Muscle RING-Finger Protein MuRF1 as a Connector of Muscle Energy Metabolism and Protein Synthesis

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Running title: MuRF1 and Muscle Protein Metabolism

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Abbreviations used: A340, absorbance at 340 nm; BCAA, branched-chain amino acid; CC, coiled coil domain; CK, creatine kinase; CSA, cross section area; D5-F, D-5-phenylalanine; GMEB1, glucocorticoid modulatory element binding protein-1; GST, Glutathione-S-transferase; HIBADH, 3-hydroxyisobutyrate dehydrogenase; KO, knock out; MARP1, muscle ankyrin repeat protein 1; MFC, MuRF family highly conserved domain; μCL, μ-calpain large subunit; PCA, perchloric acid; PMSF, phenylmethylsulfonyl fluoride; SUMO, small ubiquitin related modifier; TA, tibialis anterior; ttk, connectin/titin kinase region; WT, wild type.
Summary

During pathophysiological muscle-wasting, a family of ubiquitin ligases, including MuRF1 (Muscle RING-Finger protein-1), has been proposed to trigger muscle protein degradation via ubiquitination. Here, we characterized skeletal muscles from wild-type (WT) and MuRF1-KO mice under amino acid (AA) deprivation as a model for physiological protein degradation, where skeletal muscles altruistically waste themselves to provide AA to other organs. When WT and MuRF1-KO mice were fed a diet lacking AA, MuRF1-KO mice were less susceptible to muscle wasting, both for myocardium and skeletal muscles. Under AA depletion, WT mice had reduced muscle protein synthesis, while MuRF1-KO mice maintained non-physiologically elevated levels of skeletal muscle protein de novo synthesis. Consistent with a role of MuRF1 for muscle protein turnover during starvation, the concentrations of essential AA, especially branched-chain AA, in the blood plasma significantly decreased in MuRF1-KO mice under AA deprivation.

To clarify the molecular roles of MuRF1 for muscle metabolism during wasting, we searched for MuRF1-associated proteins using pull-downs and mass-spectrometry. Muscle-type creatine kinase (M-CK), an essential enzyme for energy metabolism, was identified among the interacting proteins. Co-expression studies revealed that M-CK interacts with the central regions of MuRF1 including its B-box domain, and that MuRF1 ubiquitinates M-CK, which triggers the degradation of M-CK via proteasomes. Consistent with a role of MuRF1 for adjusting CK activities in skeletal muscles by regulating its turnover in vivo, we found that CK levels were significantly higher in the MuRF1-KO mice than in WT mice. Glucocorticoid-modulatory-element-binding protein-1 and 3-hydroxyisobutyrate-dehydrogenase, previously identified as potential MuRF1 interacting proteins, were also ubiquitinilated MuRF1-dependently.

Taken together, these data suggest that MuRF1 multifacetedly participates in the regulation of AA metabolism, including the control of free AA and their supply to other organs under catabolic conditions, and in the regulation of ATP synthesis under metabolic stress conditions where MuRF1 expression is induced.
Keywords: MuRF1; RING-finger protein; titin/connectin; creatine kinase; muscle atrophy; E3 ubiquitin ligases; Glucocorticoid modulatory element binding protein-1; 3-hydroxyisobutyrate dehydrogenase
Introduction

Skeletal muscle, the largest organ in vertebrates, is important not only for motor function and sustaining the body shape, but also in metabolic homeostasis. For instance, under conditions of malnutrition, muscle proteins are rapidly degraded to amino acids, which are supplied through the blood to other tissues to supplement nutritional sources. Therefore, muscle protein degradation, when under appropriate control, is beneficial and necessary for the whole organism to maintain metabolic homeostasis. Muscle wasting is induced not only by malnutrition, particularly a lack of proteins, but also by muscle disuse and various pathological conditions, such as diabetes, sepsis, cancer cachexia, and sarcopenia. In these cases, muscle atrophy and the underlying disease states can exacerbate each other in vicious cycles. Therefore, the elucidation of the molecular mechanisms of muscle atrophy is also very important for improving the treatment of certain diseases.

Muscle proteins, especially myofibril proteins, are thought to be degraded by the calpain system and the ubiquitin(Ub)-proteasome system. Under conditions promoting muscle atrophy, such as immobilization, denervation, and fasting, the expression levels of two muscle-specific Ub-ligases, MuRF1 (muscle RING-finger protein 1) and atrogin-1/MAFbx (muscle atrophy F-box), are up-regulated. Furthermore, mice lacking either of these genes show partial resistance to the muscle atrophy caused by denervation, suggesting that both of these Ub-ligases participate in the induction of atrophy.

