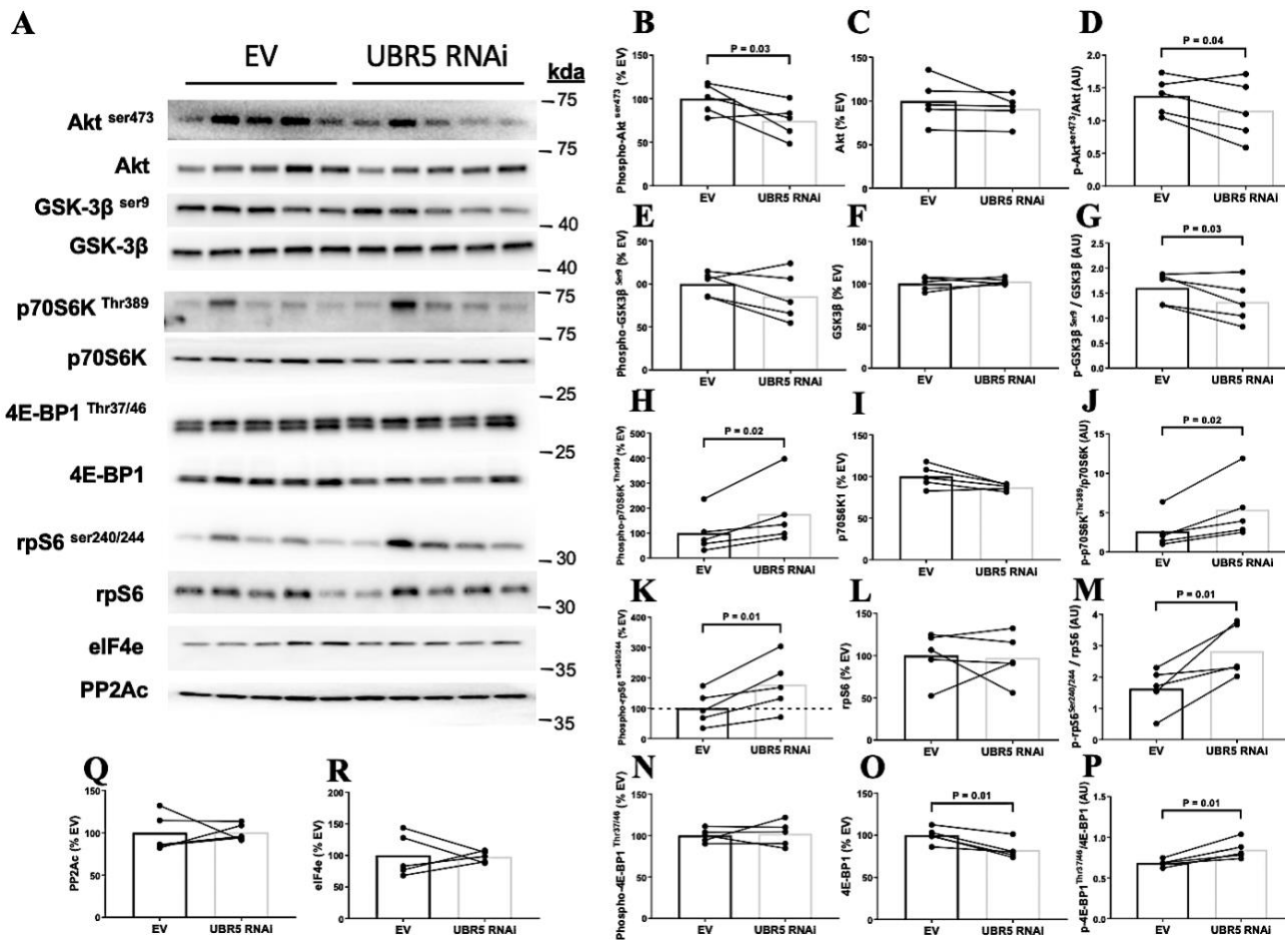
201 Along with a reduction in fiber CSA at 7 days post UBR5 RNAi, a significant reduction in total RNA
202 concentrations ($P = 0.01$, Figure 2A) and protein synthesis ($-17 \pm 3.2\%$, $P = 0.05$, Figure 2B & 3C) was
203 evident. Wishing to determine potential regulators of this process in the ERK signalling 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 Thr^{389} ($176 \pm 57\%$, $P = 0.02$; 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 $\text{rpS6}^{\text{Ser}240/244}$ 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 $\text{Thr}^{37/46}$ 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

