

1 **Inhibition of mTORC1 signaling in aged rats counteracts the decline in muscle mass and**
2 **reverses multiple parameters of muscle signaling associated with sarcopenia**

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4 Giselle A. Joseph, Sharon Wang, Weihua Zhou, Garrett Kimble, Herman Tse, John Eash, Tea
5 Shavlakadze, and David J. Glass*

6

7 Novartis Institutes for Biomedical Research, Cambridge, MA 02139

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9

10 *Correspondence: david.glass@novartis.com

11

12 **Abstract**

13 There is a lack of pharmacological interventions available for sarcopenia, a progressive age-
14 associated loss of muscle mass, leading to a decline in mobility and quality of life. We found
15 mTORC1 (mammalian target of rapamycin complex 1), a well-established critical positive
16 modulator of mass, to be hyperactivated in sarcopenic muscle. Furthermore, inhibition of the
17 mTORC1 pathway counteracted sarcopenia as determined by observing an increase in muscle
18 mass and fiber type cross sectional area, surprising because mTORC1 signaling has been shown
19 to be required for muscle mass gains in some settings. Additionally, several genes related to
20 senescence were downregulated, while gene expression indicators of neuromuscular junction
21 denervation were diminished using a low dose of a rapalog. Therefore mTORC1 inhibition may
22 delay the progression of sarcopenia by directly and indirectly modulating multiple age-associated
23 pathways, implicating mTORC1 as a therapeutic target to treat sarcopenia.

24 **Introduction**

25 Skeletal muscle size is physiologically regulated by load and activity, and can decrease when
26 load is reduced. Muscle also atrophies, or decreases in size, in pathological conditions such as
27 cancer, immobilization and denervation (1). One setting where muscle mass and function are
28 diminished is old age. This loss of muscle is called sarcopenia, and it is associated with a
29 decrease in the ability to move, leading to morbidity and ultimately mortality (2); indeed, a
30 decrease in walking speed is one of the strongest predictors of mortality in humans, and this
31 finding is associated with sarcopenia (3, 4). In addition to frailty and sarcopenia, aging of course
32 affects every tissue system and greatly increases susceptibility to other serious diseases and co-
33 morbidities, such as cancer, heart failure, chronic kidney disease, loss of vision, dementia and
34 Alzheimer's disease (1, 5, 6).

35 Experimental data strongly suggest the coordinated regulation of aging by distinct
36 molecular pathways (7); modulation of these pathways can counteract several age-related
37 diseases and co-morbidities, and prolong life (7-10). Of these signaling pathways, genetic or
38 pharmacological inhibition of the mammalian target of rapamycin (mTORC1) is thus far the
39 best-validated intervention to delay age-related pathophysiological changes (11). For instance,
40 the use of an mTORC1 inhibitor, rapamycin, even when administered at later stages in life, has
41 been shown to extend lifespan in mice (12-15). Pharmacological agents related to rapamycin are
42 called "rapalogs". Use of a rapalog for aging-like indications has recently been translated to
43 human beings, where it was shown to improve responses to vaccinations in the elderly,
44 coincident with decreasing signs of immune-senescence (16). The low dose rapalog treatment
45 used in the human study was reverse-translated to rats, where it was shown that intervention late
46 in life could prevent signs of age-related kidney pathology (17). However, there has always been

47 concern about the potential effects of rapamycin and rapalogs on skeletal muscle. For example,
48 inhibition of the mTORC1 pathway was shown to entirely block responses to compensatory
49 hypertrophy in mice (18). This certainly gave the impression that activation of mTORC1
50 signaling was desirable for the maintenance of muscle mass. Most recently it was shown that
51 rapamycin treatment inhibited muscle mass increase caused by myostatin loss (19). Thus it
52 seemed reasonable that inhibition of the pathway was not desirable in settings of muscle loss (1,
53 18, 20).

54 As to the pathway, Akt induces protein synthesis in part by activation of mTORC1
55 signaling (18, 21). mTOR exists in the distinct complexes, mTORC1 and mTORC2. mTORC1
56 is characterized by the presence of Raptor (regulatory-associated protein of mTOR) (22),
57 while TORC2 binds to Rictor (rapamycin-insensitive partner of mTOR) (23, 24). The
58 mTORC1 complex induces downstream signaling responsible for protein synthesis through
59 phosphorylation and activation of S6 Kinase 1 (S6K1), and via inhibition of 4E-BP1 (24, 25),
60 and is sensitive to inhibition by rapamycin and rapalogs. In addition to the anabolic function, Akt
61 also limits muscle protein degradation and atrophy by phosphorylating and thereby inhibiting the
62 FOXO (also known as Forkhead) family of transcription factors. Activation of FOXO3 is
63 sufficient to induce atrophy (26, 27); transgenic expression of FOXO1 also lead to an atrophic
64 phenotype (28, 29). Dephosphorylated FOXO1 and FOXO3 proteins translocate to the nucleus
65 where they induce transcription (30), upregulating the expression of the muscle-atrophy
66 associated E3 ligases, muscle RING finger 1 (MuRF1) and muscle atrophy F-box
67 (MAFbx)/Atrogin-1 (31-33). Both MuRF1 and MAFbx/Atrogin-1 are specifically upregulated in
68 atrophic conditions (34, 35), and target proteins that are critical for muscle structure and protein
69 synthesis for degradation, thereby inducing muscle loss (36-40).

70 mTORC1 inhibition has been widely suggested as a way to improve function in the
71 elderly in various tissues. However, its potential as a therapeutic intervention for the treatment of
72 sarcopenia has not been considered. Upon examination, we were surprised to learn that
73 mTORC1 signaling is upregulated rather than downregulated coincident with signs of sarcopenia
74 in rats, We therefore explored the effects of rapalog treatment in this setting. The results
75 demonstrate that the inhibition of mTORC1 is helpful in preventing pathological changes related
76 to sarcopenia.

77

78 **Results**

79 **Increased activation of the mTORC1 pathway with age**

80 Given prior reports that mTORC1 inhibition was helpful to treat a variety of age-related
81 disorders, but also the data that mTORC1 activation is required for muscle hypertrophy, we
82 conducted a time course analysis of the mTORC1 pathway to get a full scope of how its activity
83 changes with age. In laboratory settings, Sprague Dawley rats have an average lifespan of up to
84 2.5 to 3 years (41). In our study, male rats ranging from 6-months to 27-months were used.
85 Protein lysates from gastrocnemius muscles were probed for the downstream effector of
86 mTORC1, phosphorylated ribosomal protein S6 (rpS6), as a determinant of pathway activity.
87 Basal (6 hours fasted) levels of phosphorylated rpS6 gradually increased as the rats aged, with a
88 substantial increase of about 10-fold in the oldest animals aged 27-months compared with 6-
89 months. (Fig. 1A, B). The age-related increase in mTORC1 signaling coincided with a decrease
90 in muscle mass. At 21-months gastrocnemius muscle weights declined and progressively
91 atrophied at each later time point (Fig. 1C). Though muscle loss at this age is not a surprise, the
92 coincidence of this loss with mTORC1 activation was quite unexpected, given that it favors
93 muscle growth and hypertrophy.

94 **Skeletal muscle mass and quality is improved in sarcopenic rats treated with the rapalog** 95 **RAD001**

96 Experimental evidence shows that the use of rapalogs as therapeutic agents is beneficial in
97 extending lifespan and counteracting age-related morbidities in humans and other evolutionarily
98 diverse species (reviewed in (42)). We sought to determine whether rapalog treatment could
99 counter the pathophysiological changes associated with sarcopenia. Aging rats display signs of
100 sarcopenia beginning at 18-months (43). In the present study, aged rats (22-months) were dosed

101 daily with either vehicle or 0.15mg/kg RAD001 for 6-weeks. This dose of RAD001 is equivalent
102 to a clinical dose of 0.5mg in humans, ensuring therapeutic relevance (16). Vehicle treated young
103 adult rats (7-months) served as a comparative baseline for aging effects. At the end of the
104 treatment, aged and young adult rats were 24-months and 9-months old respectively, and will be
105 referred to as such.

