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Identification of MAFbx as a myogenin-engaged F-box protein in SCF ubiquitin ligase

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ABSTRACT

Myogenesis is conducted by transcription factors including MyoD and myogenin. Myogenin is known to be polyubiquitinated by SCF (Skp1/Cullin 1/F-box protein) followed by proteasomal degradation, though the participating F-box protein is remaining unidentified. In this study, we found that myogenin in differentiated myoblasts is destabilized by muscle atrophy-inducing dexamethasone and that MAFbx (muscle atrophy F-box protein) is increased in atrophying myotubes. MAFbx overexpression resulted in MG132-sensitive reduction of myogenin. Myogenin had a MAFbx-recognition motif and interacted with MAFbx. MAFbx activated polyubiquitination of myogenin. The results of this study suggest that MAFbx functions as an F-box protein for ubiquitination of myogenin.

Structured summary:
MINT-7222713: *Myogenin* (uniprotkb:P12979) *physically interacts* (MI:0914) with *MAFbx* (uniprotkb:Q9CPU7) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7222741: *Myogenin* (uniprotkb:P12979) *physically interacts* (MI:0914) with *MAFbx* (uniprotkb:Q9CPU7) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-7222726: *Myogenin* (uniprotkb:P12979) and *MAFbx* (uniprotkb:Q9CPU7) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7222760: *Myogenin* (uniprotkb:P12979) *physically interacts* (MI:0914) with *Ubiquitin* (uniprotkb:P62991) by *anti bait coimmunoprecipitation* (MI:0006)

1. Introduction

Muscle proteins are rapidly broken down in adaptation to various physiological conditions and diseases [1,2]. This protein degradation is driven by the ubiquitin proteasome system (UPS) [3]. In this system, proteins subjected to degradation are covalently linked to a ubiquitin (Ub) chain by a cascade involving Ub-activating E1 enzyme, Ub-conjugating E2 enzyme, and Ub ligase. Activated Ub is transferred to a target protein by Ub ligase and the resultant polyubiquitinated proteins are degraded by proteasome. Though E1 and E2 enzymes have no apparent target specificity, Ub ligase selectively binds to a target protein. As for multi-subunit Ub ligases, they contain a specific target-recognition subunit [4]. SCF is one such multi-subunit Ub ligase, consisting of Skp1, cullin1 and F-box protein. The F-box protein binds directly to the substrate [5].

MAFbx (muscle atrophy F-box protein)/Atrogin-1 was identified as a muscle-specific F-box protein [6,7]. Gene expression of MAFbx is elevated during muscle atrophy induced by dexamethasone (Dex) and several stresses [6]. Tintignac et al. [8] found that MyoD is degraded by MAFbx-containing SCF-mediated UPS. MyoD, which functions in an early stage of myogenesis, is a member of the family of muscle-specific bHLH (basic helix-loop-helix) transcription factors [9–11]. An EGLQALLR motif in helix 2 of MyoD functions as a MAFbx-recognizing motif, and LXXLL sequence (underlined) has been designated as a core motif [8]. MAFbx contains multiple protein-interacting motifs in addition to an F-box motif [7,8]. Myogenin, another member of the MyoD family, functions in the middle stage of myogenesis [9]. Unlike MyoD, it has not been clarified whether myogenin level alters during muscle atrophy and hypertrophy. Although we found that myogenin is degraded by SCF [12], its F-box protein has not yet been identified. In this study, we demonstrated that myogenin binds to MAFbx in cells and that MAFbx participates in polyubquitination and degradation of myogenin.

Abbreviations: UPS, ubiquitin proteasome system; Ub, ubiquitin; MAFbx, muscle atrophy F-box protein; SCF, Skp1/Cullin 1/F-box protein; MuRF1, muscle RING-finger protein-1; Dex, dexamethasone; IGF-1, insulin-like growth factor-1; HA, hemagglutinin; siRNA, small interference RNA; GM, growth medium; DM, differentiation medium; PAGE, polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA-binding protein

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2. Materials and methods

2.1. Cell culture, reagent treatment, and transfection

Mouse C2C12 myoblasts were cultured in DMEM-based growth medium (GM) supplemented with 20% fetal calf serum. For myogenic differentiation, GM was exchanged with differentiation medium (DM) consisting of DMEM and 2% horse serum [12]. Dex (Sigma–Aldrich) and insulin-like growth factor-1 (IGF-1) (Sigma–Aldrich) were added to the DM at 10 μM and 10 ng/ml, respectively, and incubated for 24 h. Cos7 cells were cultured in DMEM with 10% fetal calf serum. When required, MG132 (Biomol) was added to media 4–6 h before cell harvest. Cells were transfected with an indicated combination of expression plasmids (10 μg/100 mm dish) using a LipofectAMINE and PLUS reagent (Invitrogen).

