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1 **MuRF1 Deficiency Prevents Age-Related Fat Weight Gain, Possibly Through**
2 **Accumulation of PDK4 in Skeletal Muscle Mitochondria in Older Mice**
3

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26

27 Running title: MuRF1 regulates lipid metabolism

28

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30

31 **Abstract**

32 Recent studies show that muscle mass and metabolic function are interlinked. Muscle
33 RING finger 1 (MuRF1) is a critical muscle-specific ubiquitin ligase associated with muscle
34 atrophy. Yet, the molecular target of MuRF1 in atrophy and aging remains unclear. We
35 examined the role of MuRF1 in aging, using MuRF1-deficient (MuRF1$^{-/-}$) mice *in vivo*, and
36 MuRF1-overexpressing cell *in vitro*. MuRF1 deficiency partially prevents age-induced skeletal
37 muscle loss in mice. Interestingly, body weight and fat mass of >7-month-old MuRF1$^{-/-}$ mice
38 were lower than in MuRF1$^{+/+}$ mice. Serum and muscle metabolic parameters and results of
39 indirect calorimetry suggest significantly higher energy expenditure and enhanced lipid
40 metabolism in 3-month-old MuRF1$^{-/-}$ mice than in MuRF1$^{+/+}$ mice, resulting in suppressed
41 adipose tissue gain during aging. Pyruvate dehydrogenase kinase 4 (PDK4) is crucial for a
42 switch from glucose to lipid metabolism, and the interaction between MuRF1 and PDK4 was
43 examined. PDK4 protein levels were elevated in mitochondria from the skeletal muscle in
44 MuRF1$^{-/-}$ mice. *In vitro*, MuRF1 interacted with PDK4 but did not induce degradation through
45 ubiquitination. Instead, SUMOylation of PDK4 was detected in MuRF1-overexpressing cells,
46 in contrast to cells without the RING domain of MuRF1. MuRF1 deficiency enhances lipid
47 metabolism possibly by upregulating PDK4 localization into mitochondrial through prevention
48 of SUMOylation. Inhibition of MuRF1-mediated PDK4 SUMOylation is a potential
49 therapeutic target for age-related dysfunction of lipid metabolism and muscle atrophy.

50

51 **Keywords:** Muscle atrophy**,** Muscle RING finger 1 (MuRF1), lipid metabolism, Pyruvate
52 dehydrogenase kinase 4 (PDK4), SUMO modification

53 **Introduction**

54 In advanced societies, age-related muscle atrophy, such as sarcopenia, is attracting
55 attention as the target of research in this mechanism and becoming the therapeutic target
56 against physical frailty [1, 2]. Aging of skeletal muscle is characterized by atrophied muscle
57 mass and a loss of force-generating capacity and leads to preferential loss of glycolytic and
58 fast-twitch muscle fibers than oxidative and slow-twitch muscle [3, 4]. Hence, age-related
59 muscle atrophy affects the glycolytic function. On the contrary, many recent researches
60 indicate that metabolic dysfunctions such as diabetes, hyperlipidemia, and obesity are causes
61 of muscle atrophy [5, 6]. Thus, muscle mass and metabolic function are closely related each
62 other.

63 Protein degradation through ubiquitin-proteasome system along with the autophagy-
64 lysosome system is the most important pathway associated with the mechanism of muscle
65 atrophy. Muscle RING finger 1 (MuRF1) was first reported in 2001 as an unknown protein
66 interacting with titin, which is located in M-Line of the myofibrillar sarcomere [7].
67 Thereafter, MuRF1 was described as a striated muscle-specific ubiquitin ligase and a critical
68 mediator of muscle atrophy caused by immobilization, denervation, and unloading [8]. So far,
69 many substrates of MuRF1 ubiquitination have been identified, including myosin heavy/light
70 chains and alpha-actin [9, 10]. At present MuRF1 is recognized as one of the most important
71 atrophy-related genes, which are called “atrogenes” [11].

72 Mice lacking MuRF1 are resistant to muscle atrophy caused by such conditions [8,
73 12, 13]. Expression of MuRF1 is regulated at higher levels in glycolytic and fast-twitch
74 muscle fibers than in oxidative and slow-twitch muscle fibers under the normal condition and
75 during unloading or immobilization induced skeletal muscle atrophy [14]. However, MuRF1
76 expression is reported to be increased, decreased, or hardly affected in skeletal muscles of
77 aging rodents [15-17]. Thus, the pathological role of MuRF1 in age-related muscle atrophy

78 remains controversial.

79 Yeast two-hybrid screening showed that MuRF1 interacts with pyruvate
80 dehydrogenase kinase 4 (PDK4), suggesting that PDK4 is a target substrate [18]. PDK4
81 regulates energy production through a shift from glucose oxidation to fatty acid oxidation via
82 pyruvate dehydrogenase (PDH) complex inhibition [19, 20]. Cardiac-specific overexpression
83 of PDK4 enhanced palmitate oxidation, but not glucose oxidation, thereby preventing diet-
84 induced triglyceride accumulation in the heart [21]. Interestingly, we found that PDK4
85 protein was accumulated into the mitochondria of MuRF1-deficient skeletal muscle, whereas
86 MuRF1 failed to induce ubiquitination of PDK4. Recently MuRF1 reportedly interacted with
87 small ubiquitin-related modifier-3 (SUMO3), a ubiquitin-like protein, involved in SUMO
88 post-transcriptional modification (SUMOylation) via its RING domain [22, 23]. Furthermore,
89 PDK4 contains the conserved SUMOylation motif -ψ-K-X-E/D- (ψ : bulky hydrophobic
90 residue), ^{282}Gly-Lys-Glu-Asp, which binds SUMOs 1, 2, and 3 [24].

91 In this study, we elucidate pathological associations between MuRF1 and lipid
92 metabolism in age-related atrophied muscles through interaction with PDK4 using MuRF1
93 knockout mice.

94 **Methods**

95 **Animals**

96 MuRF1$^{-/-}$ mice, generated by inserting a neomycin-resistance gene into exon 2 of
97 *MuRF1*, were kindly provided by Dr. Sorimachi [12, 25]. MuRF1$^{-/-}$ mice were backcrossed
98 with C57BL/6 mice more than eight times. MuRF1$^{+/+}$ and MuRF1$^{-/-}$ male mice were housed
99 in a room maintained at 23 ± 2°C on a 12:12-h light/dark cycle and allowed free access to
100 standard chow and water. A small amount of blood for measuring glucose and insulin was
101 collected after overnight fast 1 week before euthanasia. Body weight (BW) was measured
102 monthly for 24 months. Fat mass of mice was quantified by computed tomography (LATheta
103 LCT-200; Aloka Inc., Tokyo, Japan).

104 Respiratory quotient (RQ) and energy expenditure (EE) were recorded every 10 min
105 for 48 h using an Ox Imax™ sensor (Covidien-Nellcor, Boulder, Colorado, USA). Mean
106 values were calculated hourly, and these values were averaged at the same Zeitgeber time
107 (ZT). Locomotor activity was measured using an ACTIMO system (Shintechno, Fukuoka,
108 Japan). The number of movements of mice recognized by infrared beams was counted for 48
109 h. Mean values were calculated at the same ZT.

110 All protocols were implemented according to the Guide for the Care and Use of
111 Laboratory Animals at Tokushima University. Experimental protocols described in this study
112 were approved by the Tokushima University Ethics Review Committee for Animal
113 Experimentation.

