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Degradation of MyoD Mediated by the SCF (MAFbx) Ubiquitin Ligase*§

Lionel A. Tintignac‡§, Julie Lagirand‡¶, Sabrina Batonnet‡¶¶, Valentina Sirri, Marie Pierre Leibovich‡, and Serge A. Leibovich‡**

From the ‡Laboratoire de Génomique Fonctionnelle et Myogénèse, UMR866 Différenciation Cellulaire et Croissance, INRA UMR II, Campus INRA/ENSA, 2 Place Pierre Viala, 34060, Montpellier, Cedex 1, France and the ¶Institut Jacques Monod, UMR 7592 CNRS, 75251 Paris, France

MyoD controls myoblast identity and differentiation and is required for myogenic stem cell function in adult skeletal muscle. MyoD is degraded by the ubiquitin-proteasome pathway mediated by different E3 ubiquitin ligases not identified as yet. Here we report that MyoD interacts with Atrogin-1/MAFbx (MAFbx), a striated muscle-specific E3 ubiquitin ligase dramatically up-regulated in atrophying muscle. A core LXXLL motif sequence in MyoD is necessary for binding to MAFbx. MAFbx associates with MyoD through an inverted LXXLL motif located in a series of helical leucine-charged residue-rich domains. Mutation in the LXXLL core motif represses ubiquitination and degradation of MyoD induced by MAFbx. Overexpression of MAFbx suppresses MyoD-induced differentiation and inhibits myotube formation. Finally the purified recombinant SCFMAFbx complex (SCF, Skp1, Cdc53/Cullin 1, F-box protein) mediated MyoD ubiquitination in vitro in a lysine-dependent pathway. Mutation of the lysine 133 in MyoD prevented its ubiquitination by the recombinant SCFMAFbx complex. These observations thus demonstrated that MAFbx functions in ubiquitinating MyoD via a sequence found in transcriptional coactivators. These transcriptional coactivators mediate the binding to liganded nuclear receptors. We also identified a novel protein-protein interaction module not yet identified in F-box proteins. MAFbx may play an important role in the course of muscle differentiation by determining the abundance of MyoD.

Ubiquitin-dependent proteolysis regulates protein abundance and serves a central regulatory function in many biological processes (1). The ubiquitination of the target protein is mediated by the ubiquitin ligases, which represent a diverse family of proteins and protein complexes. The SCF (Skp1, Cdc53/Cullin1, F-box protein) and SCF-like complexes are the largest family of ubiquitin ligases. They ubiquitinate a broad range of proteins involved in cell cycle progression, signal transduction, transcription, and development (2). The formation of ubiquitin-protein conjugates involves three components that participate in a cascade of ubiquitin transfer reactions, an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin ligase (E3) that acts at the last step of the cascade (3). The specificity of the SCF complexes derives of the variable components called the F-box proteins that serve as receptor for the target protein (4).

Myogenic differentiation is under the control of the MyoD family of basic helix-loop-helix transcription factors (MRFs) that includes MyoD, myogenin, Myf5, and MRF4/herculin/Mylf-6 (5, 6). Activation of muscle-specific genes by the MRFs occurs through their heterodimerization with the E protein basic helix-loop-helix factors that bind to an E-box DNA consensus sequence (CANNTG) to transactivate muscle specific genes and to efficiently convert non-muscle cells to a myogenic lineage (7). p300/CBP and P/CAF coactivators have acetyltransferase activities and regulate transcription, cell cycle progression, and differentiation. They are both required for MyoD activity and muscle differentiation (8, 9). In contrast, histone deacetylation inhibits gene activation, and the interaction between histone deacetylase 1 and MyoD prevents premature activation of the myogenic program in growing myoblasts (10). The MRFs contain several functionally distinct domains responsible for transcriptional activation, chromatin remodeling, DNA binding, nuclear localization, and heterodimerization (11).

The mechanism by which MyoD induces myogenesis involves both the withdrawal from cell cycle and the activation of muscle-specific gene expression. Recent data demonstrate a direct link between MyoD and cell cycle regulation (12, 13) and also its requirement for myogenic stem cell function in adult skeletal muscle (14) where MyoD protein levels decline from postnatal stage onward (15). Accurate synchronization of dividing myoblasts revealed that MyoD is subject to specific cell cycle-dependent regulation (16–18). Phosphorylation of MyoD at serine 202 plays a crucial role in modulating its half-life and transcriptional activity during myoblast proliferation (19, 20). Linking this phosphorylation to the cell cycle-dependent drop in MyoD protein before S phase leads to a mechanism implicating Cdk2-cyclin E and up-regulation of its inhibitors (p57kip2 and p21cip1) in the tight control of MyoD levels and subsequent myoblast cell cycle progression or exit into differentiation (17, 21).