2.2. DNAs and plasmids

To isolate mouse MAFbx cDNA, total RNA of differentiated and Dex-treated C2C12 cells was extracted with an RNeasy RNA extraction kit (Quiagen). RT-PCR was performed using an RNA PCR kit (Takara). Mouse MAFbx cDNA was amplified by PCR using 5′-CCATGCCGTTCCTTGGGCAG (forward) and 5′-AAGTGTTGT CGTGTGCTGGG (reverse) primers. The amplified MAFbx cDNA was cloned in pGEM T-Easy vector (Promega) to construct pGEM-MAFbx. For construction of HA-tagged (HA-MAFbx) and Flag-tagged (FLAG-MAFbx) expression plasmids, an EcoRI fragment of pGEM-MAFbx was inserted into HA and FLAG tag-carrying pcDNA vectors (Invitrogen), respectively. A BamHI–XbaI fragment of pGEM-MAFbx was inserted into pEGFP-N1 vector (Clontech) to construct GFP-MAFbx that expresses GFP-fused MAFbx protein. Other plasmids were described before [12,13].

2.3. RNA interference

Small interference RNA (siRNA) was prepared with a Silencer siRNA Construction kit (Ambion). The sequences for targeting mouse MAFbx was 5′-AAGCGCTTCTTGGATGAGAAA [14]. An siRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included in the kit. A set of 29-mer oligodeoxynucleotides for MAFbx (sense: 5′-AAGCGCTTCTTGGATGAGAAACCTGTCTC and antisense: 5′-AATTTCTCATCCAAGAAGCGCCCTGTCTC) contains a corresponding siRNA sequence and flanking sequences complementary to the T7 primer. Cells were transfected with 50 nM siRNA in molecule molarity for each gene.

2.4. Immunofluorescence staining

Cell fixation with paraformaldehyde, permealization with Triton X-100 and interaction with a primary antibody were the same as described before [12].

2.5. Preparation of cell extracts and immunoblotting

Cells were lysed with 1% Triton X-100-containing TNE buffer (10 mM Tris–HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, and 10% glycerol) [15] supplemented with protease inhibitor mixture (at final concentrations of 1 mM for benzamidine–HCl, 1 μg/ml for pepstatin A, 0.5 mM for phenylmethylsulfonyl fluoride, and 1 μg/ml for leupeptin). The whole cell extracts were collected and protein concentration was determined [16]. For immunoblotting, proteins (5–30 μg) were separated by SDS–polyacrylamide gel electrophoresis (PAGE). Procedures for immunoblotting were essentially the same as described before [16].

2.6. Immunoprecipitation

Immunoprecipitation was performed as described before [12]. To detect ubiquitination of myogenin (Fig. 4), whole cell extracts were prepared in RIPA buffer (TNE buffer containing 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing the protease inhibitor mixture, 25 μM MG132, and 20 mM *N*-ethylmaleimide and then incubated with protein G-Sepharose beads (GE Healthcare Bioscience) for 1 h. The supernatant fraction was incubated with anti-myogenin antibody (Pharmingen) overnight. The materials were incubated with protein G-Sepharose beads for 90 min followed by washing with the same buffer used in cell lysis. Immunocomplexes were eluted with SDS sample buffer by boiling for 4 min. Proteins were detected by immunoblotting with a corresponding antibody.