106 To determine if we were able to ameliorate age-related muscle loss with rapalog
107 treatment, we measured the wet weights of the tibialis anterior (TA), plantaris, gastrocnemius,
108 and soleus muscles. Consistent with previous data, all muscles, except for the soleus muscle
109 from 24-month old vehicle treated rats had considerably reduced mass compared to 9-month old
110 rats (Fig. 2A). RAD001 treatment did not lead to further atrophy in any of these muscles. On the
111 contrary, rapalog appeared to be protective for aged animals, and reduced extensive muscle mass
112 loss. Plantaris and TA muscles showed a surprising increase in mass, with the TA muscle being
113 significantly increased compared to vehicle treated animals (Fig. 2A). Our data provide strong
114 evidence that when administered to sarcopenic rats, low dose rapalog treatment is not detrimental
115 to muscle mass. Rather, it allows for animals to maintain or gain muscle.

116 Changes in muscle mass often reflect morphological alterations in tissue. We performed
117 histological analysis on H&E stained plantaris muscle cross-sections. Tissue from 9-month old
118 rats had normal morphology, typical of healthy muscle (Fig. 2B). In contrast, we detected several
119 indicators of distressed muscle in aged animals that received only vehicle. A high proportion of
120 fibers had a smaller cross-sectional area, a phenotype associated with muscle atrophy (Fig. 2D,
121 E). Moreover, about 23% of myofibers from vehicle treated 24-month old muscles presented
122 with central nuclei, indicative of prior degeneration and ongoing regeneration (Fig. 2B, C). There
123 was a striking reduction in the number of myofibers with central nuclei in 24-month old plantaris

124 muscles treated with RAD001 compared with aged matched muscles treated with vehicle (Fig.
125 2B, C). In addition, consistent with the observed trend of increased plantaris muscle mass in
126 RAD001 treated rats, the average myofiber cross-sectional area tended to increase (Fig. 2D) - the
127 most obvious change was a reduced frequency of very small, mis-shaped atrophic myofibers
128 (Fig. 2E). Taken together, these data show that a low dose rapalog treatment for 6 weeks can
129 counteract age-related morpho-pathological changes in sarcopenic skeletal muscle - especially
130 signs of degeneration requiring regeneration, as measured by the presence of central nuclei.

131 Chronic activation of the mTORC1 pathway by muscle-specific deletion of *Tsc1*, a
132 negative regulator of mTORC1, has been shown to cause a late-onset myopathy, with muscle
133 atrophy in young adult mice (44). Inhibition of mTORC1 activity using rapamycin was able to
134 reverse the observed pathological changes and normalize muscle mass in these animals (44).
135 Despite evidence of similarly sustained mTORC1 signaling in aged muscle, its inhibition has not
136 yet been studied in the context of sarcopenia. Given the positive phenotypic changes in aged
137 RAD001-treated muscle, we assessed perturbation of mTORC1 pathway activity. Western blot
138 analyses confirmed that RAD001 treatment significantly reduced phosphorylation of S6K1, a
139 downstream target of mTORC1, in the muscles of old rats (Fig. 3A, B; Fig. S1). Phosphorylation
140 of rpS6, a direct downstream target of S6K1 was also reduced with RAD001 treatment (Fig. 3A,
141 C; Fig. S1). These data confirm that the relatively low dose of the rapalog used in the present
142 study was sufficient to inhibit mTORC1 signaling in aged skeletal muscle.

143 **mTORC1 inhibition reverses molecular changes associated with sarcopenia**

144 We previously reported on age-related gene expression changes that help to demonstrate the
145 molecular pathogenesis of sarcopenia (43). These data revealed the transcriptional upregulation
146 of several pathways, including pathways related to innate inflammation and senescence, cellular

147 processes modulated by mTORC1. Because RAD001-treated animals displayed a remarkable
148 sparing of muscle morho-pathology and mass with mTORC1 inhibition, we sought to determine
149 the molecular changes that could account for this.

150 The E3 ligase MuRF1 is an important regulator of atrophy (1, 34). MuRF1 gene
151 expression was analyzed in young and old muscles treated with vehicle, and in old muscles
152 treated with RAD001 (Fig. 4A). Old muscles treated with vehicle had significantly higher level
153 of MuRF1 mRNA compared with young muscles (Fig. 4A). RAD001 treatment reduced MuRF1
154 gene expression in old muscles (Fig. 4A). MaFbx, like MuRF1, is an E3 ubiquitin ligase that is
155 transcriptionally upregulated under atrophic conditions (34). There was a significant increase in
156 MaFbx expression in muscle from 24-month vehicle-treated animals compared to young
157 controls. However, its expression was not impacted by RAD001 treatment (Fig. 4A).

158 Additionally, expression of the metallothioneins, MT1 and MT2, increases during
159 atrophy and is elevated in sarcopenic muscle (45). Genetic silencing of these genes promotes
160 muscle hypertrophy *in vivo* (45). Interestingly, RAD001 treatment suppressed the MT1 gene
161 expression level in old muscles, while levels of MT2 though variable within either treatment
162 group, remained unchanged between groups (Fig. 4A). Together with the observed increase in
163 muscle mass (Fig. 2A), these data demonstrate that mTORC1 inhibition by RAD001 can prevent
164 further muscle loss by suppressing expression of atrophy markers.

165 The onset of senescence with age is associated with the inability to efficiently repair and
166 recover muscle, a contributing factor to the progressive decline in muscle mass in sarcopenia.
167 Cell cycle proteins Cdkn1a (p21) and Cdkn2a (p16) are known cellular senescence markers that
168 are upregulated with age in several tissues, including skeletal muscle (43, 46, 47). Relative to 9-
169 month old rats, Cdkn1a (p21) and Cdkn2a (p16) are both highly expressed at the mRNA level in

170 muscle from aged vehicle treated rats (Fig. 4B). RAD001 significantly reduced Cdkn1a and
171 Cdkn2a mRNA levels in 24-month old muscles compared to age-matched vehicle treated
172 controls (Fig. 4B).

173 **RAD001 treatment protects from age-associated signs of denervation**

174 Along with the deterioration of muscle tissue, the breakdown of the neuromuscular junction
175 (NMJ) also contributes to muscle weakness; abrogation of the NMJ is associated with aging in
176 rodents (48-50). Previous work identified the transcriptional perturbation of several genes
177 associated with functional denervation and the loss of motor neurons in the rat sarcopenic muscle
178 (43). We therefore investigated whether mTORC1 inhibition could reverse these transcriptional
179 changes. The expression levels of a panel of select genes, Chrna1, Chrne, MuSK, Myogenin and
180 Gadd45a, known to be markers of functional denervation (43), were determined by RT-qPCR. In
181 agreement with our previous observations, all of these genes were significantly upregulated in
182 muscles from vehicle treated 24-month old animals compared to 9-month old controls (Fig. 5).
183 Interestingly, treatment of aged animals with RAD001 reduced the transcriptional upregulation
184 of these denervation-associated gene markers in relation to their vehicle treated age-matched
185 counterparts (Fig. 5). These data suggest that suppressing the mTORC1 pathway in aged animals
186 could be protective against age-associated denervation.