At present it is unclear how the different E3 ubiquitin ligases that are co-expressed in striated muscle tissues (such as MuRF1 and two paralogues, MuRF2 and MuRF3, which form a distinct conserved subgroup in mammals within the RBCC (RING-finger–B-box–Coiled-coil)/TRIM (Tripartite motif) protein family) functionally cooperate, for example, if they target similar or distinct sets of muscle proteins, and which muscle proteins could represent rate limiting targets for muscle turnover.
Our previous studies on the interactomes recognized by MuRFs demonstrated that MuRF1 and MuRF2 (but not MuRF3) interact with the giant muscle proteins, nebulin and connectin/titin.\(^{18; 19}\) Titin is a giant filamentous protein that forms an intra-sarcomeric filament system by connecting the Z-band and the M-line of the sarcomere.\(^{20; 21}\) It should be noted that MuRF1 interacts with the titin domain A169,\(^ {22}\) which positions MuRF1 immediately N-terminal to the connectin/titin kinase domain (ttk) and close to the binding site for skeletal-muscle-specific calpain, p94/calpain 3, of another neighboring connectin/titin molecule that is headed in the other direction.\(^ {23-25}\) This raises the possibility that MuRF1 is in the triad of ubiquitin-proteasome, calpain, and ttk signaling pathways at the M-line.\(^ {18; 26}\)

So far, most molecular insights into the signaling pathways regulated by MuRFs have been obtained from studies on cultured myocytes.\(^ {27-33}\) These studies have suggested that MuRF1 may ubiquitinate troponin I,\(^ {29}\) and may also recognize many other myofibrillar proteins and energy metabolic enzymes.\(^ {19}\) Furthermore, MuRF1 interacts with several proteins involved in SUMOylation and transcriptional regulation.\(^ {30; 34}\) Therefore, MuRF1 may have multiple functions in the regulation of muscle metabolism. However, so far, these models need to be tested \textit{in vivo}. Here, we attempted to determine MuRF1’s role for muscle metabolism by nutritionally depleting MuRF1 KO mice (constitutively lacking MuRF1 protein) and WT mice for amino acids. Results indicate that both muscle-type creatine kinase activities, identified as one of the substrates ubiquitinated by MuRF1, and muscle protein synthesis are regulated by MuRF1, suggesting that MuRF1 connects the regulation of energy metabolism and protein synthesis in muscle.

\textbf{Results}

\textit{Identification of muscle-type creatine kinase as a MuRF1-interacting protein.} We screened for
MuRF1-interacting proteins by a GST-pull-down assay using N-terminally GST-tagged recombinant MuRF1 (GST-MuRF1) and muscle lysates prepared from MuRF1 KO mice (i.e., lacking endogenous MuRF1). Several proteins were specifically co-precipitated with GST-MuRF1 (Figure 1, lane 4). Among these proteins, a 40-kDa species (Figure 1, arrowhead) was identified by mass-spectrometry analysis as M-CK (creatine kinase, muscle type), a muscle enzyme that is critical for energy metabolism. An interaction between MuRF1 and M-CK was suggested previously by yeast two-hybrid analysis, raising the possibility that these two proteins form a complex within the sarcomeric M-line region, the main site of their in vivo localization in myocytes.

To confirm the interaction between MuRF1 and M-CK in living cells, an immunoprecipitation analysis was performed. When Flag-M-CK was co-expressed with myc-MuRF1 in COS7 cells, they were co-precipitated (Figure 2B, lane 7). To examine whether the MuRF1 paralogues, MuRF2 and MuRF3, interact with M-CK, they were also co-expressed with Flag-M-CK. The myc-MuRF3 interacted with Flag-M-CK, although this interaction was weaker than that with myc-MuRF1 (Figure 2B, lane 9). No interaction between myc-MuRF2 and Flag-M-CK was detectable under the conditions used (Figure 2B, lane 8).

MuRF1 ubiquitinates M-CK, leading to M-CK degradation via proteasomes. To determine the site of MuRF1 that interacts with M-CK, Flag-M-CK and various deletion constructs of myc-MuRF1 were co-expressed in COS7 cells. As shown in Figure 2C, all the constructs containing B-box domain were co-precipitated with Flag-M-CK (lanes 13-15, 20), while MuRF1 B-box deletion mutant was not (lane 16), indicating that the B-box of MuRF1 is important for contributing to or mediating this interaction (Figure 2A). The co-precipitates contained ubiquitinated proteins probably corresponding to ubiquitinated M-CK and/or self-ubiquitinated MuRF1 (data not shown).

Since it was not possible to distinguish between self-ubiquitination of MuRF1 and MCK-ubiquitination in the above experiments, we examined whether M-CK was ubiquitinated in a
MuRF1-dependent fashion: N-terminally HA-tagged ubiquitin (HA-Ub) was co-expressed with Flag-M-CK and myc-MuRF1 in the presence of MG132, a proteasome inhibitor. Flag-M-CK was then immunoprecipitated with anti-Flag agarose after denaturation. Both mono- and oligo-ubiquitinated Flag-M-CKs were detected with anti-Flag and anti-HA antibodies when myc-MuRF1 was co-expressed (Figures 3A and 3B, lane 9). When the procedures above were performed in the absence of MG132, ubiquitated Flag-M-CK was almost undetectable (Figures 3A and B, lane 3).