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

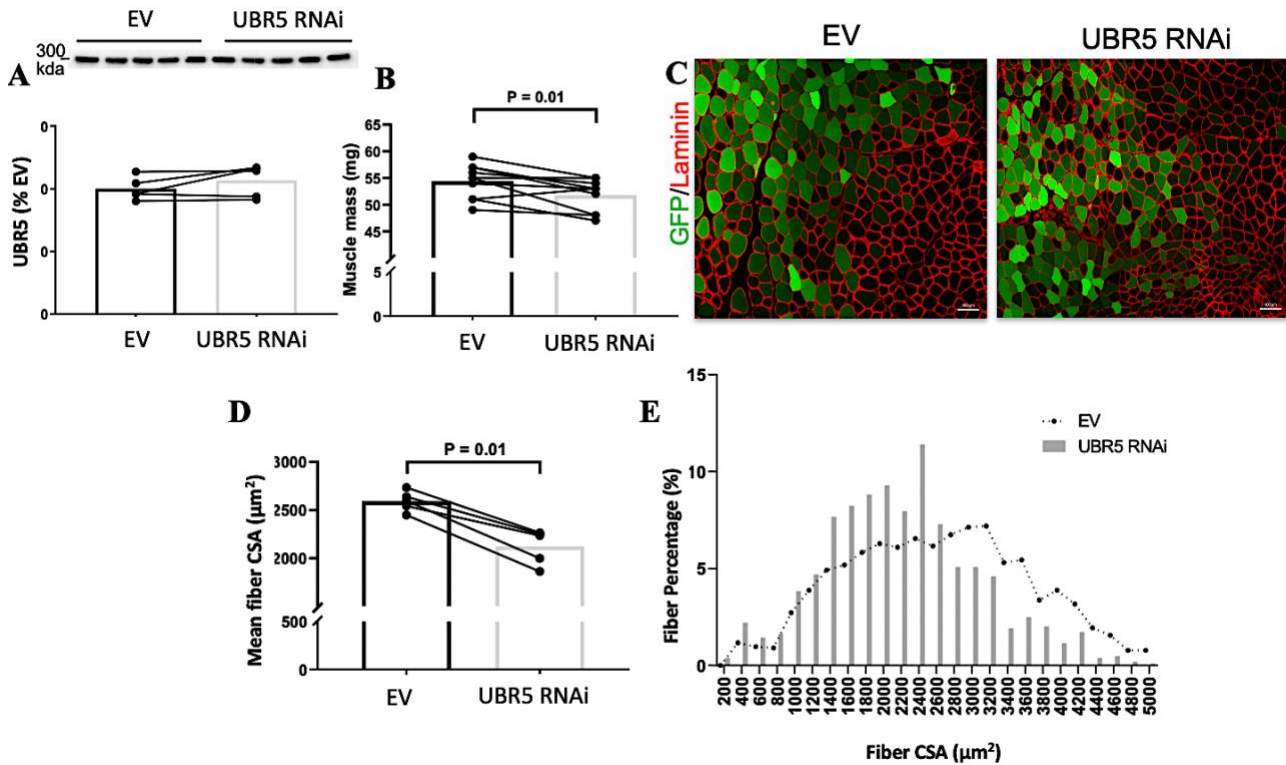


239
 240 **Figure 3. (A) Western blot analysis for the Akt signaling pathway (Akt, GSK-3 β , p70S6K, 4E-BP1, rpS6, eIF4e, PP2Ac)**
 241 **in UBR5 RNAi transfected TA muscle after 7 days. (B-P) Phosphorylation, total levels and phosphorylation / total ratios**
 242 **respectively for Akt (B-D), GSK-3 β (E-G), p70S6K (H-I), rpS6 (K-M), 4E-BP1 (N-P). (Q) PP2Ac protein levels, and (R)**
 243 **eIF4e levels. All $n = 5$ per group – EV control and UBR5 RNAi. Total protein loading of the membranes captured from images**
 244 **using stain-free gel technology was used as the normalization control. Statistical significance is depicted where present**
 245 **($P \leq 0.05$).**

246
 247 *Prolonged UBR5 RNAi transfection leads to significant loss of muscle mass and fiber CSA atrophy in*
 248 *mouse skeletal muscle*

249 Given the data over 7 days, the next question to interrogate was the impact of UBR5 RNAi
 250 transfection in TA muscle after a prolonged period (30 days), with the hypothesis that this would
 251 perhaps lead to greater muscle atrophy vs. 7 days of UBR5 suppression. In line with this hypothesis,
 252 while UBR5 protein reductions were not maintained out to this 30-day timepoint (Figure 4A), the
 253 earlier reductions in UBR5 at 7 days led to a significant reduction in muscle mass by 30 days ($-4.6 \pm$
 254 1.5%) in UBR5 RNAi transfected vs. EV muscles ($P = 0.01$; Figure 4B). Alongside the reduction in
 255 muscle mass, a significant larger reduction in GFP-positive fiber CSA ($-18.2 \pm 2.3\%$ at 30 d vs. $-9.5 \pm$

256 3.2% at 7 d) was evident after UBR5 RNAi transfection ($P = 0.01$; Figure 4C & D) vs. EV control, with
 257 the RNAi transfected muscles displaying a shift in the distribution of fiber CSA primarily towards
 258 smaller fibers ($\geq 2600 \mu\text{m}^2$) compared to the EV control muscle (Figure 4E). These observations
 259 provide further support towards UBR5 being a positive modulator of muscle mass, with its
 260 knockdown evoking considerable atrophy.