187

188 **Discussion**

189 Age-associated diseases comprise many of the most serious conditions afflicting human beings:
190 sarcopenia and frailty, cancer, heart disease, Alzheimer's disease, and chronic kidney disease.
191 The mTORC1 inhibitor rapamycin and its analogs (rapalogs) have been shown to extend lifespan
192 (12-15) and delay many of these age-related conditions (9-11). These findings have even been
193 extended to human beings, where a rapalog reversed immune-senescence and increased
194 responses to vaccines that normally decline with age in the elderly (16). One area which caused
195 some concern when it came to giving mTORC1 inhibitors to aged subjects was skeletal muscle,
196 since mTORC1 activation mediates protein synthesis (51) and mTORC1 inhibition blocks load-
197 dependent hypertrophy (52). However, when we examined mTORC1 signaling in skeletal
198 muscles in rats, at ages where sarcopenia occurs (43), we were surprised to see that signaling was
199 increased rather than decreased - there was an age-related increase in the phosphorylation of
200 rpS6, a readout of mTORC1 activity. Coincident with elevated mTORC1 signaling, there was a
201 progressive decrease in skeletal muscle mass. These findings at least established that activation
202 of mTORC1 was coincident with atrophy, and therefore was not sufficient to prevent muscle loss
203 in sarcopenic conditions. We therefore asked whether counter-regulating this age-associated
204 increase in mTORC1 signaling may perhaps be beneficial for skeletal muscle, and thus we
205 treated aged rats for six weeks with a rapalog, RAD001, at a clinically relevant low dose (we
206 reverse-translated the low dose that had been used in a human study) (16). Treatment with a
207 similar low dose of the rapalog RAD001, although with a distinct dosing regimen (intermittent
208 dosing) had recently been shown to delay age-related changes in the kidney (17).

209 We were surprised to see that skeletal muscle mass increased rather than decreased as a
210 result of mTORC1 inhibition. This was not due to adverse events such as edema; muscles -

211 particularly the TA, showed increased mass, and examination of individual myofibers showed a
212 trend towards increased cross-sectional area; very small atrophic fibers that are found with age
213 were in particular absent with rapalog treatment. With age, there is a dramatic increase in fibers
214 with central nuclei - a sign of muscle undergoing degeneration followed by regeneration.
215 Treatment with the rapalog for six weeks decreased the number of myofibers with central nuclei
216 by almost half, which is a marker that there was less functional degeneration, requiring
217 subsequent regeneration. In line with this, there were also signs that functional denervation
218 occurred with age; this impression was bolstered molecularly by the demonstration that gene
219 markers associated with denervation, including MuSK and several of the acetyl choline receptor
220 genes, were increased with age - consistent with a prior report (43). These denervation markers
221 were each counter-regulated by the rapalog, indicating that rapalog treatment prevented
222 functional denervation, providing an additional mechanism for preservation of muscle mass.

223 Rapalog treatment decreased mTORC1 signaling detected by de-phosphorylation of
224 S6K1 and its downstream target rpS6. Coincident with this, mRNA levels of the putative
225 atrophy marker MuRF1 were significantly reduced. In addition to MuRF1, the metallothionein
226 MT1 was downregulated by mTORC1 inhibition. We had previously shown that this is a high-
227 fidelity marker of atrophy, and knocking out the MT genes in mice causes muscle hypertrophy
228 (45). This finding too is consistent with the increase in mass observed in the present study, and
229 provides further mechanistic rationale. As for the senescence markers p16 and p21, they were
230 elevated in aged muscle when compared to young muscle, and reversed towards the "younger
231 level" following rapalog treatment, consistent with what had been shown previously in geriatric
232 satellite cells (46, 53). This reversal of senescent markers suggest the possibility of improved

233 satellite cell function necessary for muscle homeostasis, reflected by the positive morphological
234 changes we observed in rapalog treated muscles.

235 In summary, mTORC1 signaling is hyper-activated in aged muscle, and this apparently is
236 causal of sarcopenia, since inhibition of this signaling can increase muscle mass. The inhibition
237 of denervation and senescence markers, and the subsequent decline in atrophy markers gives a
238 therapeutic rationale for treating aged sarcopenic patients with an mTORC1 inhibitor.

239

240

241 **Materials and Methods**

242 **Animal maintenance and RAD001 treatment**

243 Male Sprague Dawley rats were obtained from Envigo (Indianapolis, IN), and housed at their
244 facility under specific pathogen-free (SPF) conditions until the appropriate age. When transferred
245 to our facility, rats continued to be maintained under SPF conditions, with regulated temperature
246 and light cycles (22°C, 12-hour light/12-hour dark cycle: lights on at 0600hours/lights off at
247 1800 hours), and unrestricted access to food (2014 Teklad Global 14% Protein diet (Envigo))
248 and water. Animals were acclimated for a minimum of 4 weeks before being used for
249 experiments. For age time course studies, rats ranging from 6 to 27-months (n = 6-8
250 animals/group) were fasted from 0600 hours to 1200 hours (during the light on cycle) before
251 being anesthetized and euthanized for end of study analysis.. Gastrocnemius muscles were
252 collected for molecular analysis. For other studies, RAD001 (Novartis) was prepared as a
253 microemulsion pre-concentrate at 2% (w/w). Prior to dosing, it was diluted to a working
254 concentration in water. Vehicle control consisted of microemulsion pre-concentrate (equivalent
255 to a dose), diluted in water. At 22-months, animals were dosed daily for 6 weeks with either
256 RAD001 or vehicle per os. In parallel, rats aged 7-months received vehicle as young adult
257 controls. Four hours after the last dose of RAD001 or vehicle, rats were anesthetized with 3.5%
258 isofluorane, and euthanized by exsanguination and thoracotomy. Gastrocnemius, soleus,
259 plantaris and tibialis anterior (TA) muscles were collected and weighed; TA and plantaris
260 muscles were processed as described below further assessment. All animal studies were done in
261 accordance with institutional guidelines for the care and use of laboratory animals as approved
262 by the Institutional Animal Care and Use Committee (IACUC) of the Novartis Institutes of
263 Biomedical Research, Cambridge MA.

264

265 **Protein extraction and Western Blot analysis**

266 Protein extracts were prepared from tibialis anterior muscles. In short, snap frozen tissue was
267 pulverized in liquid nitrogen by mortar and pestle to a fine powder. Approximately 30mg of
268 tissue powder was homogenized in MSD lysis buffer (#R60TX, Meso Scale Discovery,)
269 supplemented with Protease and Phosphatase inhibitor cocktail (Thermo Fisher Scientific, MA).
270 Following a 30 min incubation at 4°C with agitation, protein lysates containing the cytoplasmic
271 fraction were collected via microcentrifugation. Protein concentration was determined by BCA
272 protein assay (Thermo Fisher Scientific, MA), prior to Western blot analysis. Diluted proteins
273 were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a
274 4-20% gradient Criterion TGX Precast Midi Protein gel (Bio-Rad, CA), and subsequently
275 transferred onto nitrocellulose membranes (Bio-Rad, CA) with the Trans Turbo Blot system
276 (Bio-Rad). Membranes were blocked in 5% milk in TBST for 1 h at room temperature, and
277 incubated with primary antibodies overnight at 4°C. After three washes in TBST, membranes
278 were incubated in the appropriate HRP conjugated secondary antibodies (Cell Signaling
279 Technologies) for 1 h at room temperature. The following primary antibodies were used: anti-
280 GAPDH (#5174, Cell Signaling Technologies, MA) , anti-rpS6 (#2217), anti-p-rpS6(S240/244)
281 (#2215), anti-S6K1 (#2708), anti-pS6K1(T389) (#9234), all from Cell Signaling Technologies.
282 Anti-rabbit and anti-mouse IgG HRP-conjugated secondary antibodies were also from Cell
283 Signaling Technologies. Densitometric analysis was performed using Fiji 1.51n software.

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286 **Cryosectioning of frozen tissue**

287 Plantaris muscles were removed, embedded in OCT (Tissue-Tek) and flash frozen in chilled 2-
288 methylbutane (Fisher Scientific). Muscles were sectioned transversely with the Leica CM3050 S
289 microtome and 10µm thick sections were collected for Hematoxylin and Eosin (H&E) staining
290 and immunohistochemistry.

291

292 **Hematoxylin and Eosin staining**

293 Muscle sections were fixed in 4% paraformaldehyde on ice for 10 mins and rinsed briefly with
294 water five times. H&E staining was done using the Tissue-Tek Prisma automated slide stainer
295 (Sakura Finetek). Images were captured using the Aperio ScanscopeAT (Leica Biosystems), and
296 used to determine morphological changes, including the incidence of central nuclei.