3. Results

3.1. Effects of Dex and IGF-1 on differentiated C2C12 cells

First, we investigated the expression of myogenin in myotube-atrophying Dex-treated differentiated C2C12 cells. C2C12 myoblasts differentiated to myotubes in the DM, and some

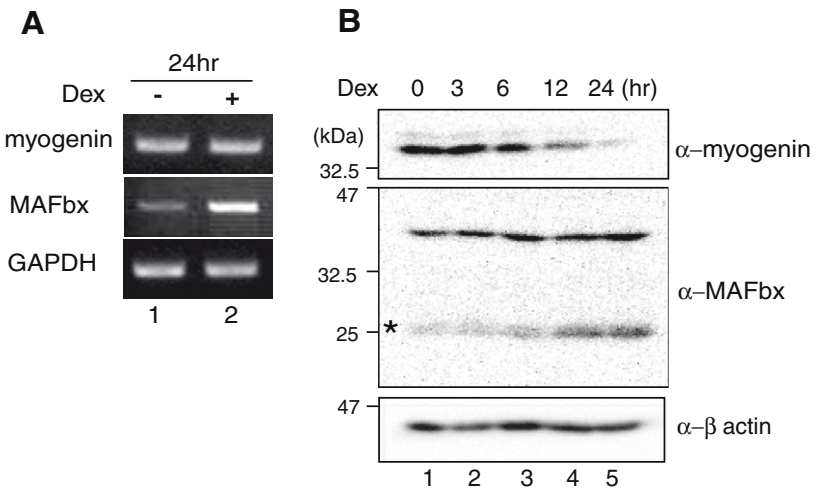

Fig. 1. Amounts of myogenin and MAFbx in reagent-treated differentiated cells. Gene expression (A) and proteins (B) of myogenin and MAFbx were determined by RT-PCR and immunoblotting, respectively. An asterisk in the middle part of panel B indicates a MAFbx isoform. C2C12 cells were treated with Dex for 24 h.

differentiated cells fused and included multiple nuclei (Supplementary Fig. S1). In this in vitro myogenesis, treatment of cells with Dex resulted in impairment of the differentiated cells (Supplementary Fig. S1), and apoptosis occurred when cells were exposed to Dex for a longer time (data not shown). IGF-1, which maintains muscles and induces muscle hypertrophy [17], showed a significant over-growth effect (Supplementary Fig. S1G and H). Next, we determined mRNA and protein of myogenin in Dex-treated differentiated cells. Dex treatment for 24 h reduced myogenin protein to an almost undetectable level (Fig. 1B, lane 5), whereas amounts of myogenin mRNA did not change (Fig. 1A). Myogenin was thus demonstrated to be degraded in atrophying myotubes,