114

115 **Isolation of Mitochondria**

116 Mitochondria from COS7 and skeletal muscles from MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice
117 were prepared following previously established protocols [26]. Briefly, cells or tissues were
118 harvested and immediately minced in ice-cold CP-1 buffer (100 mM KCl, 50 mM Tris-HCl,

119 2 mM EGTA, pH 7.4). After grinding in a glass homogenizer, supernatants were passed
120 through a 40-μm cell strainer. Mitochondria were then separated by differential
121 centrifugation and lysed with RIPA™ buffer (50 mM Tris-HCl, pH 8.0, containing 1% NP-
122 40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 5 mM
123 EDTA, and protease inhibitors) (Nacalai Tesque Inc., Kyoto, Japan).
124

125 **Plasmid Constructs**

126 Mouse MuRF1, PDK4, PDHα, PDHβ, and SUMO3 cDNAs were isolated from a
127 mouse skeletal muscle cDNA library by PCR. PCR products were subcloned into mammalian
128 expression plasmid vectors pcDNA3.1-V5, pcDNA6.1-myc, and pcDNA-FLAG (MuRF1-
129 V5, PDK4-myc, PDHα-myc, PDHβ-myc, and SUMO3-FLAG). RING domains of truncation
130 mutants of MuRF1 (ΔRING-MuRF1-V5) and mitochondrial targeting signal (MTS)
131 truncation mutants of PDK4 (ΔMTS-PDK4-myc) were constructed using a modified PCR
132 technique (Stratagene Cloning Systems), as previously described [27]. Construction of the
133 expression plasmid for green fluorescence protein (GFP) containing the MTS of PDK4
134 (MTS-GFP) used an MTS mutant of PDK4 (residues 1–10, pcDNA6.1-MTS-myc)
135 constructed as described above, and GFP was subcloned from the pCDNA3.1-GFP vector
136 and ligated in-frame into the pcDNA6.1-MTS-myc vector.
137

138 **Cell Culture and Transfection**

139 COS7 cells were maintained in Dulbecco's Modified Eagle Medium containing 10%
140 fetal bovine serum and penicillin-streptomycin (Nacalai Tesque Inc.) at 37°C in the presence
141 of 5% CO_2. Cells were transfected with plasmid vectors containing indicated genes using a
142 FuGENE HD™ lipofection reagent (Roche Diagnostics, Tokyo Japan) for 24 h prior to
143 experiments.

144

145 **SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting**

146 Cells were homogenized with a sonicator in 50 mM Tris-HCl, pH 7.5, containing
147 150 nM NaCl, 1% Triton X-100, and protease inhibitors with EDTA. Protein samples were
148 combined with 4× sample buffer (250 mM Tris-HCl, 8% SDS, 40% glycerol, 8% β-
149 mercaptoethanol, 0.02% bromophenol blue) and separated on a polyacrylamide gel. Proteins
150 were transferred to a polyvinylidene difluoride membrane and probed with the following
151 primary antibodies, following the manufacturer's instructions: anti-V5 (Invitrogen, R96025,
152 Carlsbad, CA), anti-myc (Upstate, #2276, New York, USA), anti-FLAG M2 (Sigma-Aldrich,
153 F3165, St. Louis, MI), anti-PDK4 (Abcam, ab38242, Cambridge, UK), anti-PDH (Abcam,
154 ab110416), anti-MFN2 (Abcam, ab50838), anti-mitochondrial cytochrome c oxidase subunit
155 IV (COX IV) (Upstate, #4844s), anti-β-actin (Calbiochem, A1978, San Diego, USA), and
156 anti-GAPDH (Santa Cruz Biotechnology, sc-25778, Santa Cruz, CA). Secondary antibodies
157 were donkey anti-rabbit 1:5000 and sheep anti-mouse 1:5000 (Amersham Biosciences,
158 NA934-1ML, Piscataway NA-9310, NJ). Membranes were developed using Amersham™
159 ECL™ or Amersham™ ECLTM Prime Western blotting detection reagents (GE Healthcare,
160 Chicago, IL).

161

162 **Immunoprecipitation**

163 Immunoprecipitation samples were prepared using 100 μg protein and adjusted to
164 300 μl with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, plus
165 protease inhibitors with or without EDTA). Samples were then incubated with 0.5 μl of
166 primary antibody at 4°C for 3 h under rotation. Thirty microliters of Protein G Sepharose™ 4
167 Fast Flow (GE Healthcare) was added to the samples, which were then rotated at 4°C for 16
168 h. The samples were washed in wash buffer six times and then prepared for SDS-PAGE as

169 described above.

170

171 **Quantitative Reverse-Transcription RT-qPCR**

172 Total RNA was extracted from mouse skeletal muscle with an acid guanidinium
173 thiocyanate-phenol-chloroform mixture (ISOGEN; Nippongene, Tokyo, Japan). RT-qPCR
174 was performed using gene-specific primers and SYBR™ Green dye in an ABI StepOne™
175 PCR system (Applied Biosystems, Foster City, CA), as previously described [28].
176 Oligonucleotide primers used for amplification are listed in Table 1.

177

178 **Measurement of the number of nuclei and fiber size of skeletal muscle**

179 Sections of TA were stained with hematoxylin-eosin (HE). Sections were
180 photographed using a BIOREVO BZ-9000 fluorescence microscope (Keyence, Osaka,
181 Japan). The number of nuclei per muscle fiber and fiber size-frequency were measured in
182 four sections of each muscle.

183

184 **Measurement of Biochemical Parameters**

185 Protein concentrations were determined using a bicinchoninic acid assay kit (Pierce,
186 Rockford, IL) [29]. Serum concentrations of triglycerides and non-esterified fatty acid
187 (NEFA) were measured using appropriate kits (Fuji Film Wako Pure Chemical Co., Osaka,
188 Japan), following the manufacturer's protocol. Serum glucose concentrations were measured
189 using a glucose meter for laboratory animals (Research and Innovation Japan Inc., Shiroi,
190 Chiba). Serum insulin concentration was measured using an enzyme-linked immunosorbent
191 assay kit (Funakoshi Co., Ltd., Tokyo, Japan). Serum concentrations of pyruvate and lactate
192 were determined using commercial assay kits (Funakoshi Co., Ltd.).

193

194 **Cycloheximide (CHX) Treatment**

195 Cycloheximide (CHX) is an inhibitor of protein synthesis, particularly of translation.
196 CHX is widely used for evaluation of activity in protein degradation independent of protein
197 synthesis. COS7 cells, transfected with the plasmid vectors containing the indicated genes,
198 were treated with CHX for indicated periods.

199

200 **Epoxomicin Treatment**

201 Epoxomicin is a strong and selective inhibitor of proteasomes and does not affect
202 other protein degradation systems. COS7 cells, transfected with the plasmid vectors
203 containing the indicated genes, were treated with epoxomicin for 2–4 h.

204

205 **Statistical Analysis**

206 Data are expressed as mean ± standard deviation (SD) ($n = 3–7$). Differences
207 between two groups were assessed using Scheffé's test. All statistical analyses were
208 conducted using SPSS 6.1 software (SPSS Japan, Tokyo, Japan). Differences were
209 considered significant at $p < 0.05$.

210

211

212 **Results**

213 **Depletion of MuRF1 Prevents Body Weight Gain**

214 Gene expression of MuRF1 was measured in 3-, 12-, and 24-month-old mice in

215 wild-type (MuRF1$^{+/+}$) by RT-qPCR and increased with age (Fig. 1A). Our RT-qPCR did not

216 detect MuRF1 expression in any skeletal muscles of MuRF1-deficient (MuRF1$^{-/-}$) mice (data

217 not shown), indicating complete knockout of MuRF1 expression. We measured BW of

218 MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice monthly for 2 years. Interestingly, BW of MuRF1$^{-/-}$ mice

219 more than 8-month-old was significantly less than age-matched MuRF1$^{+/+}$ mice (Fig. 1B).

220 However, no significant difference in food intake between MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice was

221 noted during the study period (Fig. 1C). We assessed body composition using CT scans to

222 explore this finding in MuRF1$^{-/-}$ mice. The percentage of fat mass was significantly lower in

223 MuRF1$^{-/-}$ mice than in MuRF1$^{+/+}$ mice (Fig. 1 D and E). The wet weight of epididymal fat in

224 12- and 24-month-old MuRF1$^{-/-}$ mice was also reduced compared with MuRF1$^{+/+}$ mice (Fig.