Deletion of RING domain of MuRF1 abolished ubiquitination of Flag-M-CK (Figures 3A and 3B, lanes 4 and 10), even though the expression levels of the proteins were in similar levels (Figure 3C, lanes 3, 4, 9, and 10). Under the same conditions, myc-MuRF2 and myc-MuRF3 also ubiquitinated Flag-M-CK (Figures 3A and 3B, lanes 11 and 12), although the levels of these expressed proteins and ubiquitination varied.

Taken together, these data from COS7 cells indicate that MuRF1’s B-box is essential for interaction between M-CK and MuRF1. Furthermore, this interaction is coupled to the ubiquitination of M-CK. Finally, MG132-sensitivity indicates its role for proteasome-dependent degradation. Further studies are needed to clarify whether MuRF2 and MuRF3 can also function as Ub-ligases for M-CK in vivo (the analysis of MuRF2 has been hampered by the existence of at least two isoforms, see ref 30).

**MuRF1 also ubiquitinates GMEB1 and HIBADH.** Previously, we have identified glucocorticoid modulatory element binding protein-1 (GMEB1) and 3-hydroxyisobutyrate dehydrogenase (HIBADH) as interacting molecules of MuRF1.\(^{19, 30}\) To investigate whether GMEB1 and HIBADH are also ubiquitinated by MuRF1, they were co-expressed with MuRF1 in COS7 cells. As shown in Figure 3D, co-expression of GMEB1 or HIBADH and MuRF1, but not MuRF1ΔRING, showed ubiquitionation signals significantly in the presence of MG132 (lanes 5 and 11). These results indicate that GMEB1 and HIBADH are also targets of MuRF1-dependent proteasome-mediated degradation.

**MuRF1 is up-regulated under amino acid deprivation.** To investigate the physiological relevance
of M-CK’s ubiquitination by MuRF1, we compared WT and MuRF1 KO mice under conditions provoking muscle atrophy. MuRF1 is reported to be up-regulated under all muscle atrophy-inducing conditions examined so far.\textsuperscript{11-14} In this study, mice were fed only 10% glucose solution and water instead of normal food for one week, to induce muscle atrophy by stimulating protein turnover. Since this condition provides mice with carbohydrates freely, it is much milder for mice than starvation, providing more focus on muscle protein turnover with less affection of total body energy homeostasis. Consistently, body weights of mice under this condition were about 90\% of the initial state on day 7 (Figure 4A), whereas they decreased to almost 80\% in 3 days under starvation conditions (data not shown). We call this feeding condition the “amino acid deprivation (–AA) condition”. Under the –AA condition, the weight and cross-sectional area (CSA) of the TA fibers of WT mice became significantly smaller (p < 0.05) than those of control WT mice fed normal food (Figures 4B and 4C), confirming the wasting of muscle under the –AA condition. Also, under this condition, the mRNA levels of MuRF1 and atrogin-1/MAFbx were elevated (Figure 4D).

When MuRF1 KO mice were fed under the same –AA condition, the weight and CSA of their TA fibers were significantly larger (p < 0.05) than those of the –AA WT mice (Figures 4B and 4C). Thus, MuRF1 plays a critical role in the muscle atrophy induced by the –AA condition, as it does in other muscle-wasting conditions.\textsuperscript{11-14} Atrogin-1/MAFbx was upregulated similarly in WT and MuRF1 KO mice, suggesting that MuRF1 and MAFbx are regulated independently, and, furthermore, that elevated atrogin-1 expression were unable to induce atrophy in the MuRF1 KO skeletal muscles (Figure 4D). Similar as in skeletal muscle, MuRF1 deficient myocardium tended to be also protected from wasting under the –AA feeding protocol (Figures 4E and 6B).

\textit{MuRF1 KO alleviates decrease of creatine kinase levels under the –AA condition.} Next, the physiological significance of the induction of MuRF1 under AA starvation as well as MuRF1-M-CK interaction was investigated by comparing the expression of M-CK in the MuRF1 KO and WT mice. To
quantify CK, the CK activity in the presence of excess phosphocreatine was measured. In skeletal muscle, there are two isoforms of CK, muscle-type (M-CK) and mitochondria-type (Mi-CK). Although this assay cannot distinguish the activities of these two isoforms, M-CK predominates in skeletal muscle.\(^3\) Therefore, the measured activity in skeletal muscle is considered to reflect the quantity of M-CK. Under normal feeding conditions, the level of CK in WT and MuRF1 KO mice did not differ significantly. In contrast, under the –AA condition, in which MuRF1 was induced in WT, the CK level in the MuRF1 KO mice was significantly higher (p < 0.05) than in WT mice (161% of the level in –AA WT) (Figure 5). These results demonstrate that M-CK activity is down-regulated by MuRF1 \textit{in vivo} under muscle-wasting conditions such as AA starvation, in which MuRF1 is up-regulated.