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‡ Present address: Growth Control Laboratory, FMI, Maulbeerstrasse 66, 4058, Basel, Switzerland.

¶ Fellows of Ministère de la Recherche et de la Technologie (MRT).

** To whom correspondence should be addressed. Tel.: 33-04-99-61-29-76; Fax: 33-04-67-54-56-94; E-mail: serge.leibovich@ensam.inra.fr.

1 The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; IRES, internal ribosome entry site; GFP, green fluorescent protein; HA, hemagglutinin; LCD, leucine-charged domain; WT, wild type; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α.
whereas specific DNA binding stabilizes MyoD (23) and proteins that first interact with human Skp1. This Skp1-enriched cDNA library was then screened to isolate F-box proteins that bind MyoD and identify positive clones. Here, we report the identification of human Atrogin-1/MAFbx (MAFbx), an E3 ubiquitin ligase up-regulated during skeletal muscle atrophy that interacts with MyoD via a novel leucine-rich interaction interface. This interface found in transcriptional co-activators mediates the binding to liganded nuclear receptors and promote its degradation. We demonstrated that the purified recombinant SCFMAFbx complex mediated ubiquitination of MyoD in vitro in a manner that appeared to depend on the presence of the lysine 133. This lysine 133 has been previously shown to play a critical role in the nuclear degradation of MyoD (26). Finally, we show that up-regulation of MAFbx in proliferating myoblasts antagonizes differentiation inducing MyoD degradation and preventing muscle specific-gene activation.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screen and DNA Manipulations**—We used the Matchmaker Two-Hybrid Kit with a human skeletal muscle cDNA library kit (Clontech). The assays were performed as recommended by the manufacturer. The full-length cDNA clone of human Skp1 (bait) was subcloned in-frame with the Gal4 DNA-binding domain in the pGBT9 vector, resulting in pGBT9-Skp1. We screened 6 × 10⁶ clones (corresponding to two library titers) and selected the positive clones as suggested by the manufacturer. The liquid β-galactosidase assay was performed according to the manufacturer’s instruction. The Skp1-enriched cDNA library was then screened against pGBT9-MyoD (bait) and we selected 100 positive clones. Oligonucleotides were synthesized by Prologo. Deletion in the F-box of MAFbx was introduced by PvuII digestion of pACT2-MAFbx and self-anneling of the resulting plasmid. The full-length coding sequences as well as mutants of MAFbx and MyoD were amplified by PCR to introduce BamHI and EcoRI sites on each side of the open reading frame and cloned as BamHI-EcoRI fragments into the eukaryotic expression vectors. The bicistronic expression plasmid for MAFbx was carrying out first by using a 1400-bp EcoRI-HindIII fragment containing the internal ribosome entry site (IRES) and GFP from the pMIGR vector and subcloned at the EcoRI and HindIII sites of pcDNA4A (Invitrogen). MAFbx and the ΔF-box mutant were then subcloned at the EcoRI site of pcDNA4A-MAFbx-ΔF-GFP.

**Cell Culture, Transfections, and Luciferase Assays**—The mouse skeletal muscle cell line C2C12 and the fibroblast cell line 10T1/2 were maintained in growth Dulbecco’s modified Eagle’s medium supplemented with antibiotics and, respectively, 20 and 15% fetal calf serum. Cells were transfected by using the Jet PEI (QBiogene). Luciferase activity was determined using 10⁻¹⁵ luciferase units as recommended by the manufacturer. The full-length cDNA clone of human Skp1 (bait) was obtained from the manufacturer. The liquid β-galactosidase assay was performed according to the manufacturer’s instruction. The Skp1-enriched cDNA library was then screened against pGBT9-MyoD (bait) and we selected 100 positive clones. Oligonucleotides were synthesized by Prologo. Deletion in the F-box of MAFbx was introduced by PvuII digestion of pACT2-MAFbx and self-anneling of the resulting plasmid. The full-length coding sequences as well as mutants of MAFbx and MyoD were amplified by PCR to introduce BamHI and EcoRI sites on each side of the open reading frame and cloned as BamHI-EcoRI fragments into the eukaryotic expression vectors. The bicistronic expression plasmid for MAFbx was carrying out first by using a 1400-bp EcoRI-HindIII fragment containing the internal ribosome entry site (IRES) and GFP from the pMIGR vector and subcloned at the EcoRI and HindIII sites of pcDNA4A (Invitrogen). MAFbx and the ΔF-box mutant were then subcloned at the EcoRI site of pcDNA4A-MAFbx-ΔF-GFP.