225 1F), indicating that aged (>7 month-old) MuRF1$^{-/-}$ mice were leaner.

226

227 **MuRF1 Deficiency Partially Protects from Age-Related Muscle Atrophy**

228 We elucidated MuRF1 deficiency on age-dependent muscle atrophy, consistent with

229 the association between muscle atrophy and ubiquitin ligase [8, 12, 13]. We measured wet

230 muscle weight of four hindlimb muscles (TA, extensor digitorum longus (EDL),

231 gastrocnemius (GA), and soleus (SO)) at 3 and 24 months of age. Age-induced decreases in

232 MW compensated by BW were significantly inhibited in TA and SO in MuRF1$^{-/-}$ mice (Fig.

233 2A).

234 We next measured the number of nuclei per muscle fiber and cross-sectional area

235 (CSA) of TA, which is sensitive to aging [3, 4, 30] in mice at 3 and 24 months of age. The

236 number of nuclei per muscle fiber significantly decreased in TA of MuRF1$^{+/+}$ mice during

237 aging. On the other hand, no significant change was observed in TA from MuRF1 deficient
238 mice during aging (Fig. 2B). In addition, the fiber size-frequency distribution of TA muscle
239 fibers was measured. The fiber size-frequency distribution shifted to larger size in MuRF1$^{-/-}$
240 mice than in age-matched MuRF1$^{+/+}$ mice (Fig 2C).
241

242 **MuRF1$^{-/-}$ Mice Preferentially Metabolize Lipid Rather than Glucose**

243 We measured serum and muscle (GA) parameters of lipid and glucose metabolism in
244 3-months-old MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice (Table 2) to determine why MuRF1 deficiency
245 prevented BW and fat mass gain in mice. Already, at 3 months of age, the serum glucose
246 levels in fasted MuRF1$^{-/-}$ mice were significantly lower than those in MuRF1$^{+/+}$ mice,
247 although the fasting serum insulin level of MuRF1$^{+/+}$ mice was similar to that of MuRF1$^{-/-}$
248 mice. In contrast, serum NEFA levels in MuRF1$^{-/-}$ mice were significantly lower than that in
249 MuRF1$^{+/+}$ mice. Further, serum triglyceride levels tended to be lower in MuRF1$^{-/-}$ mice,
250 although the difference with MuRF1$^{+/+}$ mice was not significant. Interestingly, serum and
251 muscle lactate levels were higher in MuRF1$^{-/-}$ mice than in MuRF1$^{+/+}$ mice, but serum
252 pyruvate levels were lower in MuRF1$^{-/-}$ mice. Thus, lipolysis and anaerobic glycolysis were
253 promoted in the skeletal muscle of MuRF1$^{-/-}$ mice.

254 We also analyzed whole-body lipid utilization and locomotor activity in young adult
255 mice using indirect calorimetry and an animal movement analysis system, respectively. We
256 use ZT, which shows time of lights on as ZT 0 and time of lights off as ZT 12. The RQ of 3-
257 month-old MuRF1$^{-/-}$ mice was significantly lower than that of MuRF1$^{+/+}$ mice (Fig. 3A). In
258 addition, EE of 3-month-old MuRF1$^{-/-}$ mice was significantly higher than that of MuRF1$^{+/+}$
259 mice during ZT 0 to ZT 12 (Light periods) in spite of similar locomotor activity. Even during
260 ZT 12 to ZT 0 (Dark periods), EE of MuRF1$^{-/-}$ animals was about the same as for MuRF1$^{+/+}$
261 mice in spite of less locomotor activity (Fig. 3A). These results indicate that MuRF1

262 deficiency enhanced EE, especially via lipid oxidation. Next, we measured gene expression
263 of genes involved in lipogenesis and lipolysis and associated transcription factors in GAs of
264 MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice. MuRF1 deficiency showed little effect on expression of
265 lipogenesis- and lipolysis-associated genes (Fig. 3B).
266

267 **PDK4 is Accumulated in the Mitochondria of MuRF1$^{-/-}$ Skeletal Muscle**

268 A previous study showed that MuRF1 interacts with glucose and fatty acid
269 metabolism-related molecules, such as PDHs and their negative regulators, PDKs, through
270 yeast two-hybrid screens [18]. We performed co-immunoprecipitation assays in MuRF1-V5
271 and empty (Mock)-, PDHα-, PDHβ-, or PDK4-myc co-transfected COS7 cells to confirm
272 MuRF1 binding to these molecules. The assays revealed that PDK4 specifically interacts with
273 MuRF1 (Fig. 4A). We focused on PDK4 as a crucial enzyme for switching from glucose to
274 fatty acid energy sources [19, 20].

275 No significant difference in the gene expression of PDK4 was observed between the
276 GA of MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice (Fig. 4B). Thus, we examined mitochondrial PDK4
277 protein levels in the skeletal muscle of MuRF1$^{-/-}$ mice; PDK4 translocated into the
278 mitochondria after translation. Surprisingly, PDK4 significantly accumulated in the
279 mitochondrial fraction of GA of 3-month-old MuRF1$^{-/-}$ mice (Fig. 4C). In contrast, MuRF1
280 deficiency did not affect protein levels of PDHs, including PDH E1α, E1β, E2, and E3, in the
281 mitochondrial fraction of GA (Fig. 4C). Further, the increase of PDK4 protein levels of the
282 muscle mitochondrial fraction in MuRF1$^{-/-}$ mice was sustained for 24 months. To confirm
283 our hypothesis, we examined total PDK4 level by Western blotting. We used mitofusin 2
284 (MFN2), which is one of the mitochondrial fusion proteins, as internal standard, because its
285 protein levels in human vastus lateralis muscle were not changed in a wide age range (21 to
286 88 years) [31]. We clearly demonstrated that the value of mitochondrial PDK4/total PDK4 in

287 12 and 24-month-old MuRF1$^{-/-}$ mice were higher than those in the respective month-old
288 MuRF1$^{+/+}$ mice. These results indicate that the accumulation of PDK4 was enhanced during
289 aging. (Fig. 4D). These results suggest that MuRF1 affects translocation of PDK4 into
290 skeletal muscle mitochondria in mice.
291

292 **PDK4 Interacts with MuRF1 Possibly in Cytosol**

293 PDK4 is targeted to mitochondria via its N-terminal MTS (Met-Lys-Ala-Ala-Arg-
294 Phe-Val-Met-Arg-Ser-) [32]. However, MuRF1 is in the cytosol [8, 12, 13]. We thus
295 examined the interaction of MuRF1 and PDK4 to explore mechanisms of interaction. We
296 constructed an MTS-truncated mutant of PDK4 (ΔMTS-PDK4), which exhibits appropriate
297 cytosolic localization, and a GFP containing the MTS of PDK4 (MTS-GFP), which exhibits
298 appropriate mitochondrial localization (Fig. 5A). We confirmed that PDK4 (-myc) localizes
299 in the mitochondria; PDK4 was detected in the mitochondrial fraction containing MFN2, a
300 mitochondrial protein. As expected, ΔMTS-PDK4 was detected in the cytosolic fraction
301 containing cytosolic glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Fig. 5B).