Concentrations of amino acids in the blood plasma are significantly decreased in –AA MuRF1 KO mice. When insufficient amounts of amino acids are supplied in the diet, the degradation of muscle proteins can alternatively provide amino acids to other organs via the bloodstream.\(^1\) Therefore, we investigated whether the disruption of MuRF1 affects the concentrations of amino acids in the blood plasma. Under normal feeding conditions, no difference in any of the plasma amino acid concentrations was detected between WT and MuRF1 KO mice (Table 1). Under the –AA feeding protocol, the WT mice showed a significant increase in the total and non-essential amino acid concentrations (Table 1, p < 0.05), but not in the levels of circulating essential amino acids. In contrast, in the –AA MuRF1 KO mice, the total amino acid serum concentrations did not change significantly, but the level of essential amino acids decreased (in comparison with both WT and MuRF1 KO mice; see Table 1, p < 0.05). In particular, the concentrations of branched-chain amino acids (BCAA), which are very important for protein turnover and the energy homeostasis of muscle,\(^1;\)\(^4\) were lower in the –AA MuRF1 KO mice than in the –AA WT mice (Table 1, p < 0.05). Therefore, under the –AA condition, the bloodstream is depleted of essential amino acids, especially BCAA, in MuRF1 KO mice, probably due at least in part to impaired MuRF1-mediated protein turnover.
Comparison of protein synthesis in starved WT and MuRF1 KO mice implicates MuRF1 as an inhibitor of de novo muscle protein synthesis. Depletion of amino acids from the blood stream might be caused by impaired protein degradation. Alternatively, altered muscle protein synthesis might also affect serum amino acid levels. Therefore, we compared de novo skeletal muscle protein synthesis in WT and MuRF1 KO mice by the flooding bolus injection method, using deuterium-labeled D-5-phenylalanine (D5-F) as a tracer. Significant incorporation of D5-F was not observed in 7-day –AA mice (data not shown), consistent with general deprivation of muscle protein synthesis after lack of essential amino acids. Depriving mice only for two to four days for amino acids resulted in more moderate weight losses (Figures 6A and 6B). Therefore, we injected D5-F after 48 hours amino-acid deprivation, and sacrificed the mice after 96 hours to determine fractional muscle protein synthesis rates. Under these conditions, about two-fold higher incorporation of D5-F into total TCA-insoluble quadriceps skeletal muscle proteins was detected in MuRF1 KO mice when compared to WT quadriceps (Figure 6C). These data implicate MuRF1 as a negative regulator of muscle protein synthesis under metabolic stress situations so that consumption of BCAA amino acids reservoir is confined.

Discussion

In this study, we identified M-CK as a target of MuRF1, i.e., M-CK interacts with and is ubiquitinated by MuRF1. Mice lacking MuRF1 showed resistance to the down-regulation of M-CK when fed an aproteinogenic diet, but the concentrations of essential amino acids in their bloodstream decreased. These results strongly suggest that MuRF1 is involved in the homeostatic regulation of energy and amino acid metabolism, possibly through the degradation of M-CK, under muscle atrophy-inducing conditions, such as malnutrition, as shown here (Figure 7). This is the first report that
identifies a *bona fide in vivo* target of MuRF1, M-CK.

M-CK is a critical enzyme for energy metabolism that reversibly produces phosphocreatine and ATP. Therefore, the down-regulation of M-CK by protein degradation is predicted to suppress energy consumption by muscle. Under malnutrition conditions, skeletal muscle, as the largest ATP-consuming organ, must down-regulate its energy consumption. Mechanistically, this could be achieved by the degradation of M-CK following its ubiquitination by MuRF1. Some other metabolic enzymes, such as adenylate kinase and aldolase A, are also potential substrates for MuRF1, because they have been found to interact with MuRF1 baits in the yeast two-hybrid system. Together, these data and ours suggest the novel hypothesis that MuRF1 is an energy homeostasis regulator for muscle, although further studies are required to determine the precise roles of MuRF1 in the regulation of energy homeostasis.