**Immunoprecipitation and Immunoblotting**—Two anti-MAFbx antibodies were generated by injecting rabbits with the amino acid peptide KKKKDM/LNSRTK(T/C) corresponding to amino acids 61–74 and amino acid peptide KGTDHPCTANNPE(C) corresponding to amino acids 326–339 of the human MAFbx protein. Antibodies were affinity-purified against antigenic peptides. For immunoprecipitation, cell lysates in IP buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 0.5 mM sodium-orthovanadate, 50 mM NaF, 80 μM β-glycerophosphate, 10 mM sodium-pyrophosphate, 1 mM dithiothreitol, 1 mM EDTA, and 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprotinin) were preclarified for 30 min with protein-G beads and immunoprecipitated by using standard procedures. Immunoprecipitated proteins were loaded onto 10% SDS-polyacrylamide gels before electrophoretic transfer onto a nitrocellulose membrane. For detection of MyoD-ubiquitin conjugates, cells were rinsed in phosphate-buffered saline and scraped into 800 μl of radioimmunoprecipitation assay buffer (20 mM Tris, pH7.5, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride). Cells were then spun at 15,000 × g for 15 min, and the supernatant was denatured and then precipitated with anti-MyoD antibody. Western blotting was performed by using an ECL kit (Amersham Biosciences) according to the manufacturer’s instructions. Anti-MyoD polyclonal (C20) and anti-HA polyclonal (Y-11) were purchased from Santa Cruz Biotechnology. Anti-ubiquitin (P4D1) antibodies were purchased from Calbiochem, anti-Troponin T monoclonal (JLT-12) was purchased from Sigma, anti-HA epitope (12CA5) was purchased from Roche Diagnostics, anti-MyoD monoclonal (5A8) was from Pharmingen, and anti-FLAG (M2) was from Kodak. To inhibit proteasome activity, cells were...
treated with 50 μM MG132 (Sigma) for 2 h.

In Vitro and in Vivo Ubiquitination Assay—Ubiquitination assays were determined essentially as described (17, 26) by using [35S]-methionine labeled in vitro translated HA-MyoD. The recombiant SCFMAFbx complex was produced in insect cells by infection with baculovirus encoding HA-MAFbx-His[6], Skp1, Cul1, and Rbx1, purified by nickel-agarose chromatography, and added in roughly similar amount to the reaction. In vivo ubiquitination assays have been described (28).

RNA Extraction and Reverse Transcript-PCR Analyses—Total RNA was isolated from C2C12 cells using TRI Reagent (Sigma). For semiquantitative reverse transcription-PCR analysis, 1 μg of RNA was used to perform reverse transcription with Superscript II Reverse Transcriptase (Invitrogen) and hexanucleotides (Roche Diagnostic). 2 μl of each reaction served as a template for PCR analysis. Primer pairs for the amplification of the gene products were MAFbx 5′-GGGAGAAGCTTTCAACAG-3′ forward, 5′-TGAGGCCCCCTTTGAAGGGCACG-3′ reverse and glyceraldehyde-3-phosphate dehydrogenase 5′-GAGCTGAGGGCAGCTAC-3′ forward, 5′-TTGTCACCATACGAGGATAATGG-3′ reverse.

Immunofluorescence Staining—Cells were cultured on coverslips and fixed in 3% paraformaldehyde for 10 min at 4 °C, and permeabilized with 0.25% Triton X-100 for 30 min at room temperature. The cells were treated with 5% normal goat serum and immunostained with an anti-MyoD mAb (5.A8) The Texas Red-conjugated F(ab0.25)2 fragment of donkey anti-mouse IgG was used to visualize the mouse monoclonal antibodies.