297

298 **Immunohistochemistry**

299 Myofiber cross-sectional area was measured on muscle cross-sections immuno-stained with the
300 anti-Laminin antibody. Briefly, tissue sections were fixed in 4% paraformaldehyde on ice for 10
301 mins, and washed in 1X PBS prior to permeabilization in 0.3% Triton-X 100 in PBS. Non-
302 specific sites were blocked in 16% goat serum diluted in 0.01% Triton-X 100 in PBS (blocking
303 buffer) for 1 h at room temperature. Sections were incubated in anti-Laminin (Sigma Aldrich,
304 L9393) antibody diluted at 1:1000 in blocking buffer overnight at 4°C. Primary antibody was
305 detected by a 1h incubation with AlexaFluor-conjugated goat anti-rabbit secondary antibody
306 (Life Technologies, A-11072) diluted in blocking buffer. Following a series of washes in 0.01%

307 Triton-X in PBS, slides were mounted with Fluoromount-G (SouthernBiotech). Images were
308 captured using the VS120 Virtual Slide Microscope (Olympus).

309

310 **RNA extraction, cDNA synthesis and Quantitative RT-PCR (RT-qPCR)**

311 Tibialis anterior muscle was ground to powder as described above. Approximately 30mg of
312 tissue powder was then processed using the miRNeasy Micro kit (Qiagen) according to the
313 manufacturer's protocol. RNA concentration was quantified by NanoDrop Spectrophotometer
314 (NanoDrop Technologies), and quality was confirmed by the OD260/OD280 absorption ratio
315 (>1.8). Following the manufacturer's protocol, cDNA was synthesized from 1µg of RNA using
316 the High Capacity cDNA Reverse Transcription kit (Applied Biosystems by Thermo Fisher
317 Scientific). cDNA was diluted 1:10 in Ultra Pure Distilled RNase-free water (Invitrogen) prior to
318 being used for further steps. The Standard TaqMan Gene Expression Master Mix (Applied
319 Biosystems by Thermo Fisher Scientific) was used for all RT-qPCR reactions, and samples were
320 run using a 384-well optical plate format. Reactions were performed using the ViiA 7 RT-qPCR
321 System (Life Technologies), and data analyzed by the $\Delta\Delta cT$ method. TaqMan probes were
322 optimized by Applied Biosystems: TATA-box binding protein (TBP) (Rn01455646_m1),
323 Vps26a (Rn01433541_m1), MurF1 (Rn01639111_m1), MaFbx (Rn00591730_m1), Mt1
324 (Rn00821759_g1), Mt2A (Rn01536588_g1), Cdkn1a (Rn00589996_m1), Cdkn2a
325 (Rn00580664_m1), Chrna1 (Rn01278033_m1), Chrne (Rn00567899_m1), MuSK
326 (Rn00579211_m1), Myogenin (Rn00567418_m1) and Gadd45a (Rn01425130_g1).

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329 **Statistical analysis**

330 Statistical significance was determined by a one way ANOVA followed by Dunnett's multiple
331 comparison tests. Means from all groups were compared to the mean of the aged vehicle treated
332 group, except where specified. All data are displayed as means with standard deviation.
333 GraphPad Prism 7.04 software was used for calculations and graphing.

334

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346 References

- 347 1. **Egerman MA, Glass DJ.** 2014. Signaling pathways controlling skeletal muscle mass. *Crit Rev*
348 *Biochem Mol Biol* **49**:59-68.
- 349 2. **Glass D, Roubenoff R.** 2010. Recent advances in the biology and therapy of muscle wasting.
350 *Ann N Y Acad Sci* **1211**:25-36.
- 351 3. **Studenski S, Perera S, Patel K, Rosano C, Faulkner K, Inzitari M, Brach J, Chandler J,**
352 **Cawthon P, Connor EB, Nevitt M, Visser M, Kritchevsky S, Badinelli S, Harris T, Newman**
353 **AB, Cauley J, Ferrucci L, Guralnik J.** 2011. Gait speed and survival in older adults. *JAMA*
354 **305**:50-58.
- 355 4. **Morley JE, Abbatecola AM, Argiles JM, Baracos V, Bauer J, Bhasin S, Cederholm T, Coats**
356 **AJ, Cummings SR, Evans WJ, Fearon K, Ferrucci L, Fielding RA, Guralnik JM, Harris TB,**
357 **Inui A, Kalantar-Zadeh K, Kirwan BA, Mantovani G, Muscaritoli M, Newman AB, Rossi-**
358 **Fanelli F, Rosano GM, Roubenoff R, Schambelan M, Sokol GH, Storer TW, Vellas B, von**
359 **Haehling S, Yeh SS, Anker SD, Society on Sarcopenia C, Wasting Disorders Trialist W.**
360 2011. Sarcopenia with limited mobility: an international consensus. *J Am Med Dir Assoc* **12**:403-
361 409.
- 362 5. **Armanios M, de Cabo R, Mannick J, Partridge L, van Deursen J, Villeda S.** 2015.
363 Translational strategies in aging and age-related disease. *Nat Med* **21**:1395-1399.
- 364 6. **Niccoli T, Partridge L.** 2012. Ageing as a risk factor for disease. *Curr Biol* **22**:R741-752.
- 365 7. **Kenyon CJ.** 2010. The genetics of ageing. *Nature* **464**:504-512.
- 366 8. **Hofmann JW, Zhao X, De Cecco M, Peterson AL, Pagliaroli L, Manivannan J, Hubbard GB,**
367 **Ikeno Y, Zhang Y, Feng B, Li X, Serre T, Qi W, Van Remmen H, Miller RA, Bath KG, de Cabo**
368 **R, Xu H, Neretti N, Sedivy JM.** 2015. Reduced expression of MYC increases longevity and
369 enhances healthspan. *Cell* **160**:477-488.
- 370 9. **Chen C, Liu Y, Liu Y, Zheng P.** 2009. mTOR regulation and therapeutic rejuvenation of aging
371 hematopoietic stem cells. *Sci Signal* **2**:ra75.
- 372 10. **Flynn JM, O'Leary MN, Zambataro CA, Academia EC, Presley MP, Garrett BJ, Zykovich A,**
373 **Mooney SD, Strong R, Rosen CJ, Kapahi P, Nelson MD, Kennedy BK, Melov S.** 2013. Late-
374 life rapamycin treatment reverses age-related heart dysfunction. *Aging Cell* **12**:851-862.
- 375 11. **Johnson SC, Rabinovitch PS, Kaeberlein M.** 2013. mTOR is a key modulator of ageing and
376 age-related disease. *Nature* **493**:338-345.
- 377 12. **Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE,**
378 **Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, Miller RA.** 2009. Rapamycin fed late
379 in life extends lifespan in genetically heterogeneous mice. *Nature* **460**:392-395.
- 380 13. **Miller RA, Harrison DE, Astle CM, Baur JA, Boyd AR, de Cabo R, Fernandez E, Flurkey K,**
381 **Javors MA, Nelson JF, Orihuela CJ, Pletcher S, Sharp ZD, Sinclair D, Starnes JW,**
382 **Wilkinson JE, Nadon NL, Strong R.** 2011. Rapamycin, but not resveratrol or simvastatin,
383 extends life span of genetically heterogeneous mice. *J Gerontol A Biol Sci Med Sci* **66**:191-201.
- 384 14. **Miller RA, Harrison DE, Astle CM, Fernandez E, Flurkey K, Han M, Javors MA, Li X, Nadon**
385 **NL, Nelson JF, Pletcher S, Salmon AB, Sharp ZD, Van Roekel S, Winkleman L, Strong R.**
386 2014. Rapamycin-mediated lifespan increase in mice is dose and sex dependent and
387 metabolically distinct from dietary restriction. *Aging Cell* **13**:468-477.
- 388 15. **Zhang Y, Bokov A, Gelfond J, Soto V, Ikeno Y, Hubbard G, Diaz V, Sloane L, Maslin K,**
389 **Treaster S, Rendon S, van Remmen H, Ward W, Javors M, Richardson A, Austad SN,**
390 **Fischer K.** 2014. Rapamycin extends life and health in C57BL/6 mice. *J Gerontol A Biol Sci Med*
391 *Sci* **69**:119-130.