Skeletal muscle provides the largest protein reservoir in vertebrates, and under malnutrition conditions the degradation of skeletal muscle proteins provides amino acids for other tissues, *via* the bloodstream. In the present study, MuRF1 was shown to be important for maintaining physiological levels of essential amino acids, especially BCAA, in the serum when dietary protein was lacking. Consistent with this, HIBADH, one of key enzymes involved in the Val catabolic pathway, is ubiquitinated by MuRF1. This most likely suppresses BCAA consumption as a source for energy catabolism, and thus can explain the elevated free BCAA levels. These findings suggest in turn that MuRF1 is involved in the regulation of amino acid homeostasis. One of the mechanisms could be that MuRF1 marks muscle proteins for degradation, including M-CK, troponin I, and other myofibrillar proteins, like telethonin/T-cap, myotilin, and connectin/titin. While previous studies have focused on the roles of MuRF1 and atrogin for muscle protein degradation, here we also tested if MuRF1 participates in the control of protein synthesis. Indeed, about two-fold elevated protein synthesis found in –AA MuRF1 KO mice demonstrated that MuRF1 is required to safeguard BCAA amino acid serum
levels also by inhibiting muscle protein synthesis. These findings implicate the coupling of synergistic mechanisms, protein degradation and synthesis, by MuRF1. Moreover, degradation of GMEB1, a transcriptional regulator in response to changes in cellular glucocorticoid levels, is regulated by MuRF1, suggesting that protein synthesis is also modulated by MuRF1 at the level of transcription as suggested previously. The relative contributions of the stimulation of muscle protein degradation and the inhibition/regulation of protein synthesis by MuRF1 represent one of the important issues to be addressed by future studies.

Our study showed some functional redundancy of MuRF paralogues in vitro. This is the first evidence that MuRF2 and 3 also have ubiquitin ligase activity. This finding is consistent with observations that some myofibrillar proteins are still ubiquitinated in MuRF1 KO mice and that MuRF1 KO mice have a subtle phenotype under normal conditions. However, the ubiquitin ligase activity of MuRF2 and 3 did not completely compensate for the functions of MuRF1 under muscle-wasting conditions in this study. This may be attributable to differences in the tissue-specificity and subcellular localizations of the MuRFs. In adult skeletal muscle, transcripts of MuRF1 are more abundant than those of MuRF2 and 3, suggesting MuRF1 plays the central roles over other paralogues in skeletal muscle.

The B-box region of MuRF1 was identified as a binding site for M-CK (see Figure 2). The importance of the B-box domain in other RBCC/TRIM proteins has been highlighted in previous reports. Consistent with this, the B-box regions are highly conserved among MuRFs, with about 80% identity (Figure 2A). MuRF3 is, however, more similar to MuRF1 in this region than is MuRF2, which may explain the distinct binding affinities of MuRFs for M-CK.

During the preparation of this manuscript, Zhao et al. reported the ubiquitination of M-CK by MuRF1 in vitro. Interestingly, they showed that only the oxidized form, one of two forms of M-CK, is susceptible to ubiquitination by MuRF1. In our results, relatively small amounts of M-CK were
ubiquitinated by MuRF1 in COS7 cells (see Figure 3A), and it is possible that the reduced form of M-CK predominated in COS7 cells, with only a small proportion in the oxidized form. Since the oxidized form of M-CK is less active than the reduced form, it is reasonable to selectively degrade the oxidized form as a source of amino acids under malnutrition conditions.

In conclusion, our investigation in vivo using MuRF1 KO mice demonstrated two distinct and important functions of MuRF1 in skeletal muscle: as a modulator for energy homeostasis by regulating M-CK via ubiquitination, and as a supplier of BCAA amino acids to other tissues by regulating muscle protein turnover. Intriguingly, in addition to regulation of protein degradation, we also found evidence that MuRF1 negatively regulates protein synthesis. Thus, MuRF1 may function as a potent regulator of muscle turnover by controlling both degradation and synthesis (Figure 7). The regulation of MuRF1 activity in a clinical setting, for example, by using some as-yet-unknown inhibitor, might contribute to improved treatments for pathological muscle wasting.

**Materials and Methods**

**Expression Plasmids**

Human MuRF1, MuRF2, M-CK, and mouse MuRF3 were amplified by PCR using *Pfu* DNA polymerase (Stratagene) and a skeletal muscle cDNA library (Clontech), and the sequences were verified by DNA sequencing. The cDNA fragments encoding MuRF1-RING and MuRF1-MFC (amino acids 66-340, 101-340, respectively, see Figure 2) were also generated by using PCR amplification. These cDNAs were inserted into the pGEX-6X vector containing an N-terminal GST-tag (GE Healthcare) or pcDNA3.1 containing an N-terminal Flag- or myc-tag (generous gifts from Dr. Tatsuya Maeda). MuRF1-C.C2 and MuRF1-C.C1 (amino acids 1-137, 1-225, respectively, see Figure 2) were constructed by inserting BamHI and HindIII fragments of pcDNA3.1-N-myc MuRF1 into the pSRD vector, respectively.