302 Co-immunoprecipitation assays revealed that MuRF1 interacted with ΔMTS-PDK4
303 and PDK4, but MTS-GFP failed to interact with MuRF1 (Fig. 5C). Deletion of MTS in
304 PDK4 enhanced interaction between MuRF1 and PDK4 (Fig. 5C). Since MuRF1 interacted
305 with native PDK4, which is preferentially localized in the mitochondria (Fig. 5A), MuRF1
306 likely interacted with PDK4 during translocation from cytosol to mitochondria.
307

308 **MuRF1 Induces SUMOylation of PDK4, but not Ubiquitination**

309 MuRF1 is suggested to trigger muscle protein degradation through substrate
310 ubiquitination [8, 12, 13]. PDK4 accumulation in the mitochondria in MuRF1$^{-/-}$ skeletal
311 muscle (Fig. 4) led us to hypothesize a posttranslational modification by MuRF1. Therefore,

312 we examined MuRF1 induction of ubiquitination and proteasomal degradation of PDK4. Cell
313 lysates from cells transfected with mock vector or vectors containing MuRF1 or an inactive
314 mutant with E2-binding domain deletion (ΔRING-MuRF1) were used in a PDK4
315 ubiquitination assay. Ubiquitination of PDK4 was measured in the presence of epoxomicin, a
316 selective and strong proteasome inhibitor, to enhance the accumulation of ubiquitin
317 conjugates. Overexpression of MuRF1 and ΔRING-MuRF1 failed to induce PDK4
318 ubiquitination (Fig. 6A). Western blotting for PDK4 after cycloheximide (CHX) treatment
319 showed that PDK4 degradation rate in MuRF1-expressing cells did not differ from
320 degradation in mock- and ΔRING-MuRF1-expressing cells (Fig. 6B). MuRF1 is probably not
321 involved in ubiquitin-dependent degradation of PDK4.

322 As described in introduction, MuRF1 reportedly interacts with SUMO3 involved in
323 SUMOylation via its RING domain [22, 23], and PDK4 contains the conserved
324 SUMOylation motif -ψ-K-X-E/D- (ψ: bulky hydrophobic residue), ^{282}Gly-Lys-Glu-Asp,
325 which binds SUMOs 1, 2, and 3 [24]. Thus, we examined whether MuRF1 induces
326 SUMOylation of PDK4. The RING finger domain contains the SUMO2/3 binding site [23,
327 33], and ΔRING-MuRF1 was used as a negative control. Although SUMOylated PDK4 was
328 weakly detected in the Mock and ΔRING-MuRF1 lane, we found that MuRF1 induced
329 SUMOylation of PDK4 more than Mock and ΔRING-MuRF1. (Fig. 6C).

330 **Discussion**

331 MuRF1 is a typical E3 ubiquitin ligase associated with muscle atrophy and, along
332 with muscle atrophy F-box protein 1/atrogin1 [8], functions in the progression of muscle
333 atrophy in various conditions. However, in age-related muscle atrophy, whether MuRF1
334 expression is increased, decreased, or unchanged as a function of age is controversial [15-17].

335 In this study, we first examined the critical role of MuRF1 in age-related muscle loss
336 using MuRF1-deficient mice. MuRF1 deficiency prevented age-related decrease in TA and
337 SO weights, but not EDL and GA weights. Possibly, decrease in the gain of extra- and intra-
338 muscular fat tissue during aging affected the wet MW of EDL and GA. Therefore, although
339 MuRF1 deficiency prevented age-related decrease in the number of nuclei per muscle fiber
340 and of CSA distribution in TA muscle, we suggest that MuRF1 deficiency partially prevents
341 age-induced muscle atrophy. Although the data against muscle functions such as muscle
342 force generation are lacking, much of the literature shows that MuRF1-deficient mice are
343 resistant against muscle atrophy or muscle wasting. Since this study was designed to
344 elucidate changes in lipid metabolism in MuRF1-deficient mice, there was no need for us to
345 measure the muscle force generation.

346 However, these phenomena contradict a previous finding that muscle mass and fiber
347 CSA are maintained with aging in MuRF1$^{-/-}$ mice, suggesting that MuRF1 deficiency
348 prevents age-induced wet weight loss of all hindlimb skeletal muscles in mice [13]. C/EBP-
349 homologous protein, a maladaptive ER stress marker, was unchanged in the skeletal muscle
350 of old MuRF1$^{-/-}$ mice. Authors concluded that with age, MuRF1 plays an important role in
351 the control of skeletal muscle mass and growth capacity through the regulation of cell-level
352 stress [13]. In their study, MuRF1$^{-/-}$ mice were established by insertion of *LacZ* that
353 simultaneously replaced approximately 8 kb of *MuRF1* genomic sequences, comprising
354 exons 1–4 and most of exon 5 [13]. In this study, murine *MuRF1* was disrupted by

355 homologous recombination into exon 2 [12]. This difference may explain the discrepancies
356 between findings in the two studies.

357 Interestingly, MuRF1 deficiency prevented age-induced BW gain and adipose tissue
358 accumulation. The results of indirect calorimetry and locomotor activity showed that MuRF1
359 deficiency enhanced the EE, especially through lipid metabolism. Significantly lower RQ and
360 higher resting EE in MuRF1$^{-/-}$ mice are consistent with decreased serum levels of lipid
361 metabolic parameters, such as NEFA and triglyceride. Prevention of BW gain by MuRF1
362 deficiency is thus possibly due to an increase in lipid metabolism. Obesity induces fat
363 accumulation in both adipose and non-adipose tissues, such as the skeletal muscle and liver
364 [34]. Our findings in MuRF1$^{-/-}$ mice thus suggest a link between skeletal muscle and whole-
365 body adipose tissue in the regulation of lipid metabolism. Although no past study has referred
366 to the association between MuRF1 and fat tissue accumulation, a previous study proposed
367 that the whole BW of an older MuRF1$^{-/-}$ mice represented an association [13]. Our study
368 showed significantly light BW of MuRF1$^{-/-}$ mice compared to that of MuRF1$^{+/+}$ mice after 7
369 months of age. On the other hand, a previous study showed no difference in the BW between
370 MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice during aging. This difference might have originated from the
371 deletion site of MuRF1, as described in the paragraph above. As a limitation, MuRF1 is a
372 striated muscle-specific ubiquitin ligase [35, 36]. Therefore, although a global deletion mouse
373 model of MuRF1 has been accepted as a striated muscle-specific deleted model, there is some
374 possibility of non-skeletal muscle effects, such as cardiac function, which was not evaluated
375 in this study.

376 PDK4 is a crucial enzyme for the switch from glucose to fatty acid metabolism, and
377 we focused on PDK4 function associated with MuRF1. Accumulated PDK4 was detected in
378 skeletal muscle mitochondria of MuRF1$^{-/-}$ mice. In our study using COS7 cells with
379 overexpression of MuRF1, MuRF1 likely interacted with PDK4 in cytosol and mediated

380 SUMOylation but not ubiquitination of PDK4. This activity may inhibit mitochondrial
381 translocation of precursor PDK4 protein in cytosol. We also considered that inhibition of
382 PDH activity mediated by PDK4 accumulated in mitochondria stimulated mitochondrial lipid
383 β-oxidation. This linkage is consistent with a previous finding that selective inhibition of
384 PDHs by PDK4 resulted in enhanced lipolysis in the skeletal muscle during prolonged
385 starvation [37].

386 Cytosolic chaperones, such as heat shock protein 70 and mitochondrial import-
387 stimulating factor, mediate import of proteins into mitochondria [38, 39]. Protein
388 modification in the cytosol is generally necessary for mitochondrial translocation.
389 SUMOylation regulates protein stability, cytosolic-nuclear transport, and transcription [40].
390 SUMOylation of some mitochondrial proteins such as dynamin-related protein 1 reportedly
391 affects the localization and the function of the proteins [41, 42]. PDK4 displays a consensus
392 sequence for SUMOylation [32]; we detected SUMOylated but not ubiquitinated PDK4 in
393 MuRF1-overexpressing cells. Deletion of MTS in PDK4 enhanced the interaction between
394 MuRF1 and PDK4. We thus considered that SUMOylation mediated by MuRF1 is associated
395 with localization and function of PDK4.