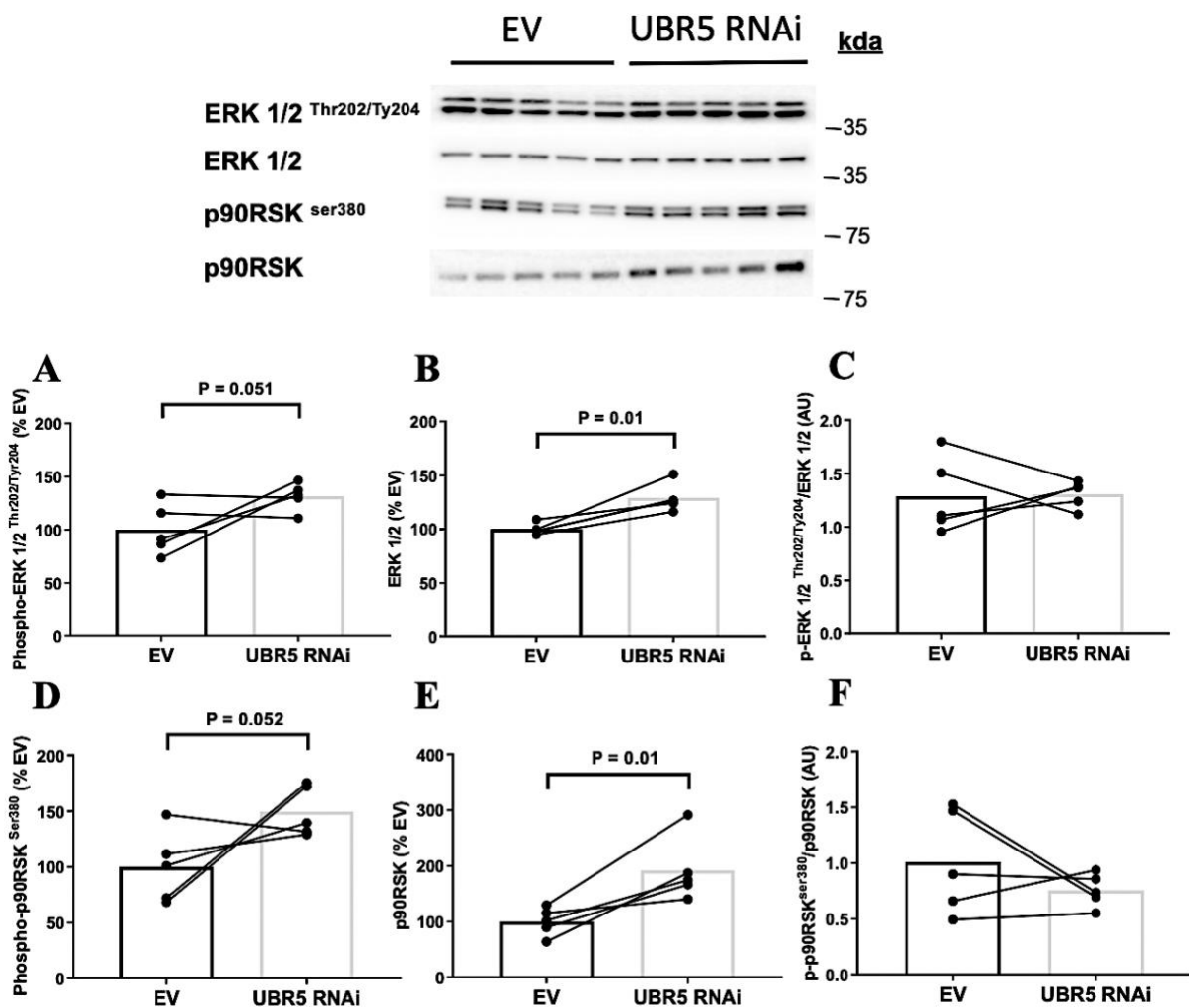


261
 262 **Figure 4. Loss of muscle mass and fiber CSA in UBR5 RNAi transfected TA muscle after 30 days.** (A) UBR5 protein after
 263 30 d UBR5 RNAi. (B) After 30 days, muscle mass was significantly reduced in UBR5 RNAi transfected vs. empty vector
 264 control (EV) TA muscles ($P = 0.01$; $n = 10$ per group). (C) Representative images (10x magnification; scale bar = 100 μm)
 265 for GFP transfected fiber identification and CSA quantification through Laminin staining. (D) A significant reduction ($P =$
 266 0.01) in mean transfected fiber CSA size was observed in the RNAi transfected vs. EV muscles ($n = 5$ per group). (E)
 267 Quantification of transfected muscle CSA revealed a leftward shift from large to smaller fiber sizes ($\geq 3400 \mu\text{m}^2$) with
 268 RNAi transfected muscles versus EV group (D). Statistical significance is depicted where present ($P \leq 0.05$).