- 392 16. **Mannick JB, Del Giudice G, Lattanzi M, Valiante NM, Praestgaard J, Huang B, Lonetto MA,**
393 **Maecker HT, Kovarik J, Carson S, Glass DJ, Klickstein LB.** 2014. mTOR inhibition improves
394 immune function in the elderly. *Sci Transl Med* **6**:268ra179.
- 395 17. **Shavlakadze T, Zhu J, Wang S, Zhou W, Morin B, Egerman MA, Fan L, Wang Y, Iartchouk**
396 **O, Meyer A, Valdez RA, Mannick JB, Klickstein LB, Glass DJ.** 2018. Short-term Low-Dose
397 mTORC1 Inhibition in Aged Rats Counter-Regulates Age-Related Gene Changes and Blocks
398 Age-Related Kidney Pathology. *J Gerontol A Biol Sci Med Sci* **73**:845-852.
- 399 18. **Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E,**
400 **Scrimgeour A, Lawrence JC, Glass DJ, Yancopoulos GD.** 2001. Akt/mTOR pathway is a
401 crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell*
402 *Biol* **3**:1014-1019.
- 403 19. **Choi DH, Yang J, Kim YS.** 2019. Rapamycin suppresses postnatal muscle hypertrophy induced
404 by myostatin-inhibition accompanied by transcriptional suppression of the Akt/mTOR pathway.
405 *Biochem Biophys Res Commun* **517**:182-190.
- 406 20. **Yoon MS.** 2017. mTOR as a Key Regulator in Maintaining Skeletal Muscle Mass. *Front Physiol*
407 **8**:788.
- 408 21. **Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD, Glass**
409 **DJ.** 2001. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and
410 PI(3)K/Akt/GSK3 pathways. *Nat Cell Biol* **3**:1009-1013.
- 411 22. **Kim do H, Sarbassov dos D, Ali SM, King JE, Latek RR, Erdjument-Bromage H, Tempst P,**
412 **Sabatini DM.** 2002. mTOR Interacts with Raptor to Form a Nutrient-Sensitive Complex that
413 Signals to the Cell Growth Machinery. *Cell* **110**:163-175.
- 414 23. **Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M,**
415 **Fitzgerald KJ, Sabatini DM.** 2006. Ablation in mice of the mTORC components raptor, rictor, or
416 mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1.
417 *Dev Cell* **11**:859-871.
- 418 24. **Sarbassov DD, Guertin DA, Ali SM, Sabatini DM.** 2005. Phosphorylation and Regulation of
419 Akt/PKB by the Rictor-mTOR Complex. *Science* **307**:1098-1101.
- 420 25. **Sarbassov DD, Sabatini DM.** 2005. Redox Regulation of the Nutrient-sensitive Raptor-mTOR
421 Pathway and Complex. *Journal of Biological Chemistry* **280**:39505-39509.
- 422 26. **Zhao J, Brault JJ, Schild A, Cao P, Sandri M, Schiaffino S, Lecker SH, Goldberg AL.** 2007.
423 FoxO3 Coordinately Activates Protein Degradation by the Autophagic/Lysosomal and
424 Proteasomal Pathways in Atrophying Muscle Cells. *Cell* **130**:472-483.
- 425 27. **Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del Piccolo P, Burden SJ, Di**
426 **Lisi R, Sandri C, Zhao J, Goldberg AL, Schiaffino S, Sandri M.** 2007. FoxO3 Controls
427 Autophagy in Skeletal Muscle In Vivo. *Cell Metab* **6**:458-471.
- 428 28. **McLoughlin TJ, Smith SM, DeLong AD, Wang H, Unterman TG, Esser KA.** 2009. FoxO1
429 induces apoptosis in skeletal myotubes in a DNA binding-dependent manner. *Am J Physiol Cell*
430 *Physiol* doi:10.1152/ajpcell.00502.2008:00502.02008.
- 431 29. **Southgate RJ, Neill B, Prelovsek O, El-Osta A, Kamei Y, Miura S, Ezaki O, McLoughlin TJ,**
432 **Zhang W, Unterman TG, Febbraio MA.** 2007. FOXO1 Regulates the Expression of 4E-BP1 and
433 Inhibits mTOR Signaling in Mammalian Skeletal Muscle. *J Biol Chem* **282**:21176-21186.
434 doi:10.1074/jbc.M702039200
- 435 30. **Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J,**
436 **Greenberg ME.** 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead
437 transcription factor. *Cell* **96**:857-868.
- 438 31. **Rüegg MA, Glass DJ.** 2011. Molecular Mechanisms and Treatment Options for Muscle Wasting
439 Diseases. *Annual Review of Pharmacology and Toxicology* **51**:373-395.

- 440 32. **Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker**
441 **SH, Goldberg AL.** 2004. Foxo transcription factors induce the atrophy-related ubiquitin ligase
442 atrogin-1 and cause skeletal muscle atrophy. *Cell* **117**:399-412.
- 443 33. **Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyeva Y, Kline WO, Gonzalez M, Yancopoulos**
444 **GD, Glass DJ.** 2004. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-
445 induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol Cell* **14**:395-403.
- 446 34. **Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro**
447 **FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD,**
448 **Glass DJ.** 2001. Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science*
449 **294**:1704-1708.
- 450 35. **Gomes MD, Lecker SH, Jagoe RT, Navon A, Goldberg AL.** 2001. Atrogin-1, a muscle-specific
451 F-box protein highly expressed during muscle atrophy. *Proc Natl Acad Sci U S A* **98**:14440-
452 14445.
- 453 36. **Clarke BA, Drujan D, Willis MS, Murphy LO, Corpina RA, Burova E, Rakhilin SV, Stitt TN,**
454 **Patterson C, Latres E, Glass DJ.** 2007. The E3 Ligase MuRF1 degrades myosin heavy chain
455 protein in dexamethasone-treated skeletal muscle. *Cell Metab* **6**:376-385.
- 456 37. **Lagirand-Cantaloube J, Offner N, Csibi A, Leibovitch MP, Batonnet-Pichon S, Tintignac LA,**
457 **Segura CT, Leibovitch SA.** 2008. The initiation factor eIF3-f is a major target for atrogin1/MAFbx
458 function in skeletal muscle atrophy. *EMBO J* **27**:1266-1276.
- 459 38. **Tintignac LA, Lagirand J, Batonnet S, Sirri V, Leibovitch MP, Leibovitch SA.** 2005.
460 Degradation of MyoD mediated by the SCF (MAFbx) ubiquitin ligase. *J Biol Chem* **280**:2847-
461 2856.
- 462 39. **Bodine SC, Baehr LM.** 2014. Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1 and
463 MAFbx/atrogin-1. *Am J Physiol Endocrinol Metab* **307**:E469-484.
- 464 40. **Csibi A, Leibovitch MP, Cornille K, Tintignac LA, Leibovitch SA.** 2009. MAFbx/Atrogin-1
465 controls the activity of the initiation factor eIF3-f in skeletal muscle atrophy by targeting multiple
466 C-terminal lysines. *J Biol Chem* **284**:4413-4421.
- 467 41. **Sengupta P.** 2013. The Laboratory Rat: Relating Its Age With Human's. *Int J Prev Med* **4**:624-
468 630.
- 469 42. **Lamming DW, Ye L, Sabatini DM, Baur JA.** 2013. Rapalogs and mTOR inhibitors as anti-aging
470 therapeutics. *J Clin Invest* **123**:980-989.
- 471 43. **Ibembunjo C, Chick JM, Kendall T, Eash JK, Li C, Zhang Y, Vickers C, Wu Z, Clarke BA, Shi**
472 **J, Cruz J, Fournier B, Brachat S, Gutzwiller S, Ma Q, Markovits J, Broome M, Steinkrauss**
473 **M, Skuba E, Galarneau JR, Gygi SP, Glass DJ.** 2013. Genomic and proteomic profiling reveals
474 reduced mitochondrial function and disruption of the neuromuscular junction driving rat
475 sarcopenia. *Mol Cell Biol* **33**:194-212.
- 476 44. **Castets P, Lin S, Rion N, Di Fulvio S, Romanino K, Guridi M, Frank S, Tintignac LA,**
477 **Sinnreich M, Ruegg MA.** 2013. Sustained activation of mTORC1 in skeletal muscle inhibits
478 constitutive and starvation-induced autophagy and causes a severe, late-onset myopathy. *Cell*
479 *Metab* **17**:731-744.
- 480 45. **Summermatter S, Bouzan A, Pierrel E, Melly S, Stauffer D, Gutzwiller S, Nolin E, Dornelas**
481 **C, Fryer C, Leighton-Davies J, Glass DJ, Fournier B.** 2017. Blockade of Metallothioneins 1
482 and 2 Increases Skeletal Muscle Mass and Strength. *Mol Cell Biol* **37**.
- 483 46. **Sousa-Victor P, Gutarra S, Garcia-Prat L, Rodriguez-Ubrea J, Ortet L, Ruiz-Bonilla V, Jardi**
484 **M, Ballestar E, Gonzalez S, Serrano AL, Perdiguero E, Munoz-Canoves P.** 2014. Geriatric
485 muscle stem cells switch reversible quiescence into senescence. *Nature* **506**:316-321.
- 486 47. **Baar MP, Perdiguero E, Munoz-Canoves P, de Keizer PL.** 2018. Musculoskeletal senescence:
487 a moving target ready to be eliminated. *Curr Opin Pharmacol* **40**:147-155.