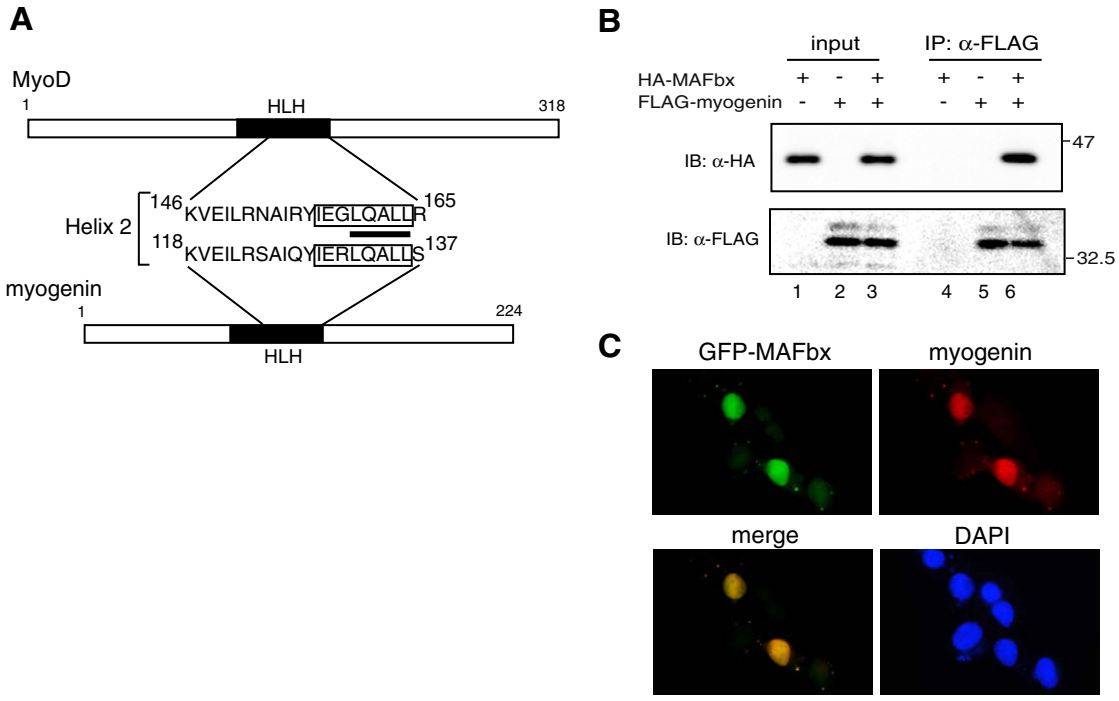

Fig. 2. Interaction of myogenin and MAFbx. (A) Schematic representation of structures of mouse MyoD and myogenin. Amino acid sequences of helix 2 in the helix-loop-helix domain (solid box) of MyoD and myogenin were aligned. MAFbx-recognizing motif with seven amino acids of MyoD [8] and its corresponding region of myogenin are enclosed with boxes. The core in the MAFbx-recognizing motif is shown as a thick bar. (B) Interaction between exogenous myogenin and MAFbx in C2C12 cells. Extracts of FLAG-myogenin- or HA-MAFbx-overexpressing cells were immunoprecipitated with anti(α)-FLAG antibody, and MAFbx and myogenin were detected with a corresponding antibody (lanes 4–6). Lanes 1–3 represent results without immunoprecipitation. (C) Localization of exogenously expressed myogenin and MAFbx. C2C12 cells transfected with FLAG-myogenin- and GFP-MAFbx-expressing plasmids were incubated in GM for 24 h, treated with 10 μM MG132 for 2 h, immunostained with α-myogenin antibody, and observed with a fluorescent microscope. A merged image from GFP-MAFbx and myogenin panels and nucleus staining with DAPI (diamidino phenylindole) are also shown.

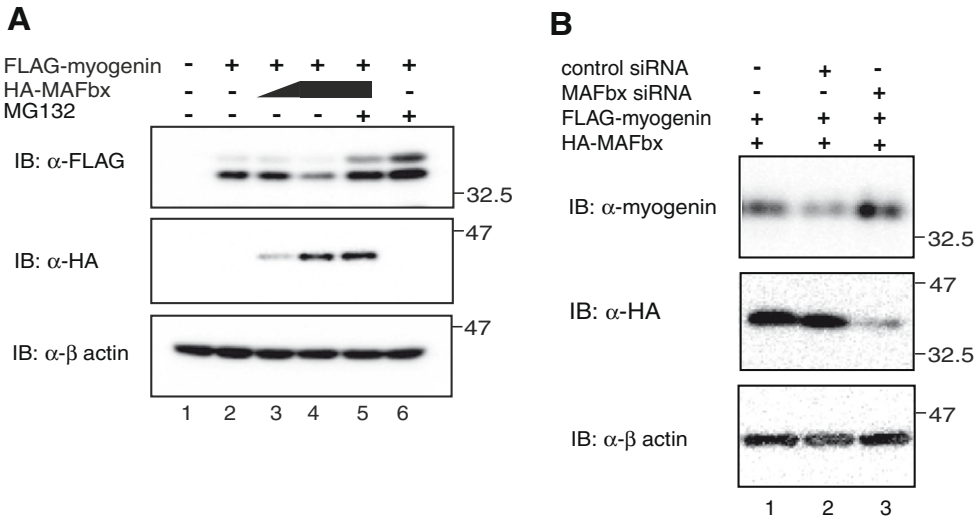

Fig. 3. Destabilization of myogenin by MAFbx. (A) FLAG-myogenin-expressing C2C12 cells were transfected with HA-MAFbx-expressing plasmid, and these two proteins were detected by immunoblotting. MG132 was also added (lanes 5 and 6). (B) Stabilization of myogenin in MAFbx knock-down cells. Experiments were performed by using siRNA of MAFbx.

which can mimic physiological muscle atrophy. In contrast to Dex, myogenin was increase by IGF-1, whereas its mRNA level did not change (data not shown). We also found that MAFbx gene expression was remarkably stimulated and repressed by Dex and IGF-1, respectively (data not shown). MAFbx protein increased in atrophying cells (Fig. 2B) as previously reported [7].