396 The effect of MuRF1 deficiency on the muscle weight is a local action, while PDK4-
397 mediated metabolic changes on BW, total fat ratio, and lipid accumulation are systemic. The
398 latter are preferentially dependent on volume, rather than kind, of skeletal muscles. Therefore
399 we used GA, a large muscle in the hindlimb, to elucidate changes in metabolic parameters,
400 gene expression, and protein levels. Metabolic changes, including PDK4 SUMOylation in
401 GA, are likely reflected in BW, total fat ratio, and lipid accumulation differences between
402 MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice. Also, previous literature suggests that MuRF1 suppresses
403 glucose-related EE in the muscle by degradation of muscle creatine kinase [25]. This
404 outcome is consistent with our finding of decreased fasting serum glucose levels in 3-month-

405 old MuRF1$^{-/-}$ mice. Thus, MuRF1 is also associated with regulation of lipid-related EE.

406 We used GA in 3-month-old young adult mice but not elder mice in the above

407 analyses recognizing that changes in BW or adipose tissue accumulation in itself affects

408 metabolism. We analyzed 3-month-old MuRF1$^{-/-}$ mice with BWs similar to MuRF1$^{+/+}$ mice

409 to eliminate the possibility that reduced BW and adipose tissue affect metabolism,

410 independent of MuRF1 function. Also, PDK4 accumulation into mitochondria of MuRF1$^{-/-}$

411 mice is already notable at 3 months of age (Fig. 4C). Occasionally, an obvious phenotype,

412 such as obesity and leanness, requires several months after organ-level metabolic changes,

413 although the mechanism of such a time lag is unknown.

414 Differences in food intake between MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice might influence

415 BW during aging, even if such differences are not significant. However, gain in BWs in

416 MuRF1$^{-/-}$ mice fed with a high-fat diet for 10 weeks was smaller than MuRF1$^{+/+}$ mice fed the

417 same diet, although their food intakes were similar (data not shown). Therefore, it seems

418 unlikely that differences in daily food intake caused differences in body weight between

419 MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice.

420 We first detected impaired body weight gain in striated muscle-specific MuRF1-

421 deficient mice during aging. Therefore, we investigated the effect of increased MuRF1 during

422 aging by using MuRF1-deficient mice. An *in vivo* experiment using MuRF1-deficient mice

423 was important for evaluating local reaction, such as the phenotype of skeletal muscle as well

424 as systemic reaction, such as BW, accumulation of fat tissue, and metabolic function like RQ

425 or EE against the deletion of MuRF1. It is exceedingly difficult to observe such a linkage

426 only via an *in vitro* model. Thus, we suggested the possibility of the effect of MuRF1 against

427 lipid metabolism via PDK4 localization. To investigate this association between the presence

428 of MuRF1 and PDK4 localization in detail, we performed an *in vitro* experiment using

429 transfection of the plasmid vector with mutant ΔMTS-PDK4 and ΔRING-MuRF1. From

430 these experiments, we suggested the possibility that MuRF1 suppressed lipid metabolism by
431 inhibiting PDK4 transfer into the mitochondria, possibly through posttranslational
432 SUMOylation of PDK4. Similarly, we considered that the MuRF1 knockout model is
433 especially useful for researching the inter-organ linkage associated with changes in the
434 muscle phenotype.

435 A part of our study used *in vitro* experiments with COS7 cells overexpressing
436 MuRF1, because we could not adequately transfect the plasmid vector into differentiated
437 C2C12 myotubes. In addition, when we transfected the plasmid vector with Mock, MuRF1,
438 and ΔRING-MuRF1 into the cells. Cells that did not express MuRF1 originally were of
439 interest in this study. Because non-SUMOylated PDK4 and changes in PDH activity and
440 phosphorylation were not detected in skeletal muscles of MuRF1$^{-/-}$ mice, could possibly
441 indicate a smaller ratio of SUMOylated PDK4 to non-SUMOylated PDK4. Further studies
442 are necessary to elucidate these issues.

443 In this study, we newly discovered that increasing MuRF1 expression during aging
444 is associated with muscle mass decrease through ubiquitin-proteasome system as well as lipid
445 metabolism dysfunction through the regulation of localization of PDK4 protein. Muscle mass
446 maintenance is important against the homeostasis of energy metabolism, and the converse is
447 also true. Hence, MuRF1 might be a key protein that regulates age-related sarcopenia and
448 metabolic diseases. In the near future, inhibitors or repressors of MuRF1might be developed
449 as a therapeutic target against locomotive and metabolic dysfunction during aging.

Conclusion

MuRF1 deficiency partially prevented age-related weight loss of skeletal muscle. However, BW and body fat tissue of >7-month-old MuRF1$^{-/-}$ mice were lower than those of age-matched MuRF1$^{+/+}$ mice, indicating that aged MuRF1$^{-/-}$ mice were leaner. The lower RQ and higher EE of 3-month-old MuRF1$^{-/-}$ mice suggest that MuRF1$^{-/-}$ mice preferentially metabolize lipid as the energy source. PDK4 was accumulated in the skeletal muscle mitochondria of 3-month-old MuRF1$^{-/-}$ mice compared with age-matched MuRF1$^{+/+}$ mice. *In vitro* study using MuRF1 overexpressing COS7 cells, MuRF1 interacted with PDK4 in cytosol, and mediated SUMOylation of PDK4. Therefore, we suggest that MuRF1 deficiency enhances lipid metabolism by upregulating localization of PDK4 into mitochondria through the inhibition of SUMOylation. Inhibition of MuRF1-mediated PDK4 SUMOylation is a potential therapeutic target to the age-related dysfunction of lipid metabolism and obesity in addition to age-related muscle atrophy.

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Author Contributions

Conceptualization, K.S., K.H., and T.N.; Data curation, K.S., K.H., T.M., T.U., K.K., M.O., and A.U.; Formal analysis, K.H.; Funding acquisition, T.N.; Methodology, K.S., K.H., M.O., I.O., R.M., S.L.; Project administration, T.N.; Validation, K.H., and K.S; Writing—original draft, K.S. and K.H.; Writing—review and editing, S.L., and T.N. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

BW: body weight; COX IV: mitochondrial cytochrome c oxidase subunit IV; CSA: cross-sectional area; EDL: extensor digitorum longus muscle; EE: energy expenditure; GA: gastrocnemius muscle: GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GFP: green fluorescent protein; HE: hematoxylin-eosin; MFN2: mitofusin 2; MM: molecular mass; MTS:

490 mitochondrial targeting signal; MuRF1: muscle RING finger 1; MW: muscle weight; NEFA:
491 non-esterified fatty acid; RT-qPCR: quantitative reverse-transcription polymerase chain
492 reaction; PDHs: pyruvate dehydrogenase complex; PDK4: pyruvate dehydrogenase kinase 4;
493 PLA2: phospholipase A2; RQ: respiratory quotient; SO: soleus muscle; SUMO: small
494 ubiquitin-related modifier; TA: tibialis anterior muscle; ZT: Zeitgeber time
495