RESULTS

Identification of MAFbx as an F-box Protein Interacting with MyoD—The CDC34 ubiquitin-conjugating enzyme activity (E2) has been implicated in MyoD ubiquitination (19) suggesting the requirement of an Skp1/Cul1/F-box protein complex (E3). To identify candidate molecular mediators of MyoD ubiquitination and degradation, we used a human skeletal muscle library enriched for F-box-expressing genes and MyoD as bait in the yeast two-hybrid screen. We identified two positive clones with the sequence and schematic representation of mutant MyoD L164Q generated by site-directed mutagenesis. Amino acid regions corresponding to the putative MAFbx-MyoD interacting motif in MAFbx are indicated in MAFbx L164Q generated by site-directed mutagenesis. B, empty plasmid (lane 1) or expression plasmids encoding HA-MyoD-L164Q (lane 2) or HA-MyoD-wt (lane 3) were cotransfected with an expression plasmid encoding FLAG-MAFbx into 10T1/2 cells. Cell lysates were processed for immunoprecipitation with anti-FLAG antibodies (M2). Immunoprecipitates were then analyzed by Western blotting with anti-FLAG and anti-HA, respectively (top panel). Note that anti-FLAG antibodies failed to immunoprecipitate HA-MyoD. D, identification of the sequence in MAFbx required for MyoD interaction. 10T1/2 cells were cotransfected with MyoD and wild type (full) and/or deletion mutants of MAFbx and (-) indicate whether or not the various mutants interact with MyoD. Representation of the putative MyoD-binding domain in MAFbx. Highly conserved LCD in mammals is underlined, and leucine residues are in bold. E, empty vector (lane 1) or expression plasmids encoding FLAG-MAFbx-wt (lane 2) or FLAG-MAFbx-L164Q (lane 3) were transfected into 10T1/2 cells together with an expression plasmid encoding HA-MyoD. Whole cell lysates were processed for immunoprecipitation with anti-FLAG antibodies. Immunoprecipitates were then analyzed by Western blotting with anti-FLAG and anti-HA, respectively (top panel). WCE, whole cell extract.
**FIG. 3. MAFbx increases MyoD turnover.** A, 10T1/2 cells were cotransfected using the expression vector encoding HA-MyoD and various FLAG-tagged F-box proteins as indicated. The cells were treated with cycloheximide (CHX) 36 h after transfection to block protein synthesis. Cell lysates were prepared at the indicated time and analyzed by Western blotting with anti-HA and anti-FLAG, respectively (left panel). Quantitation of MyoD turnover following cycloheximide treatment based on densitometric scanning of experiments is shown (right panel). Note that the ∆F-box mutant stabilized MyoD. B, effects of mutations of LXXLL motif in MAFbx on MyoD degradation. MyoD turnover was analyzed in 10T1/2 cells transfected with the indicated expression vectors followed by pulse chase to measure MyoD stability in the presence of MAFbx wild type and/or the mutant L169Q. Quantitation of MyoD turnover following pulse chase based on densitometric scanning is shown (right panel). C, pulse-chase analysis was used to measure MyoD stability in wild type and mutant MyoD L164Q in the absence or in the presence of MAFbx as in B.
which are conserved between human, rat, and mouse species (supplemental Fig. 1). The presence of various protein-protein interacting domains suggests that MAFbx could potentially recognize different substrates.

Expression of MAFbx and MyoD during C2C12 Muscle Differentiation—Because little is known about the expression of MAFbx in myogenic differentiation, we initially characterized MAFbx expression during early myogenic differentiation using