269
 270 *Prolonged UBR5 RNAi at 30 days and reductions in muscle mass and fiber size are associated with*
 271 *increased PP2Ac, reduced eIF4e and inappropriate chronic elevation of p70S6K and rpS6*

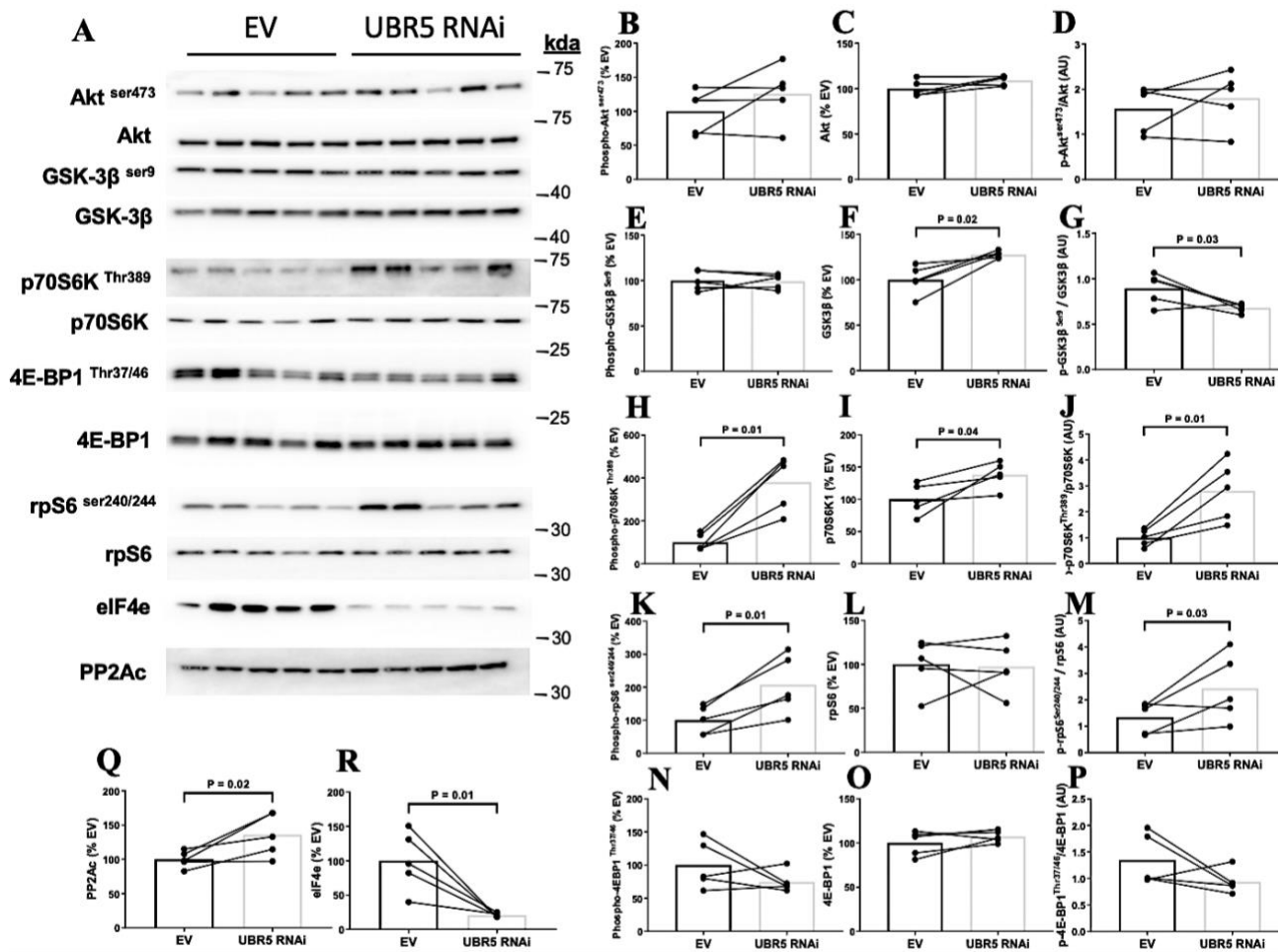
272 At 30 days post RNAi transfection phosphorylated ERK1/2 was significantly increased (Figure 5A), as
 273 were its total levels (Figure 5B) resulting in no significant change in its activity ratio (Figure 5C). A
 274 similar trend was observed for p90RSK (Figures 5D, E, F). As with 7 days, we observed no differences
 275 in the phosphorylation of MEK 1/2^{Ser 217/221} or MSK-1^{Thr581} between RNAi and EV transfected TA
 276 muscles after 30 days (data not depicted). For the Akt signalling pathway (Figure 6A), by 30 days
 277 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).