496 **References**

497 1. Larsson L., Degens H., Li M., Salviati L., Lee Y.I., Thompson W., Kirkland J.L., Sandri
498 M. 2019. Sarcopenia: aging-related loss of muscle mass and function. Physiol Rev. 99:427-
499 511.
500 2. Li C.W., Yu K., Shyh-Chang N., Li G.X., Jiang L.J., Yu S.L., Xu L.Y., Liu R.J., Guo Z.J.,
501 Xie H.Y., Li R.R., Ying J., Li K., Li D.J. 2019. Circulating factors associated with
502 sarcopenia during ageing and after intensive lifestyle intervention. J Cachexia Sarcopenia
503 Muscle. 10:586-600.
504 3. Greig, C.A.; Gray, C.; Rankin, D.; Young, A.; Mann, V.; Noble, B.; Atherton, P.J 2011.
505 Blunting of adaptive responses to resistance exercise training in women over 75y. Exp
506 Gerontol. 46:884-890.
507 4. Frontera, W.R.; Suh, D.; Krivickas, L.S.; Hughes, V.A.; Goldstein, R.; Roubenoff, R.
508 Skeletal muscle fiber quality in older men and women. 2000. Am J Physiol Cell Physiol.
509 279:C611-C618.
510 5. Kalinkovich A, Livshits G. 2017. Sarcopenic obesity or obese sarcopenia: A cross talk
511 between age-associated adipose tissue and skeletal muscle inflammation as a main
512 mechanism of the pathogenesis. Ageing Res Rev. 35:200-221.
513 6. Chhetri K.J., Barreto S.P., Fougère B., Rolland Y., Vellas B., Cesari M. 2018. Chronic
514 inflammation and sarcopenia: A regenerative cell therapy perspective. Exp Gerontol.
515 103:115-123.
516 7. Centner, T.; Yano, J.; Kimura, E.; McElhinny, A.S.; Pelin, K.;Witt, C.C.; Bang, M.L.;
517 Trombitas, K.; Granzier, H.; Gregorio, C.C.; Sorimachi, S; Labeit, S. 2001. Identification
518 of muscle specific ring finger proteins as potential regulators of the titin kinase domain. J.
519 Mol Biol. 306: 717-726.
520 8. Bodine, S.C.; Latres, E.; Baumhueter, S.; Lai, V.K.; Nunez, L.; Clarke, B.A.; Poueymirou,

521 W.T.; Panaro, F.J.; Na, E.; Dharmarajan, K.; Pan, Z.Q.; Valenzuela, D.M.; DeChiara,
522 T.M.; Stitt, T.N.; Yancopoulos, G.D.; Glass, D.J. 2001. Identification of ubiquitin ligases
523 required for skeletal muscle atrophy. Science. 294:1704-1708.
524 9. Clarke, B.A.; Drujan, D.; Willis, M.S.; Murphy, L.O.; Corpina, R.A.; Burova, E.; Rakhilin,
525 S.V.; Stitt, T.N.; Patterson, C.; Latres, E.; Glass, D. 2007. The E3 ligase MuRF1 degrades
526 myosin heavy chain protein in dexamethasone-treated skeletal muscle. Cell Metab. 6: 376–
527 385.
528 10. Fielitz, J.; Kim, M.-S.; Shelton, J.M.; Latif, S.; Spencer, J.A.; Glass, D.J.; Richardson,
529 J.A.; Bassel-Duby, R.; Olson, E.N. 2007. Myosin accumulation and striated muscle
530 myopathy result from the loss of muscle RING finger 1 and 3. J. Clin. Investig. 117: 2486–
531 2495.
532 11. Moreno, D.P.; Taillandier, D.; Polge, C. 2020. MuRF1/TRIM63, master regulator of
533 muscle mass. Int J Mol Sci. 21: 6663.
534 12. Witt, C.C.; Witt, S.H.; Lerche, S.; Labeit, D.; Back, W.; Labeit, S. 2008.
535 Cooperative control of striated muscle mass and metabolism by MuRF1 and MuRF2.
536 EMBO J. 27:350-360.
537 13. Hwee, D.T.; Baehr, L.M.; Philp, A.; Baar, K.; Bodine, S.C. 2014. Maintenance of muscle
538 mass and load-induced growth in Muscle RING Finger 1 null mice with age. Aging Cell
539 13:92-101.
540 14. Okamoto, Y.; Machida S. 2017. Changes in FOXO and proinflammatory cytokines in the
541 late stage of immobilized fast and slow muscle atrophy. Biomed Res. 38:331-342.
542 15. Clavel, S.; Coldefy, A.S.; Kurkdjian, E.; Salles, J.; Margaritis, I.; Derijard, B. 2006.
543 Atrophy-related ubiquitin ligases, atrogin-1 and MuRF1 are up-regulated in aged rat
544 tibialis anterior muscle. Mech Ageing Dev. 127:794-801.
545 16. Deruisseau, K.C.; Kavazis, A.N.; Powers, S.K. 2005. Selective downregulation of

546 ubiquitin conjugation cascade mRNA occurs in the senescent rat soleus muscle. Exp
547 Gerontol. 40:526-531.
548 17. Whitman, S.A.; Wacker, M.J.; Richmond, S.R.; Godard, M.P. 2005. Contributions of the
549 ubiquitin-proteasome pathway and apoptosis to human skeletal muscle wasting with age.
550 Pflugers Arch. 450:437-446.
551 18. Hirner, S.; Krohne, C.; Schuster, A.; Hoffmann, S.; Witt, S.; Erber, R.; Sticht, C.; Gasch,
552 A.; Labeit, S.; Labeit, D. 2008. MuRF1-dependent regulation of systemic carbohydrate
553 metabolism as revealed from transgenic mouse studies. J Mol Biol. 379:666-677.
554 19. Sugden, M.C.; Holness, M.J. 2003. Recent advances in mechanisms regulating glucose
555 oxidation at the level of the pyruvate dehydrogenase complex by PDKs. Am J Physiol
556 Endocrinol Metab. 284:E855-E862.
557 20. Wu, P.; Sato, J.; Zhao, Y.; Jaskiewicz, J.; Popov, K.M.; Harris, R.A. 1998. Starvation and
558 diabetes increase the amount of pyruvate dehydrogenase kinase isoenzyme 4 in rat heart.
559 Biochem J. 329:197-201.
560 21. Chambers, K.T.; Leone, T.C., Sambandam, N.; Kovacs, A.; Wagg, C.S.; Lopaschuk, G.D.;
561 Finck, B.N.; Kelly, D.P. 2011. Chronic inhibition of pyruvate dehydrogenase in heart
562 triggers an adaptive metabolic response. J Biol Chem. 286:11155-11162.
563 22. Heras, G.; Namuduri, A.V.; Traini, L.; Shevchenko, G.; Falk, A.; Bergström, Lind, S.; Jia,
564 M.; Tian, G.; Gastaldello, S. 2019. Muscle RING-finger protein-1 (MuRF1) functions and
565 cellular localization are regulated by SUMO1 post-translational modification. J Mol Cell
566 Biol. 11:356-370.
567 23. Dai, K.S.; Liew, C.C. 2001. A novel human striated muscle RING zinc finger protein,
568 SMRZ, interacts with SMT3b via its RING domain. J Biol Chem. 76:23992-23999.
569 24. Lescasse, R.; Pobiega, S.; Callebaut, I.; Marcand, S. 2013. End-joining inhibition at
570 telomeres requires the translocase and polySUMO-dependent ubiquitin ligase Uls1.