**Figure 4.** Overexpression of MAFbx degrades MyoD and suppresses myogenic differentiation. A, effect of MAFbx on MyoD-dependent transcriptional transactivation of muscle-specific genes. 10T1/2 cells were cotransfected with 0.5 µg of MCK-Luc reporter plasmid together with 2 µg of pEMSV-MyoD in combination with 0.5 and 2 µg of expression vector encoding FLAG-tagged MAFbx. Expression vector without insert was included to normalize DNA in all transfections. Luciferase levels were determined 48 h after transfections in proliferating medium and represent the average of three independent experiments (errors bars, S.D.). Protein concentrations were equalized by Bradford assay, and aliquots were analyzed by Western blotting for MyoD and MAFbx expression using specific antibodies (upper panel). B, overexpression of MAFbx suppressed MyoD in C2C12 myoblasts (a–o). Myoblasts were transfected with empty pcDNA4-IREs-GFP vector (a–e) and either MAFbx-IREs-GFP (f–j) or the mutant MAFbx AF-IREs-GFP (k–o). Forty-eight hours later, cells were fixed and stained with anti-MyoD and revealed with goat anti-mouse antibody conjugated to Texas Red (d, i, and n). Hoechst 33258 dye shows nuclei (b, g, and i). Merged images are shown in e, j, and o. Images of a representative field were obtained by indirect immunofluorescence microscopy (400×). C, overexpression of MAFbx suppresses myogenic differentiation. C2C12 myoblasts were transfected with pcDNA4-IREs-GFP expression plasmid (a–e), the mutant MAFbx AF-IREs-GFP (f–j), or MAFbx-IREs-GFP (k–o). Twenty-four hours later, cells were switched to differentiation medium (DM) for 72 h, fixed, and stained with anti-MyoD as in (B) and with Hoechst 33258 dye (b, g, and i). Merged images are shown in e, j, and o. Images of a representative field were obtained by indirect immunofluorescence microscopy (400×). DAPI, 4,6-diamidino-2-phenylindole.
the C2C12 cell line, a well defined model for ex vivo differentiation. These cells proliferate as myoblasts in high serum concentrations and can be induced to differentiate by reducing the serum concentration from 20 to 2%. Under our conditions, C2C12 myoblasts fuse into myotubes within 48–60 h with an efficiency of 75–80%. A semiquantitative reverse transcription-PCR analysis showed that the MAFbx mRNA was up-regulated (2–3-fold) in the course of C2C12 differentiation (Fig. 1B). Anti-MAFbx antibodies were developed in our laboratory (see “Experimental Procedures”), and Western blot analysis revealed that the MAFbx protein is barely detectable in myoblasts and accumulates in C2C12 myotubes. By this time, MyoD is up-regulated after switching cells into the differentiation medium (DM) and then decreases during differentiation; troponin T, a skeletal muscle marker, is observed only in differentiated cells (Fig. 1C). Thus, during the course of C2C12 differentiation MyoD and MAFbx showed a contrasting pattern of expression.

Phosphorylation or Acetylation of MyoD Is Not Necessary for Interaction with MAFbx—We first determined the binding of MyoD with MAFbx in mammalian cells by using a comimmunoprecipitation assay. In C2C12 myogenic cells, endogenous MyoD coimmunoprecipitated with endogenous MAFbx, and an increase in MyoD/MAFbx association was observed when proteasome activity was inhibited (Fig. 2A). In transiently transfected 10T1/2 cells, MyoD and MAFbx have a nuclear localization, and MyoD coimmunoprecipitated with MAFbx but did not associate with other F-box proteins such as Skp2 or FBX03 (supplemental Fig. 2A and B). Furthermore, Myf-5, the second determination factor that is also expressed in myoblasts, did not coimmunoprecipitate with MAFbx in cotransfection experiments (supplemental Fig. 2C).

Posttranslational modifications of target proteins are implicated in the recognition by certain SCF-type E3 ubiquitin ligases and subsequent degradation by the 26 S proteasome (4, 31, 32). On the other hand, ubiquitination of substrates requires direct binding of specific domains of the F-box protein and the substrate (33). The main posttranslational modifications of MyoD that implicate phosphorylation of Ser-5 and Ser-200 (18, 20) and/or acetylation by CBP/p300 (8) and p/CAF (9) were tested for their interaction with MAFbx. 10T1/2 cells were transiently transfected with plasmids encoding MAFbx alone or with MyoD-wt, MyoD S200D, which mimics constitutive serine 200 phosphorylation, MyoD SASA/S200A, and MyoD/K → R in which all lysines were mutated to arginines. Surprisingly MyoD-wt and the MyoD mutants all interacted with MAFbx suggesting that major phosphorylation and acetylation are not implicated in MyoD/MAFbx interaction (supplemental Fig. 2D). Altogether, these results show that independent of the phosphorylation and/or acetylation, MyoD and MAFbx form complexes in vivo.

LCID of MAFbx Interacts with a Highly Conserved LXXLL Motif in MyoD—The results outlined above show that MAFbx binds to MyoD, but the lack of known protein binding motifs found in other F-box proteins prompted us to search for regions of MAFbx important for substrate recognition. The domains of both proteins required for interaction were mapped by in vivo protein binding experiments. 10T1/2 cells were cotransfected with expression vectors encoding MyoD-wt and/or MyoD deletion or substitution mutants together with MAFbx, and the immunoprecipitates were then examined for the presence of MAFbx by an immunoblot analysis (supplemental Fig. 3A). Stable association between MyoD and MAFbx was mediated by the helix 2 domain of MyoD (Fig. 2B). Sequence analysis of amino acids 143–172 revealed a LXXL consensus sequence that mediates protein-protein interactions and was originally found in a variety of coactivators (34). To assess the contribution of this motif to the interaction, we mutated the leucine 164 to glutamine (L164Q) and tested its interaction with MAFbx as described above. Mutation of the LXXLL motif was sufficient to reduce MyoD interaction with MAFbx (Fig. 2C).