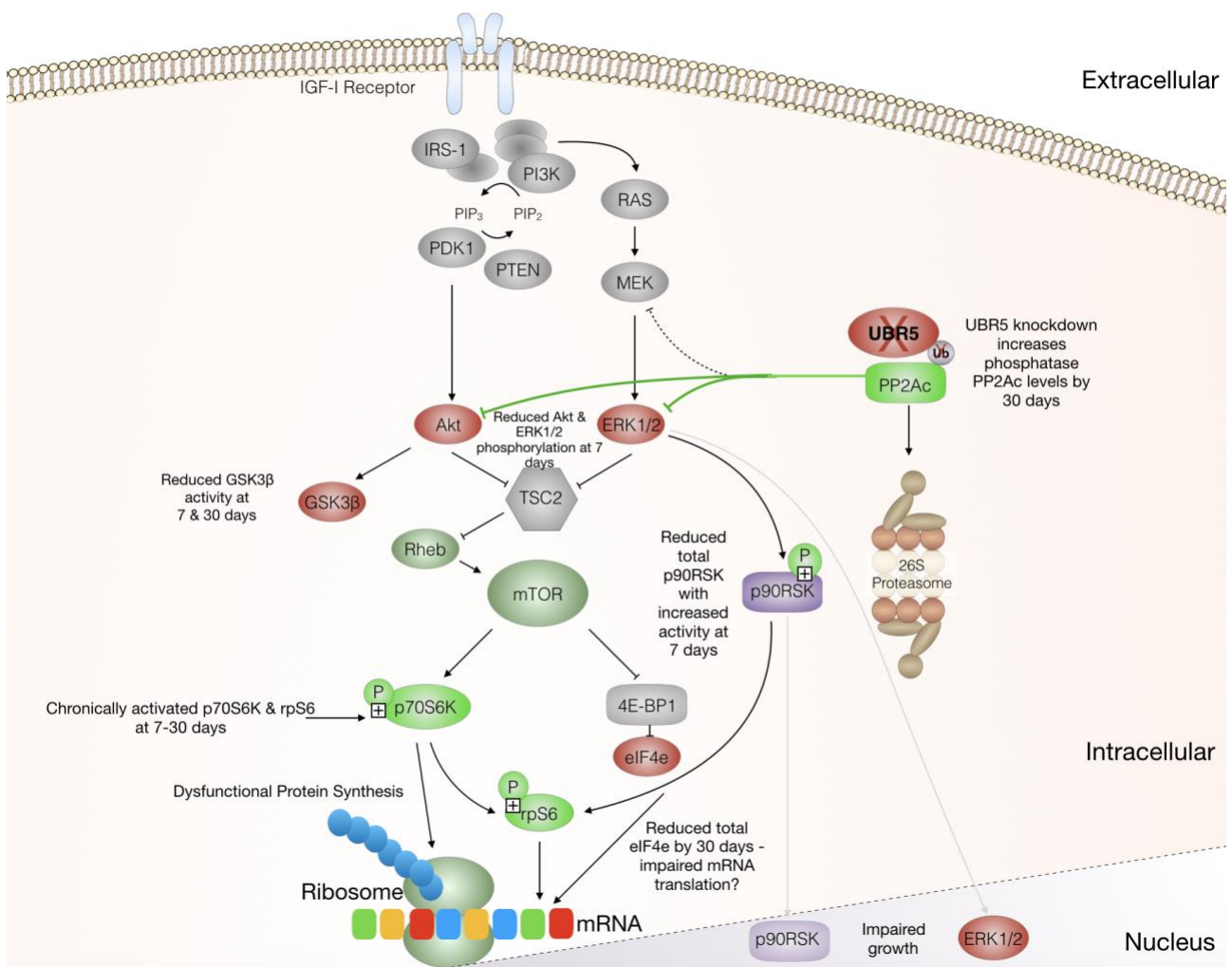
287
 288 **Figure 5. ERK pathways signaling in UBR5 RNAi transfected TA muscle after 30 days.** (A) Phosphorylated ERK1/2, (B)
 289 total ERK1/2, (C) ratio of phosphorylated ERK1/2 to total ERK1/2. (D) Phosphorylated p90RSK, (E) total p90RSK, (F)
 290 ratio of phosphorylated p90RSK to total p90RSK. All $n = 5$ 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



294
295
296
297
298
299
300
301
302

Figure 6. (A) Western blot analysis for the Akt signaling pathway (Akt, GSK-3 β , 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-3 β (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 ($P \leq 0.05$).



303

304 **Figure 7. Schematic of cell signaling events after UBR5 knockdown in skeletal muscle in-vivo.** UBR5 knockdown (X)
 305 increases phosphatase PP2Ac by 30 days; reduces ERK1/2, Akt phosphorylation at 7 days and GSK-3β activity at 7 and
 306 30 days (RED); reduces total p90RSK at 7 days and eIF4e at 30 days (PURPLE); chronically increases the phosphorylation
 307 of p70S6K and rpS6 (GREEN).