571 EMBO J. 32:805-815.
572 25. Koyama, S.; Hata, S.; Witt, C.C.; Ono, Y.; Lerche, S.; Ojima, K.; Chiba, T.; Doi, N.;
573 Kitamura, F.; Tanaka, K.; Abe, K.; Witt, S.H.; Rybin, V.; Gasch, A.; Franz, T.; Labeit, S.;
574 Sorimachi, H. 2008. Muscle RING-finger protein-1 (MuRF1) as a connector of muscle
575 energy metabolism and protein synthesis. J Mol Biol. 376:1224-1236.
576 26. Sprague, J.E.; Yang, X.; Sommers, J.; Gilman, T.L.; Mills, E.M. 2007. Roles of
577 norepinephrine, free fatty acids, thyroid status, and skeletal muscle uncoupling protein 3
578 expression in sympathomimetic-induced thermogenesis. J Pharmacol Exp Ther. 320:274-
579 280.
580 27. Arezi, B.; Xing, W.; Sorge, J.A.; Hogrefe, H.H. 2003. Amplification efficiency of
581 thermostable DNApolymerases. Anal Biochem. 321:226-235.
582 28. Nakao, R.; Hirasaka, K.; Goto, J.; Ishidoh, K.; Yamada, C.; Ohno, A.; Okumura, Y.;
583 Nonaka, I.; Yasutomo, K.; Baldwin, K.M.; Kominami, E.; Higashibata, A.; Nagano, K.;
584 Tanaka, K.; Yasui, N.; Mills, E.M.; Takeda, S.; Nikawa, T. 2009. Ubiquitin ligase Cbl-b
585 is a negative regulator for insulin-like growth factor 1 signaling during muscle atrophy
586 caused by unloading. Mol Cell Biol. 29:4798-4811.
587 29. Noble, J.E.; Bailey, M.J. 2009. Quantitation of protein. Methods Enzymol. 463:73-95.
588 30. Akasaki, Y.; Ouchi, N.; Izumiya, Y.; Bernardo, B.L.; Lebrasseur, N.K.; Walsh, K. 2014.
589 Glycolytic fast-twitch muscle fiber restoration counters adverse age-related changes in
590 body composition and metabolism. Aging Cell. 13:80-91.
591 31. Distefano, G.; Standley, R.A.; Dubé J.J.; Carnero, E.A.; Ritov, V.B.; Stefanovic-Racic,
592 M.; Toledo, F.G.S.; Piva, S.R.; Goodpaster, B.H.; Coen, P.M. 2017. Chronological age
593 does not influence ex-vivo mitochondrial respiration and quality control in skeletal muscle.
594 J Gerontol A Biol Sci Med Sci. 72:535-542.
595 32. Mootha, V.K.; Bunkenborg, J.; Olsen, J.V., Hjerrild, M.; Wisniewski, J.R.; Stahl, E.;

596 Bolouri, M.S.; Ray, H.N.; Sihag, S.; Kamal, M.; Patterson, N.; Lander, E.S.; Mann, M.
597 2003. Integrated analysis of protein composition, tissue diversity, and gene regulation in
598 mouse mitochondria. Cell. 115:629-640.
599 33. Liang, L.; Zhang, Z.; Li, J.; Wu, J.; Wang, L.; Huang, W.; Gao, S. 2017. Direct binding of
600 RNF8 to SUMO2/3 promotes cell survival following DNA damage. Mol Med Rep.
601 16:8385-8391.
602 34. Schrauwen-Hinderling, V.B.; Hesselink, M.K.; Schrauwen, P.; Kooi, M.E. 2006.
603 Intramyocellular lipid content in human skeletal muscle. Obesity (Silver Spring) 14:357-
604 367.
605 35. Bodine, S.C.; Baehr, L.M. 2014. Skeletal muscle atrophy and the E3 ubiquitin ligases
606 MuRF1 and MAFbx/atrogin-1. Am J Physiol Endocrinol Metab. 307: E469-484.
607 36. Willis, M.S.; Parry, T.L.; Brown, D.I.; Mota, R.I.; Huang, W.; Beak, J.Y.; Sola, M.; Zhou,
608 C.; Hicks, S.T.; Caughey, M.C.; D'Agostino Jr, R.B.; Jordan, J.; Hundley, W.G.; Jensen,
609 B.C. 2019. Doxorubicin exposure causes subacute cardiac atrophy dependent on the
610 striated muscle-specific ubiquitin ligase MuRF1. Circ Heart Fail.12: e005234o.
611 37. Li, R.Y.; Zhang, Q.H.; Liu, Z.; Qiao, J.; Zhao, S.X.; Shao, L.; Xiao, H.S.; Chen, J.L.; Chen,
612 M.D.; Song, H.D. 2006. Effect of short-term and long-term fasting on transcriptional
613 regulation of metabolic genes in rat tissues. Biochem Biophys Res Commun. 344:562-570.
614 38. Azem, A.; Oppliger, W.; Lustig, A.; Jenö, P.; Feifel, B.; Schatz, G.; Horst, M. 1997. The
615 mitochondrial hsp70 chaperone system. Effect of adenine nucleotides, peptide substrate,
616 and mGrpE on the oligomeric state of mhsp70. J Biol Chem. 272:20901-20906.
617 39. Hachiya, N.; Komiya, T.; Alam, R.; Iwahashi, J.; Sakaguchi, M.; Omura, T.; Mihara, K.
618 1994. MSF, a novel cytoplasmic chaperone which functions in precursor targeting to
619 mitochondria. EMBO J. 13:5146-5154
620 40. Gareau, J.R.; Lima, C.D. 2010. The SUMO pathway: emerging mechanisms that shape

621 specificity, conjugation and recognition. Nat Rev Mol Cell Biol. 11:861-871.

622 41. Anderson, C.A.; Blackstone, C. 2013. SUMO wrestling with Drp1 at mitochondria.
623 EMBO J. 32:1496-1498.

624 **42.** Guerra de Souza, A.C.; Prediger, R.D.; Cimarost, H. SUMO. 2016. -regulated
625 mitochondrial function in Parkinson's disease. J Neurochem. 137:673-686.

626 **Figure legends**

627 **Fig 1. Depletion of MuRF1 prevents body weight gain in mice through suppressing fat**
628 **tissue accumulation.** (A) Gene expression of MuRF1 in MuRF1$^{+/+}$ mice at 3, 12, and 24
629 months (n = 7). The expression of MuRF1 increased with age, and significantly increased in
630 24-month MuRF1$^{+/+}$ mice (Data are means ± SD. **$P < 0.01$ vs. 3-month MuRF1$^{+/+}$ mice).
631 (B) Body weight (BW) over 24 months of aging in MuRF1$^{+/+}$ (n = 7) and MuRF1$^{-/-}$ mice (n =
632 6). The weight of the mice was measured monthly, and BW of MuRF1$^{-/-}$ mice was
633 significantly lighter compared to that of MuRF1$^{+/+}$ mice at the same age (Data are means ±
634 SD. *$P < 0.05$ vs. MuRF1$^{+/+}$ mice). (C) Food intake of MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice (n = 6–
635 7). Food intake was measured at 3, 12, and 24 months. The amount of food intake between
636 MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice was not significantly different (Data are means ± SD). (D)
637 Representative CT scans of 12-month-old MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice. Red, yellow, and
638 blue represent visceral fat, subcutaneous fat, and bone, respectively. (E) Percentage of fat
639 mass analyzed based on CT scans of MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice. The fat tissue ratio of
640 MuRF1$^{-/-}$ mice at 10 and 12 months of age was significantly lower compared to that of
641 MuRF1$^{+/+}$ mice (Data are means ± SD. *$P < 0.05$ vs. MuRF1$^{+/+}$ mice, n = 6–7). (F) White
642 adipose tissue weight of MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice. Wet weight of epididymal adipose
643 tissue was measured at 3, 12, and 24 months of age. The weight of white adipose tissue of
644 MuRF1$^{-/-}$ mice was significantly lighter compared to that of MuRF1$^{+/+}$ mice at 12 and 24
645 months of age (Data are means ± SD, *$p < 0.05$ vs. MuRF1$^{+/+}$ mice, n = 6–7).

646

647 **Fig 2. MuRF1 depletion partially protects from aging muscle atrophy.** (A) Wet muscle
648 weights (MW) of hindlimb skeletal muscles compensated by body weight (BW). Wet weights
649 of tibialis anterior (TA), extensor digitorum longus (EDL), gastrocnemius (GA), and soleus
650 (SO) muscles of MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice were measured at 3 and 24 months of age.