In vivo reciprocal binding experiments were performed to define the sequence in MAFbx that contributes to complex interactions by testing a series of MAFbx deletion mutants. The results (Fig. 3A) showed that the interaction depends on the presence of the helix 2 domain and that alterations in the LXXLL motif (L164Q) led to a significant decrease in association. When this motif was decreased to the L164A mutant, no interaction was observed between MyoD and MAFbx (lane 4).

The results also suggest that the motif is necessary and sufficient, as the LXXL motif alone was sufficient to reconstitute in vivo interaction, as shown by cotransfection experiments in 10T1/2 cells (Fig. 3B). Thus, our data indicate that the LXXL motif may mediate important protein-protein interactions.
The SCFMAFbx complex was assayed for the ability to mediate the ubiquitination of MyoD. To avoid confusion between the IgG band and the MAFbx mutants, we used a mammalian expression plasmid encoding GST-MyoD (supplemental Fig. 3B). Overlapping deletion mutant analysis showed that the central part of MAFbx (amino acids 140–222) was implicated in the MyoD binding (Fig. 2D). This region contains a LCD that shares a consensus inverted LXXLL motif flanked by LXXX and LXXL sequences highly conserved in mammals. We found that mutation (L169Q) in the inverted LXXLL motif dramatically reduced the binding to MyoD (Fig. 2F). These results demonstrated that complex formation depends upon the integrity of the inverted LXXLL motif in MAFbx.

Accelerated Degradation of MyoD by MAFbx—The effect of MAFbx expression on the MyoD half-life was investigated after blocking protein synthesis with cycloheximide and/or in pulse-chase experiments. As expected the empty vector did not affect MyoD stability (half-life was to ~45 min). Overexpression of MAFbx resulted in enhanced degradation of MyoD by decreasing its half-life to ~30 min. By contrast Skp2 and/or FBL5 was ineffective on MyoD degradation. In cells with compromised SSCPMAFbx activity, MyoD degradation was affected; the MAFbx mutant ΔF-box (deleted of the F-box domain required for Skp1 interaction) showed an increased MyoD half-life (~150 min) (Fig. 3A). In cells with compromised LXXLL motif interaction between MyoD and MAFbx, the degradation rate of MyoD remained similar to that of MyoD-wt alone (Fig. 3B and C). It appears that destabilization of MyoD is increased in cells with SSCPMAFbx E3-ligase activity and highlights the LXXLL motif that mediates MyoD/MAFbx interaction.

Overexpression of MAFbx in C2C12 myotubes results in atrophy (28), and on the other hand, during the course of C2C12 differentiation MyoD and MAFbx showed a contrasting pattern of expression (Fig. 1C). Together these data suggested that MAFbx-inducing C2C12 atrophy implicated MyoD degradation. We observed that increasing amounts of MAFbx on MyoD-mediated myogenic conversion of 10T1/2 cells caused a gradual decrease of MCK-Luc activity and MyoD protein abundance (Fig. 4A). To locate and assess the effect of MAFbx overexpression on MyoD proteins, C2C12 myoblasts were transfected with pcDNA4-MAFbx-IRES-GFP or the mutant MAFbx-ΔF-box-IRES-GFP, and 48 h later C2C12 myoblasts (Fig. 4B) were stained with anti-MyoD antibodies and/or incubated to differentiate in low serum medium (Fig. 4C). In C2C12 myoblasts transfected with MAFbx (green), MyoD staining was selectively lost, whereas in C2C12 myoblasts transfected with the empty vector and/or the ΔF-box mutant, MyoD staining was observed in the nucleus (Fig. 4B). Furthermore overexpression of MAFbx-IRES-GFP was never observed during myotube formation, and the great majority of the transfected cells were lost. The few MAFbx-expressing C2C12 myoblasts (green) maintained the mononucleated, non-differentiated phenotype and did not express MyoD. In contrast, C2C12 cells transfected with empty vector expressed MyoD and fused into multinucleated myotubes, with a higher level in the presence of the mutant ΔF-box (Fig. 4C). Altogether these data suggest that high levels of MAFbx are incompatible with MyoD protein expression.