- 488 48. **Valdez G, Tapia JC, Kang H, Clemenson GD, Jr., Gage FH, Lichtman JW, Sanes JR.** 2010.
489 Attenuation of age-related changes in mouse neuromuscular synapses by caloric restriction and
490 exercise. *Proc Natl Acad Sci U S A* **107**:14863-14868.
- 491 49. **Valdez G, Tapia JC, Lichtman JW, Fox MA, Sanes JR.** 2012. Shared resistance to aging and
492 ALS in neuromuscular junctions of specific muscles. *PLoS One* **7**:e34640.
- 493 50. **Chai RJ, Vukovic J, Dunlop S, Grounds MD, Shavlakadze T.** 2011. Striking denervation of
494 neuromuscular junctions without lumbar motoneuron loss in geriatric mouse muscle. *PLoS One*
495 **6**:e28090.
- 496 51. **Ma XM, Blenis J.** 2009. Molecular mechanisms of mTOR-mediated translational control. *Nat Rev*
497 *Mol Cell Biol* **10**:307-318.
- 498 52. **Goodman CA, Frey JW, Mabrey DM, Jacobs BL, Lincoln HC, You JS, Hornberger TA.** 2011.
499 The role of skeletal muscle mTOR in the regulation of mechanical load-induced growth. *J Physiol*
500 **589**:5485-5501.
- 501 53. **Garcia-Prat L, Martinez-Vicente M, Perdiguero E, Ortet L, Rodriguez-Ubreva J, Rebollo E,**
502 **Ruiz-Bonilla V, Gutarra S, Ballestar E, Serrano AL, Sandri M, Munoz-Canoves P.** 2016.
503 Autophagy maintains stemness by preventing senescence. *Nature* **529**:37-42.

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505

506 **Figure legends**

507 **Figure 1.** mTORC1 signaling is hyper-activated in sarcopenic skeletal muscle. (A) Immunoblots
508 for phosphorylated (p) and total (t) protein for rpS6 in gastrocnemius muscles of rats aged 6 (n =
509 5), 21 (n = 6), 24 (n = 6) and 27 (n = 8) months. Glyceraldehyde-3-phosphate dehydrogenase
510 (GAPDH) is shown as a loading control. (B) p-rpS6(S240/244) protein amounts were quantified
511 relative to their respective total rpS6 protein amounts by densitometry. (C) Gastrocnemius
512 muscle weights in rats aged 6 (n = 12), 9 (n = 8), 12 (n = 10), 18 (n = 11), 21 (n = 8), 24 (n = 8)
513 and 27 (n = 10) months. Data are mean \pm standard deviation of the mean. Statistical significance
514 was determined by a one way ANOVA followed by Dunnett's multiple comparison tests. Means
515 from all groups were compared to the mean of 12-month old animals. Asterisk (*) denotes
516 significance at **P< 0.01; ***P< 0.001 and ****P< 0.0001. Y-axis in (B) represents arbitrary
517 units, and in (C), milligrams (mg).

518

519 **Figure 2.** Skeletal muscle mass and quality is improved with RAD001 treatment. (A) Weights of
520 tibialis anterior (TA), plantaris, gastrocnemius and soleus muscles from 9- and 24-month old rats
521 treated with vehicle and 24-month old rats treated with RAD001 (n = 7-12 animals per group).
522 Y-axes represent weight units in milligrams (mg). (B) Representative images of transverse
523 sections of plantaris muscles stained with H&E from 9- and 24-month old rats treated with
524 vehicle (n = 4 and n = 5 animals per group respectively) and 24 month old rats treated with
525 RAD001 (n = 5 animals). Open arrows depict mis-shaped, flattened myofibers. Closed arrows
526 show central nuclei. Scale bar is 100 μ m. (C) Quantification of myofibers with central nuclei in
527 plantaris muscles. Myofibers with central nuclei are shown as a percentage of total myofibers (n
528 > 1200 myofibers assessed per animal). (D) Average myofiber cross-sectional area (CSA) in

529 plantaris muscles ($n > 1200$ myofibers assessed per animal). Y-axes represent area units in
530 microns squared (μm^2). (E) Histogram depicting the distribution of myofiber cross-sectional
531 areas from data shown in (D). Myofiber cross-sectional area frequencies are shown as a
532 percentage of total myofibers in the given treatment group. All other data are mean \pm standard
533 deviation of the mean. Asterisk (*) denotes significance at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and
534 **** $p < 0.0001$.

535

536 **Figure 3.** Confirmed mTORC1 inhibition following rapalog treatment. (A) Representative
537 immunoblots for phosphorylated (p) and total (t) protein for S6K1 and rpS6 in tibialis anterior
538 muscles of 9- and 24-month old rats treated with vehicle and 24-month old rats treated with
539 RAD001. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control.
540 (B) p-S6K1(T389) and (C) p-rpS6(S240/244) protein amounts were quantified relative to their
541 respective total S6K1 and rpS6 protein amounts by densitometry ($n = 10 - 12$ animals per
542 group). Data are mean \pm standard deviation of the mean. Asterisk (*) denotes significance at
543 *** $p < 0.001$. Y-axes represent arbitrary units.

544

545 **Figure 4.** mTORC1 inhibition blunts molecular changes associated with sarcopenia. (A) mRNA
546 amounts for MuRF1, MaFbx, MT1 and MT2, and (B) Cdkn1a and Cdkn2a in tibialis anterior
547 muscles of 9- and 24- month old rats treated with vehicle and 24-month old rats treated with
548 RAD001 ($n = 10 - 12$ animals per group). mRNA amounts were standardized to a geometric
549 mean of TBP and Vps26a, used as reference genes (A and B). Data are mean \pm standard