3.2. Interaction between myogenin and MAFbx

The above results imply a negative correlation between alterations in myogenin and MAFbx. MyoD has been demonstrated to bind to MAFbx via the EGLQALLR motif within helix 2 (Fig. 2A), and a core region of EGLQALLR (LXXLL motif, underlined) is designated as a MAFbx-binding signature [8]. Since this signature motif was detected in helix 2 of myogenin (Fig. 2A), we investigated the interaction of MAFbx and myogenin in C2C12 cells by immunoprecipitation and immunoblotting in an overexpression condition. It was found that MAFbx-including immunocomplexes contain myogenin (Fig. 2B, lane 6). Furthermore, we examined the localization of MAFbx in cells and found that exogenously expressed GFP-MAFbx and FLAG-myogenin were co-localized (Fig. 2C). It was clearly demonstrated that both MAFbx and myogenin were localized in nuclei (Fig. 2C). GFP alone was distributed throughout a cell and did not co-localized with myogenin (data not shown). The findings thus suggested that MAFbx is physically associated with myogenin in C2C12 cells.

3.3. MAFbx-dependent reduction of myogenin in differentiated cells

Myogenin in C2C12 cells was determined in an overexpression condition. FLAG-myogenin was decreased when HA-MAFbx was expressed (Fig. 3A, lanes 2 vs. 3 and 4). Furthermore, a high myogenin level was maintained when the proteasome inhibitor MG132 was added (Fig. 3A, lanes 5 and 6). We further applied MAFbx siRNA in this assay and found that MAFbx knockdown restored the level of myogenin (Fig. 3B). Taken together, the results suggested that MAFbx destabilizes intracellular myogenin. Since myogenin was considered to be ubiquitinated by MAFbx-containing SCF, we investigated in vivo ubiquitination of exogenously expressed myogenin. Cells overexpressing HA-Ub and FLAG-myogenin were co-transfected with increased amounts of FLAG-MAFbx. HA signals were clearly observed in high-molecular-weight regions of the gel (Fig. 4, lanes 14 vs. 12), whereas these bands were not observed when HA-Ub was omitted (lanes 4–6). These results suggest polyubiquitination of myogenin.

4. Discussion

Although MAFbx works as a target-recognizing component of SCF in muscles [6,7], no protein has so far been reported as a target of MAFbx except for MyoD [8]. Since MAFbx interacts with myogenin and enhances its ubiquitination (Figs. 2 and 4) and since MAFbx stimulates MG132-sensitive myogenin degradation (Fig. 3), it is thought that MAFbx is a myogenin-engaged F-box protein of SCF. Consistently, MAFbx gene expression was increased and decreased when differentiated C2C12 cells were treated with Dex (Fig. 1) and IGF-1 (data not shown), respectively. Myogenin and MyoD are presumably degraded by a common mechanism. We speculate that physiologically occurring Dex-induced muscle atrophy is also involved in MAFbx-related degradation of myogenin in addition to MyoD.

Since myogenin belongs to the MyoD family [9], we expected myogenin to also be a target of MAFbx. However, Myf-5 does not bind to MAFbx [8] even though it also belongs to the MyoD family. EGLQALLR sequence has been identified as a MAFbx-recognition