308

309 Discussion

310 The present study aimed to investigate the impact of reduced UBR5 on ERK and Akt signalling
 311 pathways in SkM tissue using our miR-based RNAi for UBR5 electroporated into the tibialis anterior
 312 (TA) of mice. Indeed, transfection of UBR5 RNAi into the TA muscle of mice after 7 days caused a
 313 significant reduction in fiber CSA, total RNA, global protein synthesis and ERK1/2, Akt
 314 phosphorylation and GSK3β activity. However, p90RSK, P70S6K and rps6 phosphorylation
 315 significantly increased, perhaps suggestive of a potential compensatory mechanism triggered by the
 316 reduction in UBR5 or inappropriate chronic activation of these pathways, like that observed in aged
 317 muscle (discussed below). Furthermore, the increases in p90RSK phosphorylation were on a
 318 background of considerably reduced total p90RSK levels. Finally, the changes observed above at 7
 319 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 MLL 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

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

365

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

375

376 Despite the exciting and novel findings surrounding UBR5's mechanistic role in skeletal muscle, it is
377 worth acknowledging the limitations of the study, where UBR5 protein levels were not reduced at
378 30 d to the same degree as the 7d time point and this may be reflective of the effectiveness of the
379 RNAi construct being reduced and also the influence of non-transfected muscle fibers in the
380 biochemical analyses. However, the earlier reductions in UBR5 at 7 days still evoked altered
381 signalling and greatest loss of muscle mass by this 30-day timepoint. Suggesting the earlier
382 reductions in UBR5 at 7 days still culminated in later atrophy by 30 days. Lastly, it would also be
383 prudent in future experiments to also evoke hypertrophy (e.g. via synergistic ablation) in rodents in
384 the presence or absence of the UBR5 RNAi plasmid.

385

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

399 **Funding**

400 DCT was funded via PhD studentships from Keele University and Liverpool John Moores University
401 (LJMU) via APS. DCTs project was further supported by the Society for Endocrinology equipment
402 grant and the North Staffordshire Medical Institute awarded to APS. RAS was funded by Doctoral
403 Alliance/ LJMU funded PhD studentship via APS and further supported by grants awarded to APS
404 from GlaxoSmithKline. PG was funded by EPSRC/MRC (UKRI) PhD studentship via APS and Keele
405 University doctoral training centre and is now supported by the Norwegian School of Sport Sciences.
406 DCH & LMB was supported by the University of Iowa in the laboratory of SCB.

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

416 **Acknowledgments**

417 We wish to thank Dr. David S. Waddell (University of North Florida) and Mr. George R. Marcotte
418 (University of Iowa) for technical assistance with the project.

419 **References**

- 420 1. **Baar K, Torgan CE, Kraus WE, Esser K.** Autocrine Phosphorylation of p70 S6k in Response to
421 Acute Stretch in Myotubes. *Mol Cell Biol Res Commun* 4: 76–80, 2000.
- 422 2. **Bentzinger CF, Lin S, Romanino K, Castets P, Guridi M, Summermatter S, Handschin C,**
423 **Tintignac LA, Hall MN, Rüegg MA.** Differential response of skeletal muscles to mTORC1
424 signaling during atrophy and hypertrophy. *Skelet Muscle* 3: 1–16, 2013.
- 425 3. **Bodine SC, Baehr LM.** Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1 and
426 MAFbx/atrogen-1. *Am J Physiol - Endocrinol Metab* 307: E469–E484, 2014.
- 427 4. **Bodine SC, Latres E, Baumhueter S, Lai VKM, Nunez L, Clarke BA, Poueymirou WT, Panaro**
428 **FJ, Erqian Na, Dharmarajan K, Pan ZQ, Valenzuela DM, Dechiara TM, Stitt TN, Yancopoulos**
429 **GD, Glass DJ.** Identification of ubiquitin ligases required for skeletal Muscle Atrophy. *Science*
430 (80-) 294: 1704–1708, 2001.
- 431 5. **Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E,**
432 **Scrimgeour A, Lawrence JC, Glass DJ, Yancopoulos GD.** Akt/mTOR pathway is a crucial
433 regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell*
434 *Biol* 3: 1014–1019, 2001.
- 435 6. **Cai C, Masumiya H, Weisleder N, Matsuda N, Nishi M, Hwang M, Ko J, Lin P, Thornton A,**
436 **Zhao X, Pan Z, Komazaki S, Brotto M, Takeshima H, Ma J.** MG53 nucleates assembly of cell
437 membrane repair machinery Chuanxi. *Nat Cell Biol* 11: 56–64, 2009.
- 438 7. **Cao PR, Kim HJ, Lecker SH.** Ubiquitin-protein ligases in muscle wasting. *Int J Biochem Cell*
439 *Biol* 37: 2088–2097, 2005.
- 440 8. **Castets P, Lin S, Rion N, Fulvio S Di, Romanino K, Guridi M, Frank S, Tintignac LA, Sinnreich**
441 **M, Ruegg MA.** Sustained Activation of mTORC1 in Skeletal Muscle Inhibits Constitutive and
442 Starvation-Induced Autophagy and Causes a Severe, Late-Onset Myopathy. *Cell Metab* 17:
443 731–744, 2013.