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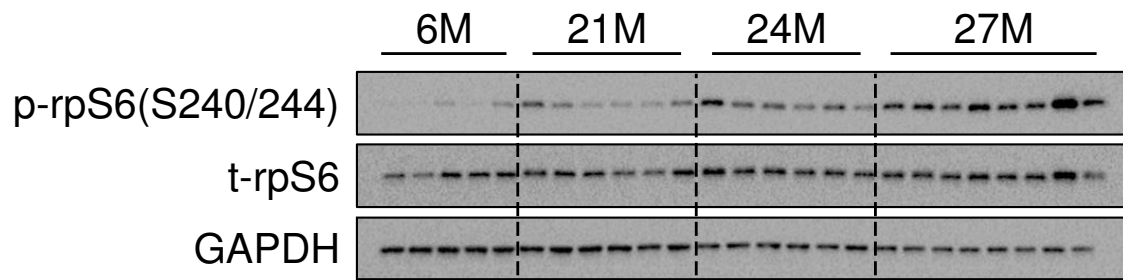
553 **Figure 5.** mRNA expression of denervation markers are reduced by rapalog treatment. (A)
554 mRNA amounts for Chrna1, Chrne, Musk, MyoG and Gadd45a in tibialis anterior muscles of 9-
555 and 24-month old rats treated with vehicle and 24-month old rats treated with RAD001 (n = 10 –
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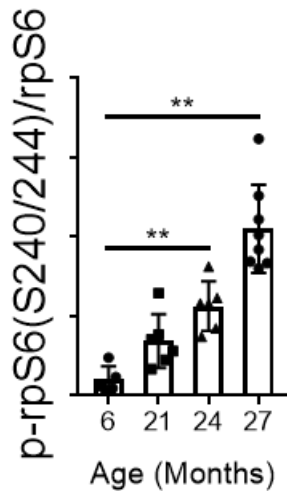
561 **Figure S1.** Rapalog treatment blocks mTORC1 pathway activity. Immunoblots of additional
562 samples that were included in the analysis of phosphorylated (p) and total (t) protein for S6K1
563 and rpS6 in tibialis anterior muscles of 9- and 24-month old rats treated with vehicle and 24-
564 month old rats treated with RAD001 in Fig. 3B, C. Glyceraldehyde-3-phosphate dehydrogenase
565 (GAPDH) is shown as a loading control.

566

A



B



C

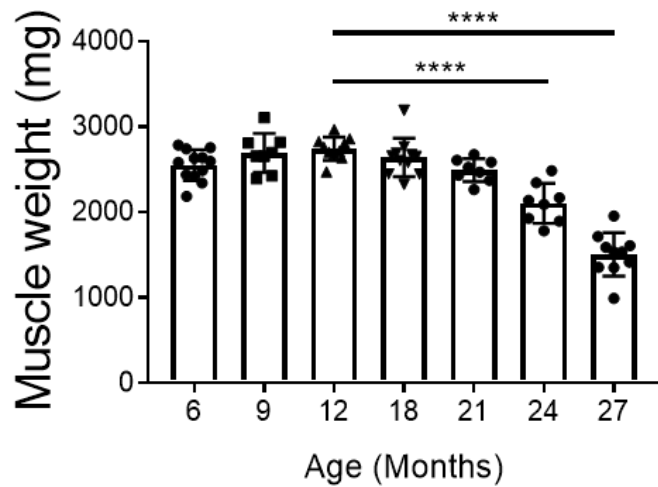


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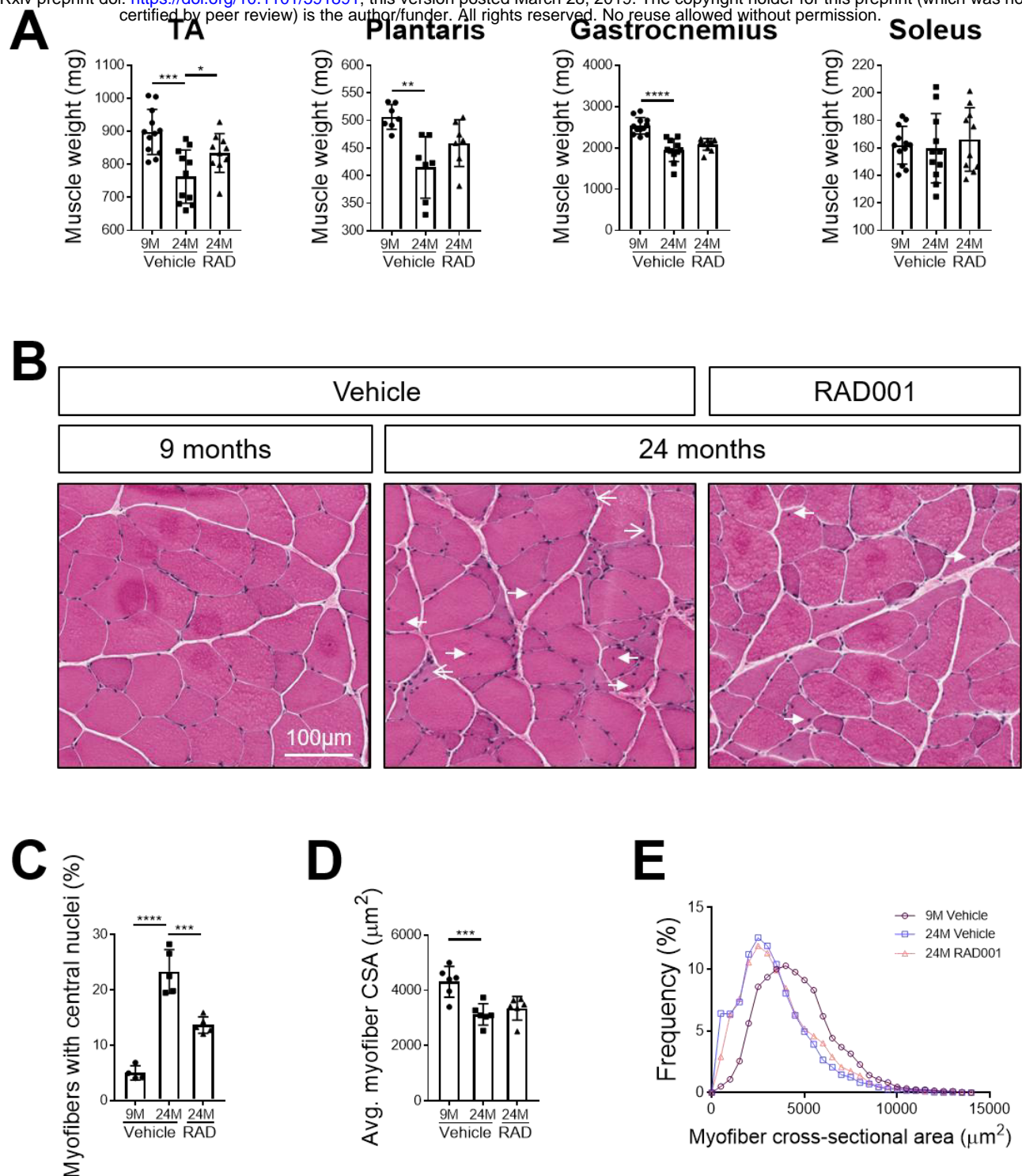
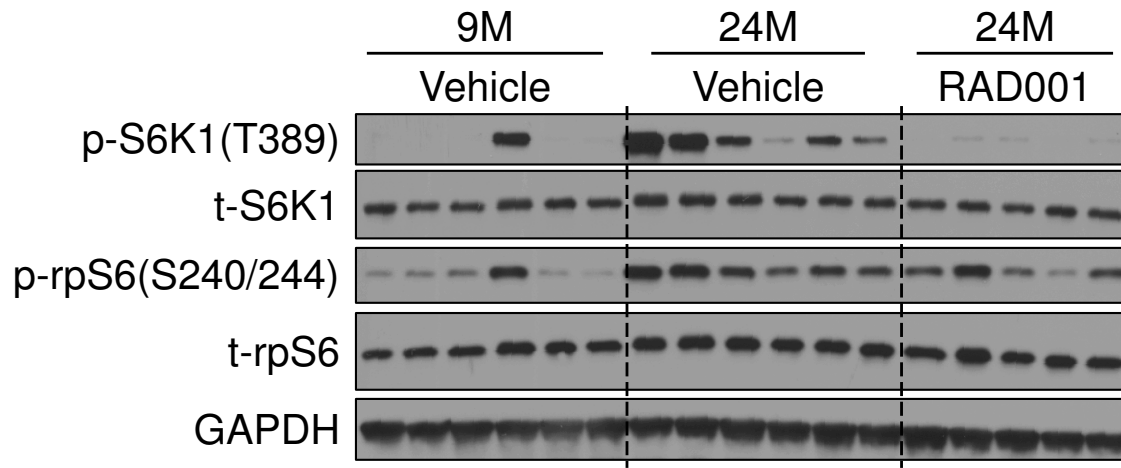
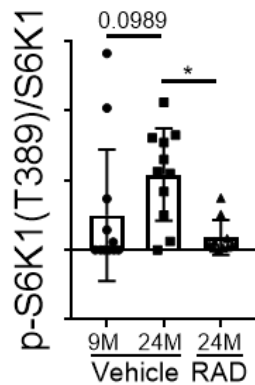