Mediation of MyoD Ubiquitination by the SSCPMAFbx—Because MAFbx mRNA was identified as a marker that is up-regulated in multiple models of skeletal muscle atrophy in mice, MAFbx protein expression was first tested in two models of in vivo atrophy, aging and food deprivation. Then the association of MAFbx with the essential Skp1 and Cul1 proteins, specific components of an E3 ubiquitin-protein ligase, was tested by using MyoD as a substrate in the ubiquitination assays. The level of MAFbx protein (42 kDa) in skeletal muscle....
increased during aging and/or food deprivation (Fig. 5A). Total extracts from the hind leg muscles were then analyzed for the association of Cul1 and Skp1 with MAFbx. Coimmunoprecipitation of MAFbx, Cul1, and Skp1 were observed in normal mouse skeletal muscle, and increased amounts were found in atrophic mice. These complexes were not observed after immunoprecipitation with a preimmune serum (Fig. 5B). In an in vitro ubiquitination assay using a MyoD mutant with the amino-terminal three amino acids deleted (HA-tagged MyoD) as the physiological substrate, the SCFMAFbx from normal skeletal muscle exhibited ubiquitination activity, which was higher in atrophic muscle (Fig. 5C). Finally to determine in vitro whether the SCFMAFbx complex mediates ubiquitination of MyoD, we expressed the four subunits of the complex (Skp1, Cul1, Rbx1, and MAFbx) in Sf9 cells. We then purified the recombinant complex to near homogeneity (Fig. 6A) and assayed it for its ability to catalyze the ubiquitination of HA-tagged MyoD in vitro in the presence of E1, Cdc34 (E2), and ubiquitin. A significant ubiquitination was detected with E1, E2, ubiquitin, and SCFMAFbx (Fig. 6B) but the polyubiquitination of the HA-tagged MyoD mutant L164Q was dramatically reduced (Fig. 6C). We also examined whether the SCFMAFbx complex mediated the ubiquitination of a MyoD mutant K133R that has been recently shown to play a critical role in the nuclear degradation of MyoD (26). As observed in Fig. 6D, the MyoD mutant K133R showed a dramatically reduced polyubiquitination strongly suggesting the implication of this internal lysine in MyoD ubiquitination by the SCFMAFbx.

Finally, to test whether MAFbx could promote MyoD ubiquitination in vivo, expression vectors encoding MAFbx or the mutant ΔF-box were cotransfected with MyoD and HA-ubiquitin into 10T1/2 cells. MyoD protein was immunoprecipitated with anti-MyoD antibodies and was probed with anti-HA to detect ubiquitinated MyoD proteins. In the presence of MAFbx, MyoD showed a higher degree of ubiquitination compared with the mock transfectant (Fig. 7A). The mutant ΔF-box did not promote the ubiquitination of MyoD, although it remained associated with MyoD. In C2C12 myoblasts, overexpression of MAFbx increased MyoD ubiquitination whereas the MAFbx mutant L169Q like the empty vector were ineffective (Fig. 7B). This was also observed in 10T1/2 cells transiently cotransfected by expression plasmids encoding HA-tagged MyoD-wt, FLAG-tagged MAFbx-wt, and/or the MAFbx mutant L169Q (Fig. 7C, lanes 1–3). The ubiquitination levels of the mutant MyoD L164Q were roughly similar to those of MyoD in the absence or presence of MAFbx (Fig. 7C, lanes 1, 4, and 5). These data
suggest that MAFbx via the LXXLL core motif may control the destabilization of MyoD. Altogether, these experiments thus demonstrated that MyoD is a target of the SCFMAFbx complex.

**DISCUSSION**

By using a modified version of the yeast two-hybrid screen to identify F-box proteins that are able to interact with MyoD, we identified MAFbx, a muscle-specific ubiquitin ligase that dramatically increases during skeletal muscle atrophy. We have provided direct biological and biochemical evidence that MAFbx interacts with MyoD and thereby induces its ubiquitin-dependent proteolysis. MAFbx contains a LCD in which the central part is an inverted LXXLL motif that mediates direct binding to a LXXLL motif found in all MyoD species. The α-helical LXXLL is described as a signature motif, which mediates the recruitment of coactivators by the nuclear hormone receptors (34, 35) as well as ligand-independent recruitment of steroid receptor coactivators to estrogen receptor by cyclin D1 (36). Recently the cardiac-enriched nuclear receptor ERRα has been shown to be coactivated by the transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α). The PGC-1α/ERRα binding interface revealed that among the three potential LXXLL motifs (L1–L3), only L3, which is an inverted motif, plays a major role in binding the nuclear receptor and represents a novel LXXLL-type of nuclear receptor binding motif on PGC-1α (37). The LXXLL motif in MyoD (EGLQALLLR) is not observed in the E proteins basic helix-loop-helix factors and E12 did not coimmunoprecipitate with MAFbx (data not shown). Myf-5 did not coimmunoprecipitate with MAFbx, although it contains the motif (ESLQELLLR) suggesting that variant residues in LXXLL core motifs influence the affinity and selectivity of MAFbx for the substrates (38). MAFbx contains other three domains known to be implicated for protein-protein interaction, the PDZ-binding motif, cytochrome c family heme-binding site, and the leucine zipper domain (Fig. 1A). It seems likely that these domains are dispensable for binding of MyoD and could mediate the interaction with additional substrates. MAFbx is the first example of an E3 ligase that involves a conserved LCD domain containing an LXXLL inverted motif for substrate recognition.