- 444 9. **Cho JH, Kim SA, Seo YS, Park SG, Park BC, Kim JH, Kim S.** The p90 ribosomal S6 kinase–
445 UBR5 pathway controls Toll-like receptor signaling via miRNA-induced translational
446 inhibition of tumor necrosis factor receptor–associated factor 3. *J Biol Chem* 292: 11804–
447 11814, 2017.
- 448 10. **Demonbreun AR, McNally EM.** Plasma Membrane Repair in Health and Disease. *Curr Top*
449 *Membr* 77: 67–96, 2016.
- 450 11. **Demonbreun AR, Quattrocelli M, Barefield DY, Allen M V., Swanson KE, McNally EM.** An
451 actin-dependent annexin complex mediates plasma membrane repair in muscle. *J Cell Biol*
452 213: 705–718, 2016.
- 453 12. **Drummond MJ, Fry CS, Glynn EL, Dreyer HC, Dhanani S, Timmerman KL, Volpi E,**
454 **Rasmussen BB.** Rapamycin administration in humans blocks the contraction-induced
455 increase in skeletal muscle protein synthesis. *J Physiol* 587: 1535–1546, 2009.
- 456 13. **Ebert SM, Dyle MC, Kunkel SD, Bullard SA, Bongers KS, Fox DK, Dierdorff JM, Foster ED,**
457 **Adams CM.** Stress-induced skeletal muscle Gadd45a expression reprograms myonuclei and
458 causes muscle atrophy. *J Biol Chem* 287: 27290–27301, 2012.
- 459 14. **Eblen ST, Kumar NV, Shah K, Henderson MJ, Watts CKW, Shokat KM, Weber MJ.**
460 Identification of novel ERK2 substrates through use of an engineered kinase and ATP
461 analogs. *J Biol Chem* 278: 14926–14935, 2003.
- 462 15. **Goldberg A.** Work-induced growth of skeletal muscle in normal and hypophysectomized
463 rats. *Am J Physiol* 213: 1193–8, 1967.
- 464 16. **Gomes MD, Lecker SH, Jagoe RT, Navon A, Goldberg AL.** Atrogin-1, A Muscle-Specific F-Box
465 Protein Highly Expressed during Muscle Atrophy Author(s): *PNAS* 98: 14440–14445, 2001.
- 466 17. **Goodman CA, Frey JW, Mabrey DM, Jacobs BL, Lincoln HC, You JS, Hornberger TA.** The role
467 of skeletal muscle mTOR in the regulation of mechanical load-induced growth. *J Physiol* 589:
468 5485–5501, 2011.

- 469 18. **Goodman CA, Mabrey DM, Frey JW, Miu MH, Schmidt EK, Pierre P, Hornberger TA.** Novel
470 insights into the regulation of skeletal muscle protein synthesis as revealed by a new
471 nonradioactive in vivo technique . *FASEB J* 25: 1028–1039, 2011.
- 472 19. **Hammarström D, Øfsteng S, Koll L, Hanestadhaugen M, Hollan I, Apró W, Whist JE,**
473 **Blomstrand E, Rønnestad BR, Ellefsen S.** Benefits of higher resistance-training volume are
474 related to ribosome biogenesis. *J Physiol* 598: 543–565, 2020.
- 475 20. **Hornberger TA, Armstrong DD, Koh TJ, Burkholder TJ, Esser KA.** Intracellular signaling
476 specificity in response to uniaxial vs . multiaxial stretch : implications for
477 mechanotransduction. *Am J Physiol Physiol* 288: C185–C194, 2005.
- 478 21. **Hughes DC, Baehr LM, Discroll JR, Lynch SA, Waddell DS, Bodine SC.** Identification and
479 Characterization of Fbxl22, a novel skeletal muscle atrophy-promoting E3 ubiquitin ligase.
480 *Am. J. Physiol. - Cell Physiol.* (2020). doi: 10.1152/ajpcell.00253.2020.
- 481 22. **Hunt LC, Stover J, Haugen B, Shaw TI, Li Y, Pagala VR, Finkelstein D, Barton ER, Fan Y,**
482 **Labelle M, Peng J, Demontis F.** A Key Role for the Ubiquitin Ligase UBR4 in Myofiber
483 Hypertrophy in Drosophila and Mice. *Cell Rep* 28: 1268–1281, 2019.
- 484 23. **Joseph GA, Wang SX, Jacobs CE, Zhou W, Kimble GC, Tse HW, Eash JK, Shavlakadze T, Glass**
485 **DJ.** Partial Inhibition of mTORC1 in Aged Rats Counteracts the Decline in Muscle Mass and
486 Reverses Molecular Signaling Associated with Sarcopenia. *Mol Cell Biol* 39: 1–16, 2019.
- 487 24. **Labuzan SA, Lynch SA, Cooper LM, Waddell DS.** Inhibition of protein phosphatase
488 methylesterase 1 dysregulates MAP kinase signaling and attenuates muscle cell
489 differentiation. *Gene* 739: 1–14, 2020.
- 490 25. **Macdonald TM, Thomas LN, Daze E, Marignani P, Barnes PJ, Too CK.** Prolactin-inducible
491 EDD E3 ubiquitin ligase promotes TORC1 signalling , anti-apoptotic protein expression , and
492 drug resistance in breast cancer cells. *Am J Cancer Res* 9: 1484–1503, 2019.
- 493 26. **Mcdonald WJ, Sangster SM, Moffat LD, Henderson MJ, Too CKL.** a4 Phosphoprotein