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A



B



C

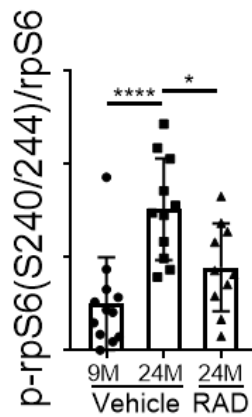


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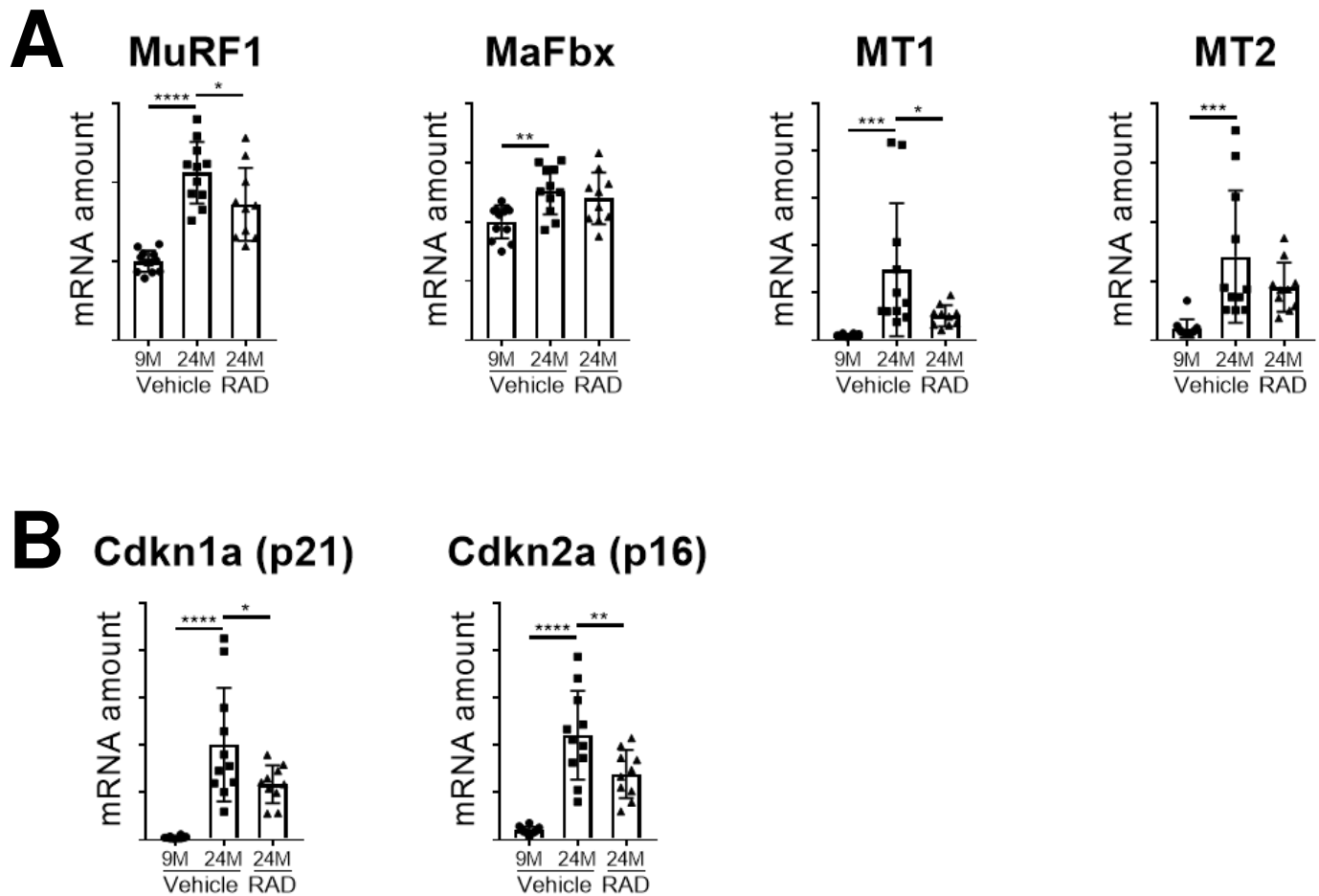


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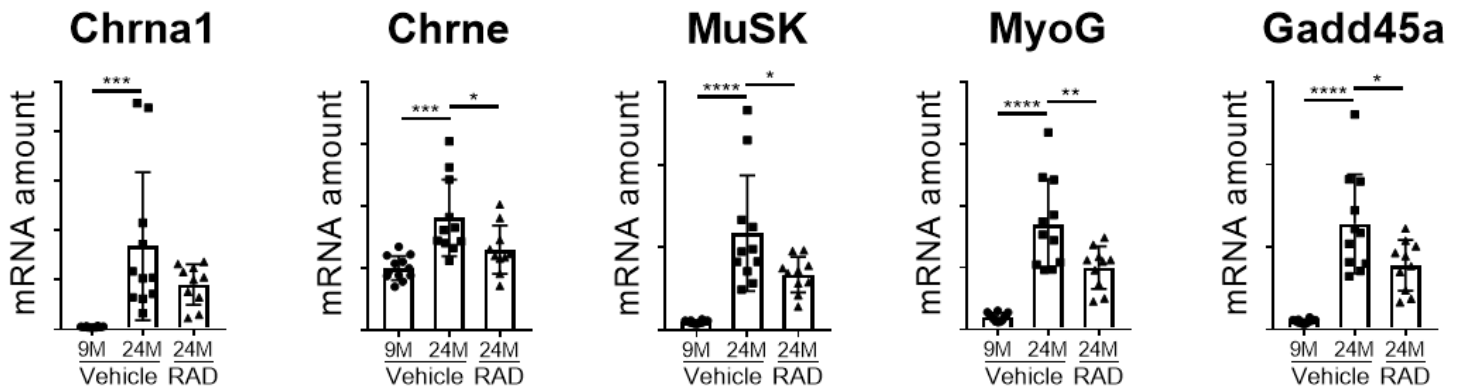


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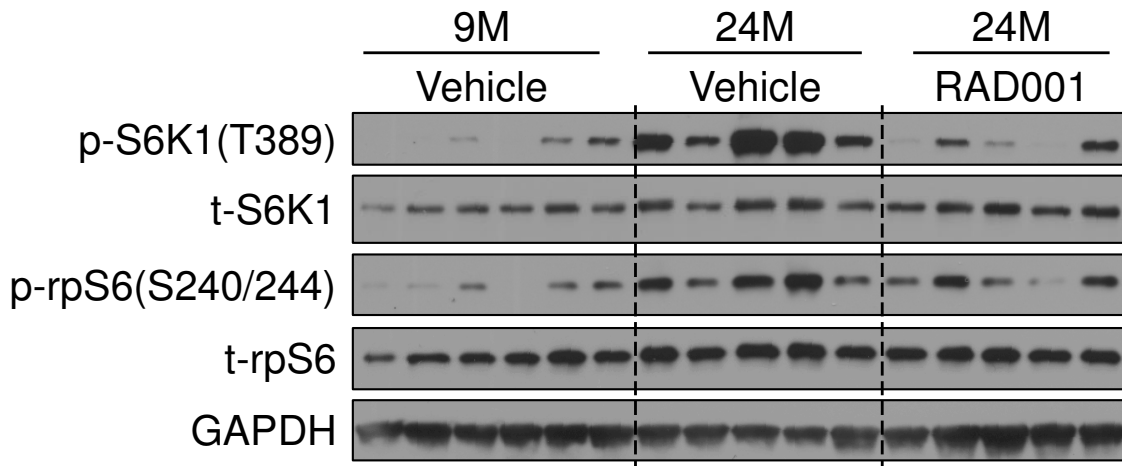


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