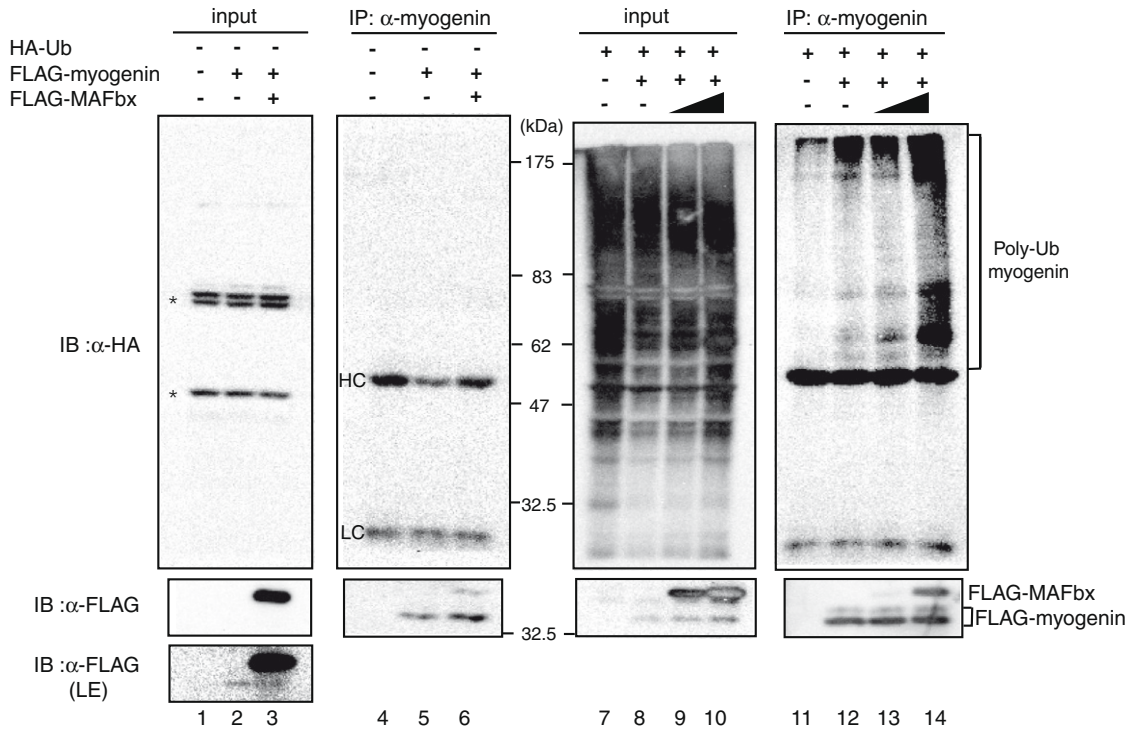

Fig. 4. Increase in polyubiquitinated myogenin in MAFbx-overexpressing cells. Cos7 cells were transfected with the indicated combination of expression plasmids. Forty-eight hours after transfection, cells were treated with 25 μM MG132 for 4 h, lysed, and immunoprecipitated with α-myogenin antibody. Proteins in the whole cell extract (lanes 1–3 and 7–10) and in immunoprecipitates (lanes 4–6 and 11–14) were detected with α-HA (for Ub) and α-FLAG (for myogenin and MAFbx) antibodies. Positions of polyubiquitinated myogenin, non-specific band (asterisks), and immunoglobulin heavy chain (HC) & light chain (LC) are indicated. FLAG-myogenin signals can be seen in lanes 2 and 3 when the blot was exposed for longer time. Long exposure (LE) data of the lower panel for lanes 1–3 were also shown.

motif of MyoD (Fig. 2A). The core motif (LQALL) within the MAFbx-recognition motif is thought to be critical for MyoD to bind to MAFbx. This core motif is also present in the MyoD-binding region of MAFbx. Both myogenin (ERLQALLS) and Myf-5 (ESLQELLR) have the consensus core motif in the hypothetical MAFbx-recognition motif. However, alanine in the core motif of MyoD, which is also conserved for myogenin, is exchanged with glutamic acid in Myf-5 (Fig. 2A). Consequently, the entire sequence of the core motif may be required for MAFbx binding. Alternatively, the second amino acid in the recognition motif might participate in target recognition. We found in data bases that the core motif of MyoD and myogenin is conserved in mammals, whereas those in lower vertebrates and invertebrates exhibit less conservation and no conservation, respectively. On the other hand, those of Myf-5 and MRF4 are essentially not conserved. Therefore, MyoD family proteins can be classified into two groups in a sense of protein degradation.

Nuclear localization of MAFbx (Fig. 2C) is consistent with the fact that targets of MAFbx identified previously (*i.e.*, MyoD) [8] and in this study (*i.e.*, myogenin) are both nucleus-localizing transcription factors. MuRF1 (muscle RING-finger protein-1) is another class of muscle-specific Ub ligase [6]. MuRF1 interacts with several cytoplasmic enzymes and some of them are actually degraded by MuRF1-mediated UPS [18]. Moreover, MuRF1 degrades many muscle-related myofibrillar proteins [19–21]. These lines of evidence imply that MuRF1 functions mainly in the cytoplasm, and MAFbx-containing SCF complex and MuRF1 might have a specific role in degradation of muscle proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.07.033.

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