651 The decease of MW per BW with age was significantly prevented in TA and SO muscles in
652 MuRF1$^{-/-}$ mice. (Data are means ± SD (n = 6–7). * $P < 0.05$, **$P < 0.01$ vs. 3-month
653 MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice). (B) Hematoxylin-eosin staining (HE) of TA. Sections of TA
654 from indicated mice were stained with HE. The number of nuclei and muscle fiber of TA was
655 measured using the image software. The decrease in the number of nuclei per muscle fiber
656 with age was significantly prevented in MuRF1$^{-/-}$ mice. (Data are means ± SD (n = 5). * $p <$
657 0.05 vs. 3-month MuRF1$^{+/+}$ mice). Scale bar = 100 µm. (C) Distribution of CSA. Values
658 indicate the ratio of the number of muscle fibers with the indicated area to the number of total
659 muscle fibers in the section. Data are means ± SD (n = 5). * $P < 0.05$ vs. 3-month-old
660 MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice, respectively.
661
662 **Fig 3. MuRF1$^{-/-}$ mice preferentially metabolize lipid rather than glucose.** (A) RQ, EE,
663 and locomotor activity in MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice. The RQ and EE of MuRF1$^{+/+}$ and
664 MuRF1$^{-/-}$ mice were measured by indirect calorimetry at 3-month-old. Locomotor activity of
665 age-matched mice was measured using an animal movement analysis system. Lower RQ in
666 MuRF1$^{-/-}$ mice during both periods suggested that MuRF1$^{-/-}$ mice depended on more lipid
667 through energy synthesis than MuRF1$^{+/+}$ mice. Higher resting EE during the light period in
668 MuRF1$^{-/-}$ mice suggested that MuRF1$^{-/-}$ mice expended more energy. EE during the dark
669 period was similar between both groups, although locomotor activity was lower in MuRF1$^{-/-}$
670 mice. Data are means ± SD (n = 4). **$p < 0.01$ vs. MuRF1$^{+/+}$ mice. Gray color behind the
671 graphs indicates the dark period. (B) Expression of lipogenesis- and lipolysis-associated
672 genes in the skeletal muscle of MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice. Gene expression in GA from
673 3-month-old MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice was analyzed by RT-qPCR. The gene
674 expressions associated lipogenesis and lipolysis were not different between the groups. Data
675 are means ± SD (n = 3). FAS, fatty acid synthase; DGAT1, diacylglycerol O-acyltransferase

676 1; SCD1, stearoyl-CoA desaturase-1; CPT1b, carnitine palmitoyltransferase 1b; ACC2,
677 acetyl-CoA carboxylase 2; CD36, cluster of differentiation 36; PPARδ, peroxisome
678 proliferator-activated receptor δ; SREBP1c, sterol regulatory element binding protein 1c;
679 HSL, hormone-sensitive lipase; ATGL, adipose triglyceride lipase
680
681 **Fig 4. PDK4 is accumulated in mitochondria of the skeletal muscle in MuRF1$^{-/-}$ mice.**
682 (A) Interaction of MuRF1 with PDHα, PDHβ, and PDK4. We co-transfected myc-tagged
683 PDHα, PDHβ, or PDK4 and V5-tagged MuRF1 (MuRF1-V5) into COS7 cells. An empty
684 vector was used as a mock control. Lysates of the COS7 cells were immunoprecipitated with
685 anti-V5 antibodies and analyzed by immunoblotting to detect with anti-V5 and anti-myc
686 antibodies. The bands without immunoprecipitation (IP) indicated the pure molecular mass of
687 each protein tagged with myc. The band with a black arrow in PDK4-myc with IP suggested
688 the specific band, and the larger bands in Mock/PDHα/PDHβ/PDK4-myc just above the band
689 with a black arrow suggested a nonspecific band. MM, molecular mass. (B) Expression of
690 PDK4 in the skeletal muscle. Gene expression in GA from 3-month-old MuRF1$^{+/+}$ and
691 MuRF1$^{-/-}$ mice was analyzed by RT-qPCR. Data are means ± SD (n = 3). (C) Accumulation
692 of PDK4 in the mitochondria. Mitochondrial fractions were isolated from the GA muscle of
693 MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice at 3 months of age. Mitochondrial extracts (20 μg/lane) were
694 subjected to SDS-PAGE followed by immunoblotting with anti-PDK4, anti-PDH, and anti-
695 COX IV. The ratio of PDK4 or PDHs to COXIV was calculated by densitometry. Only
696 PDK4 proteins significantly accumulated in the mitochondria in GA from MuRF1$^{-/-}$ mice.
697 Data are means ± SD (n = 3). *$p < 0.05$ vs. MuRF1$^{+/+}$ mice. MM, molecular mass. (D) Age-
698 dependent changes of PDK4 level in the whole cell protein and mitochondria fractions
699 isolated from the GA of MuRF1$^{+/+}$ and MuRF1$^{-/-}$ mice at 3, 12, and 24 months of age. Whole
700 cell lysate and mitochondrial extracts (20 μg/lane) were subjected to SDS-PAGE followed by

701 immunoblotting with anti-PDK4, anti-MFN2, and anti-COX IV antibodies. PDK4
702 accumulated in the mitochondria in MuRF1$^{-/-}$ mice during aging. Data are means ± SD (n =
703 3). *$p < 0.05$ vs. MuRF1$^{+/+}$ mice.
704
705 **Fig 5. PDK4 interacts with MuRF1 *in vitro*.** (A) Schema of PDK4 and its mutants. (B)
706 Localization of PDK4 and its mutants. Mitochondrial and cytosolic fractions were isolated
707 from COS7 cells transfected with PDK4/ΔMTS-PDK4-myc. Mitochondrial and cytosolic
708 extracts (20 μg/lane) were subjected to SDS-PAGE followed by immunoblotting with anti-
709 myc, anti-MFN2, and GAPDH antibodies. ΔMTS-PDK protein could not be transferred to
710 the mitochondrial fraction. (C) Interaction of intact PDK4 with MuRF1. COS7 cell extracts
711 (100 μg) transfected with PDK4/ΔMTS-PDK4 or MTS-GFP were immunoprecipitated with
712 anti-V5 antibody and analyzed by immunoblotting to detect myc and V5. The absence of
713 MTS in PDK4 did not affect the interaction of MuRF1 and PDK4. * Indicates nonspecific
714 bands. MM, molecular mass
715
716 **Fig 6. MuRF1-mediated SUMOylation of PDK4.** (A) Ubiquitination assay of PDK.
717 Lysates from PDK4-myc-, FLAG-ubiquitin-, and MuRF1/ΔRING-MuRF1-V5-expressing
718 COS7 cells treated with 100 nM epoxomicin for 2–4 h were immunoprecipitated with an anti-
719 myc antibody. Immunoprecipitates were subjected to immunoblotting for indicated proteins.
720 COS7 cells were transfected with PDK4-myc, Mock, or MuRF1/ΔRING-MuRF1-V5. The
721 transfection of MuRF1/ΔRING-MuRF1 did not affect the amount of ubiquitination of PDK4
722 compared to that of Mock. MM, molecular mass. (B) Degradation of PDK4. To analyze the
723 ubiquitination-mediated degradation rate of MuRF1, lysates from PDK4-myc- and
724 MuRF1/ΔRING-MuRF1-V5-expressing COS7 cells treated with 100 μg/ml cycloheximide
725 for the indicated periods were subjected to immunoblotting for the indicated proteins. β-Actin

726 was used as an internal standard. The transfection of MuRF1/ΔRING-MuRF1 did not
727 upregulate the degradation of PDK4-myc compared to that of Mock. Data are means ± SD.
728 **$p < 0.01$ vs. 0 h among each group. (C) SUMOylation assay of PDK4. Lysates from
729 PDK4-myc-, FLAG-SUMO3-, and MuRF1/ΔRING-MuRF1-V5-expressing COS7 cells were
730 immunoprecipitated with an anti-myc antibody. Immunoprecipitates were subjected to
731 immunoblotting for indicated proteins. The transfection of MuRF1 upregulated SUMOylated
732 PDK4, although ΔRING-MuRF1 did not upregulate SUMOylated PDK4 compared to the
733 Mock. MM, molecular mass