**GST Pull-down experiment**

GST-MuRF1 or GST was expressed in *Escherichia coli* BL21(DE3) and purified with glutathione-Sepharose 4B (GE Healthcare), according to the manufacturer’s instructions. Frozen
sections from tibialis anterior (TA) muscles of MuRF1 KO mice (see “Mouse experiments” below) were homogenized in 10 volumes (w/v) of lysis buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 1% Nonidet P-40) for 1 hr. After ultra-centrifugation (20,000 x g for 20 min), the supernatant (1 mg of total protein amounts) was incubated for 3 hr with 3 μg of GST-tagged MuRF1 immobilized on glutathione-Sepharose 4B. After three washes with lysis buffer, the precipitates were boiled with SDS-PAGE sample buffer, and the solubilized proteins were electrophoresed and silver-stained without adding glutaraldehyde (Silver Stain Kit, Protein, GE Healthcare).

Identification of MuRF1-Interacting Proteins
Protein bands above obtained were excised and subjected to in-gel digestion followed by tandem mass-spectrometry analysis, as described previously. Trypsin digestion was performed overnight at 37°C. The peptides were extracted from the gel using 5% trifluoroacetic acid and 50% acetonitrile. The eluate was deionized using a ZipTip (Millipore), recovered in matrix solution (2.5 mg/ml α-cyano-4-hydroxycinnamic acid, 50% acetonitrile, and 0.1% trifluoroacetic acid), and spotted onto a matrix-assisted laser desorption ionization (MALDI) target plate. After the spots were air-dried, MALDI-time-of-flight (TOF) mass-spectrometry was performed using an ABI 4800 analyzer. The protein was identified using the Mascot program (Matrix Science).

Immunoprecipitation and detection of ubiquitination
COS7 cells were transiently transfected with expression vector(s) by electroporation, as previously described. Forty-eight hours after transfection, the cells were treated with 25 μM MG132 added in culture medium for 2.5 hr. The cells were then harvested and lysed by sonication in TNE buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5 mM EDTA) supplemented with 1% Nonidet P-40 and inhibitors (10 μg/ml Aprotinin, 0.1 mM PMSF, and 25 μM MG132). The lysates were subjected to ultracentrifugation at 20,000 x g for 30 min. The protein concentrations of the supernatants were determined by the RC-DC protein assay (BioRad), and equal amounts of protein were used for immunoprecipitation with an anti-myc antibody or anti-Flag (M2)-agarose (Sigma), as previously described. For detection of ubiquitination status, the same procedures were performed with TNE buffer supplemented with 0.1% SDS.

CK activity Assay
The amount of creatine kinase was represented by its activity measured, as previously described. Briefly, TA muscles (ca. 1 mg) were homogenized in 1,000 volume (v/w) of 26 mM Tris/Cl (pH 8.0), 0.3 M sucrose, 1% NP-40, and 20 mM 2-mercaptoethanol, aliquots of 50 μl were incubated with 1 ml of 10 mM Tris/Cl (pH 7.4), 130 mM KCl, 1 mM MgCl2, 2 mM AMP, 50 μM diadenosine pentaphosphate, 5 mM glucose, 0.7 mM NADP, 1.5 mM ADP, 9 mM phosphocreatine, and 1.3 and 0.5 units of hexokinase
and glucose-6-phosphate dehydrogenase, respectively, at 25°C for 20 min, and A340 was measured. All the reagents were purchased from Sigma. The values were normalized to the amount of protein, which was determined by the RC-DC protein assay (BioRad).

**Mouse experiments**

All procedures using experimental animals were approved by the Experimental Animal Care and Use Committee of the Tokyo Metropolitan Institute of Medical Science. For inactivation of MuRF1, the gene was targeted by homologous recombination (described more in detail in ref. 53). WT and MuRF1 KO mice (7-weeks old) were divided into two groups: mice in the control group were fed normal food *ad libitum*, and mice in the experimental group were fed only 10% glucose solution and water, for one week. Whole blood collected from the heart with heparin was spun (10,000 x g, 5 min), and the supernatant was used for the measurement of amino acid concentrations in the blood plasma (measured by SRL Inc., Japan). The cross-sectional area of the TA muscle fibers was determined as the field area divided by the number of myofibers in Hematoxylin-Eosin-stained transverse sections.

For the determination of D5-F incorporation, mice were fed with 10% glucose and water for 4 days. Forty-eight hr after the start of starvation, 50 µmol/100g D5-F were injected intraperitoneally. After another 48 hr, mice were sacrificed for the following analysis. The bound amino acids were basically determined as described before.54,55 The quadriceps was powdered with a mortar and homogenized in 8 volumes of 90 mM perchloric acid (PCA). Homogenates were centrifuged and the insoluble materials were washed in 4 volumes 0.2 M PCA. The pellets were resuspended in 0.3 M NaOH and lysed for 2h at 37°C. Proteins were precipitated with 2 M PCA overnight at 4°C. The protein pellet was hydrolyzed in 6M HCl at 110°C for 24h. HCl was removed by evaporation. The pellet was resuspended in H2O and purified with a cation-exchange column (AG50 W-X8, BioRad). The purified amino acids were eluted from the column by 4 M NH4OH, dried, and resuspended in methanol. Relative content of D5-F to F was determined by mass-spectrometry by comparing ion counts at m/Z of 171.24 (D5-F) and 166.24 (F) using D5-F and F as standards (Sigma).