- 494 Interacts With EDD E3 Ubiquitin Ligase and Poly(A)-Binding Protein. *J Cell Biochem* 110:
495 1123–1129, 2010.
- 496 27. **Miyazaki M, Mccarthy JJ, Fedele MJ, Esser KA.** Early activation of mTORC1 signalling in
497 response to mechanical overload is independent of phosphoinositide 3-kinase/Akt
498 signalling. *J Physiol* 589: 1831–1846, 2011.
- 499 28. **Moore DR, Atherton PJ, Rennie MJ, Tarnopolsky MA, Phillips SM.** Resistance exercise
500 enhances mTOR and MAPK signalling in human muscle over that seen at rest after bolus
501 protein ingestion. *Acta Physiol* 201: 365–372, 2011.
- 502 29. **Muñoz-Escobar J, Matta-Camacho E, Kozlov G, Gehring K.** The MLLE domain of the
503 ubiquitin ligase UBR5 binds to its catalytic domain to regulate substrate binding. *J Biol Chem*
504 290: 22841–22850, 2015.
- 505 30. **Nakada S, Ogasawara R, Kawada S, Maekawa T, Ishii N.** Correlation between ribosome
506 biogenesis and the magnitude of hypertrophy in overloaded skeletal muscle. *PLoS One* 11:
507 1–14, 2016.
- 508 31. **Seaborne R., Hughes DC, Turner DC, Owens DJ, Baehr LM, Gorski P, Semenova EA, Borisov**
509 **O V., Larin AK, Popov D V., Generozov E V., Sutherland H, Ahmetov II, Jarvis JC, Bodine SC,**
510 **Sharples AP.** UBR5 is a novel E3 ubiquitin ligase involved in skeletal muscle hypertrophy and
511 recovery from atrophy. *J Physiol* 597: 3727–3749, 2019.
- 512 32. **Seaborne R., Strauss J, Cocks M, Shepherd S, O'Brien T., van Someren K., Bell P.,**
513 **Murgatroyd C, Morton J., Stewart C., Mein C., Sharples A.** Data Descriptor : Methylome of
514 human skeletal muscle after acute & chronic resistance exercise training , detraining &
515 retraining. *Sci Data* 5: 1–9, 2018.
- 516 33. **Seaborne R., Strauss J, Cocks M, Shepherd S, O'Brien T., Van Someren K., Bell P.,**
517 **Murgatroyd C, Morton J., Stewart C., Sharples A.** Human Skeletal Muscle Possesses an
518 Epigenetic Memory of Hypertrophy. *Sci Rep* 8: 1–17, 2018.

- 519 34. **Seshacharyulu P, Pandey P, Datta K, Batra SK.** Phosphatase : PP2A structural importance ,
520 regulation and its aberrant expression in cancer. *Cancer Lett* 335: 9–18, 2013.
- 521 35. **Stec MJ, Kelly NA, Many GM, Windham ST, Tuggle SC, Bamman MM.** Ribosome biogenesis
522 may augment resistance training-induced myofiber hypertrophy and is required for
523 myotube growth in vitro. *Am J Physiol - Endocrinol Metab* 310: E652–E661, 2016.
- 524 36. **Wen Y, Murach KA, Vechetti IJ, Fry CS, Vickery C, Peterson CA, McCarthy JJ, Campbell KS.**
525 Myo Vision: Software for automated high-content analysis of skeletal muscle
526 immunohistochemistry. *J Appl Physiol* 124: 40–51, 2018.
- 527 37. **West DWD, Baehr LM, Marcotte GR, Chason CM, Tolento L, Gomes A V., Bodine SC, Baar**
528 **K.** Acute resistance exercise activates rapamycin-sensitive and -insensitive mechanisms that
529 control translational activity and capacity in skeletal muscle. *J Physiol* 594: 453–468, 2016.
- 530 38. **Williamson D, Gallagher P, Harber M, Hollon C, Trappe S.** Mitogen-activated protein kinase
531 (MAPK) pathway activation: Effects of age and acute exercise on human skeletal muscle. *J*
532 *Physiol* 547: 977–987, 2003.
- 533 39. **Xie J, Kozlov G, Gehring K.** The “tale” of poly(A) binding protein: The MLL domain and
534 PAM2-containing proteins. *Biochim Biophys Acta - Gene Regul Mech* 1839: 1062–1068,
535 2014.
- 536 40. **Yu M, Blomstrand E, Chibalin A V., Krook A, Zierath JR.** Marathon running increases ERK1/2
537 and p38 MAP kinase signalling to downstream targets in human skeletal muscle. *J Physiol*
538 536: 273–282, 2001.
- 539