**RT-PCR**

Total RNA was extracted from frozen muscles with TRIzol reagent (Invitrogen). First-strand cDNA was synthesized with a First-strand cDNA synthesis kit (GE Healthcare), and used as a template for RT-PCR. RT-PCR was performed using ExTaq DNA polymerase (TaKaRa) and the following primers: 5’-gactcctgcagagtgaccaag-3’ and 5’-cttctacaatgctcttgatgagc-3’ for MuRF1; 5’-gaatagcatccagatcagcag-3’ and 5’-gagaatgtggcagtgtttgca-3’ for atrogin-1/MAFbx; 5’-gaattggaataccacattttacagg-3’ and 5’-tcaaaggtgcaacacatccagg-3’ for μCL.
Acknowledgements

We would like to thank Dr Kenji Takehana (Ajinomoto Co., Inc.), Dr Ichiro Matsumoto and Dr Takumi Misaka (The University of Tokyo), Dr Tatsuya Maeda (The University of Tokyo), Dr Shigeo Murata (Rinshoken), Dr Choji Taya (Rinshoken), and Dr Hiromichi Yonekawa (Rinshoken), and all of our laboratory members, for experimental support and valuable discussions. This work was supported in part by JSPS Research Fellowships for Young Scientists 1811508 (to S.K.), by MEXT.KAKENHI 18076007 (to H.S.), by JSPS.KAKENHI 18770124 (to Y.O.), 18700392 (to K.O.), 17780115 (to S.H.), 18380085 and 19658057 (to H.S.), by the Sasagawa Scientific Research Grant from The Japan Science Society (to S.H. and K.O.), by a Research Grant (17A-10) for Nervous and Mental Disorders from the Ministry of Health, Labor and Welfare, by a Takeda Science Foundation research grant (to H.S.), by the DFG (La668/10-1 and 11-1 to S.L.; Wi3278/2-1 to C.W.), and by the NAR-initiative of the Landesstiftung Baden-Württemberg.
References


FIGURE LEGENDS

Figure 1. Identification of MuRF1-interacting proteins. Recombinant GST-MuRF1 (lanes 4 and 5) or GST (lanes 2 and 3) was incubated with (lanes 1, 2, and 4) or without (lanes 3 and 5) TA muscle lysate of MuRF1 KO mice, and pulled down by glutathione Sepharose beads. Precipitates were subjected to SDS-PAGE, and visualized with silver staining. Among the MuRF1-specific bands shown in lane 4, the band indicated by an open arrowhead was identified as creatine kinase muscle type (M-CK).

Figure 2. Interactions between MuRFs and M-CK. (A) Schematic structures of MuRFs.16 18 The regions of MuRF1 deletion mutants used in Figs 2C and 3 are represented by bars. (B) FLAG-tagged M-CK and myc-tagged MuRF1, 2, or 3 (lanes 2 and 7, 3 and 8, or 4 and 9, respectively), or a mock vector (lanes 1 and 6) or myc-tagged muscle ankyrin-repeat protein 1 (lanes 5 and 10) as negative controls were co-expressed in COS7 cells. Cell lysates were immunoprecipitated (IP) with an anti-myc antibody, and the precipitated proteins were subjected to western blot analysis using anti-FLAG or anti-myc antibodies. (C) Myc-tagged MuRF1 deletion mutants and FLAG-tagged M-CK (lanes 5-8, 13-16, 18, and 20) or a mock vector (lanes 1-4, 9-12, 17, and 19) were co-expressed in COS7 cells. Immunoprecipitation and western blots were performed as in B using the antibodies indicated. (B) and (C): Arrowheads and asterisks indicate M-CK and non-specific bands (immunoglobulin originating from the antibody used for IP), respectively.

Figure 3. MuRF1 ubiquitinated M-CK, GMEB1, and HIBADH in COS7 cells. (A-C) HA-tagged ubiquitin was co-expressed in COS7 cells with FLAG-tagged M-CK (lanes 2-6 and 8-12) and myc-tagged MuRF1, MuRF1ΔRING, MuRF2, or MuRF3 (lanes 3 and 9, 4 and 10, 5 and 11, or 6 and 12, respectively) or mock vector (lanes 2 and 8), or with mock vector alone (lanes 1 and 7). Cells were separated into two groups: after 48 hr of transfection, one group was treated with 25 mM MG132 for 2.5 hr (lanes 1-6), but the other was not (lanes 7-12). The cells were then harvested and lysed. Lysates were analyzed by western blots using an anti-myc antibody (C) or immunoprecipitated with an anti-FLAG antibody under denaturing conditions (A, B). Precipitated proteins were subjected to western blot analysis using anti-FLAG (A) or anti-HA antibodies (B). Open and closed arrowheads, open and closed arrows indicate Ub-M-CK and M-CK, MuRF1-3 and MuRF1 ΔRING, respectively. (D) HA-tagged ubiquitin, FLAG-tagged GMEB1 (lanes 1-6) or HIBADH (lanes 7-12), and myc-tagged MuRF1 (lanes 2, 5, 8, 11) or MuRF1ΔRING (lanes 3, 6, 9, 12) or mock vector (lanes 1, 4, 7, 10) were co-expressed, pulled-down, and tested for ubiquitination similarly as (A-C).

Figure 4. MuRF1 inactivation protects from muscle atrophy induced by –AA deprivation. (A) The body weight of each mouse was measured and represented as a percentage to the average body weight of the
mice on day 0. Error bars indicate the average ± standard deviation (n = 3). (B) The weight of the tibialis anterior (TA) and gastrocnemius (GC) of WT or KO mouse under normal or –AA conditions. Error bars indicate the average ± standard deviation (n = 3 x 2 (right and left legs)). (C) Cross-sectional area of the TA muscle was measured and is shown as the average ± standard deviation (n = 2). (D) Amounts of mRNA for MuRF1, atrogin-1/MAFbx, and μCL as a standard were examined by RT-PCR. Data are shown for two mice in each group. (E) The weight of the heart of each mouse was measured and presented as ratios to the body weight on day 7. Error bars indicate the average ± standard deviation (n = 3). * indicates significant difference (p < 0.05) between WT –AA and KO –AA.

Figure 5. MuRF1 inactivation maintains creatine kinase activity during –AA deprivation. Significantly higher creatine kinase level in MuRF1 KO than in WT mice under the –AA condition. The amount of creatine kinase (CK) in the TA muscles was measured as the activity in the presence of excess phosphocreatine, and normalized to the amount of protein in the TA. Data are represented by arbitrary units. *, p < 0.05.

Figure 6. Muscle protein synthesis in WT and MuRF1 KO skeletal muscles during amino acid deprivation. Body (A) and muscle (B) weight loss during the 4-day amino acid deprivation. MuRF1 KO muscles are more resistant to the –AA condition. (C) After 4-day amino acid deprivation MuRF1 KO quadriceps muscles maintain two-fold higher protein synthesis rates when compared to WT mice.

Figure 7. Critical multiple roles of MuRF1 in muscle cells under malnutrition conditions. Signals for muscle atrophy such as malnutrition, immobilization, steroid administration, denervation, etc., lead to the up-regulation of MuRF1, which ubiquitinates muscle proteins, including M-CK, GMEB1, HIBADH, troponin I, and other myofibrillar proteins. At the same time, MuRF1 suppresses synthesis of muscle proteins. The degradation of M-CK results in the suppression of energy consumption, while the degradation of muscle proteins (including M-CK) generates free amino acids, which are transferred to other organs through the bloodstream. The degradation of GMEB1 and HIBADH alter expression of genes governed by glucocorticoid-responsive elements (GME) and Val catabolism, respectively. All these functions are part of the emergency response of muscles to maintain homeostasis of the whole body.
Figure 1

The figure shows a gel electrophoresis experiment with muscle lysates treated with GST and GST-MuRF1. The gel contains markers for molecular weight (kDa) ranging from 20 to 97. Lane 1 shows the muscle lysate with GST, lane 2 shows the muscle lysate with GST-MuRF1, lane 3 shows the muscle lysate without GST, lane 4 shows the muscle lysate with GST, and lane 5 shows the muscle lysate without GST-MuRF1.
**Figure 2**

A

![Diagram showing MuRF1, MuRF2, and MuRF3 domains with % identity to MuRF1](Image)

- **MuRF1**: 100% identity to MuRF1
- **MuRF2**: 80.7% identity to MuRF1
- **MuRF3**: 82.5% identity to MuRF1

B

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**I.B:** anti-myc (MuRFs) 45

C

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Figure 3

A. (kDa)

B. (kDa)

C. (kDa)

D. (kDa)
Figure 4

A

Relative body weight (% to day 0)

KO cont
WT cont
KO -AA
WT -AA

Day

B

Muscle weight (mg)

KO cont
WT cont
KO -AA
WT -AA

TA
GC

C

Cross section area ($x 10^3 \mu m^2$)

KO cont
WT cont
KO -AA
WT -AA

D

WT
KO

MuRF1
atrogin1/MAFbx
\(\mu CL\)

E

Heart/body weight (%)

KO cont
WT cont
KO -AA
WT -AA

Day
Figure 5
Signals for muscle atrophy

Muscle proteins

protein synthesis

MuRF1

Muscle cells

GMEB-1

M-CK

ADP

ATP

energy consumption

amino acids catabolism

HIBADH

GMEB-1

transcriptional regulation

Gene

nucleus

Blood stream

Other organs