MyoD is degraded in the nucleus by the ubiquitin-proteasome system implicating at least two distinct pathways, an N terminus-dependent pathway (39) and a lysine-dependent pathway (40). In vivo studies also implicate a link between the phosphorylation of MyoD Ser-200 and the ubiquitin-proteasome degradation pathway in myoblasts (17, 19, 20). Altogether these data suggest that MyoD may be targeted by different E3 ligases. To avoid confusion between the N terminus-dependent pathway and the lysine-dependent pathway, we introduced modifications to the N terminus of MyoD protein (the first three amino acids have been replaced by three HA epitopes), and we have recently shown that in MyoD, lysine 133 is targeted for ubiquitination and rapid degradation in the lysine-dependent pathway and plays an integral role in compromising MyoD activity in the nucleus (26). We showed that the SCFMAFbx recombinant complex and the SCFMAFbx from the skeletal muscles induced ubiquitin-dependent proteolysis of HA-tagged MyoD, leading to the conclusion that MAFbx is probably not implicated in the N terminus-dependent ubiquitination of MyoD. Indeed we observed that the mutation K133R in MyoD that dramatically reduced its ubiquitination by SCFMAFbx strengthened the implication of this internal lysine in MyoD ubiquitination. Altogether our data show that MyoD is ubiquitinated by the SCFMAFbx via the internal lysines in which lysine 133 may be the major ubiquitination site. On the other hand, the Δbox mutant binds to and represses the ubiquitination and degradation of MyoD probably by blocking and/or masking the binding sites for other ligase(s). It appears that the ubiquitin-proteasome pathway via phosphorylation-dependent and -independent mechanisms regulates the turnover of MyoD suggesting that MyoD ubiquitination on the N terminus and/or on lysine 133 could be mediated by different ubiquitin ligases during myogenesis.

The expression of MAFbx in normal skeletal muscle tissues (27, 28) and its up-regulation during the course of C2C12 differentiation suggest that MAFbx is not involved in regulating typical cell cycle progression. MAFbx could help maintain myotubes in a postmitotic state and/or regulate proteins in pathways that typically couple signal transduction to cell cycle control in proliferating cells. MyoD is known to control the exit from the cell cycle as well as muscle-specific gene expression (41). It would be critical for myofibers that maintain MyoD and signaling pathways that control proliferation in undifferentiated myoblasts to have mechanisms protecting against attempts to enter the cell cycle (42). In healthy muscles, there is a continual process of muscle protein production and breakdown. Skeletal muscle contains not only differentiated cells but also undifferentiated quiescent cells (satellite cells) that retain differentiation potential and contribute not only to the myotubes but also to the satellite pools (43). In the quiescent state, satellite cells are negative not only for differentiation markers but also for MyoD, and in the activated state they proliferate with high levels of MyoD protein in their nuclei. MyoD expression is induced from satellite cells and is required for these cells to proliferate and to reinitiate skeletal muscle differentiation necessary for the repair process (14). MyoD+/− satellite cells are differentiation-defective, highlighting the dependence of MyoD expression in the muscle fiber regeneration (44). Accelerated proteolysis via the ubiquitin-proteasome pathway is a major cause of muscle atrophy induced by denervation, disuse, and various pathological conditions (45). In atrophy, the muscle-specific ubiquitin-ligase MAFbx dramatically increases, and protein breakdown occurs more rapidly than protein production, leading to loss of muscle weight. The subsequent effect on MyoD turnover in myogenesis and regeneration after injures suggests that up-regulation of MAFbx is likely to affect skeletal muscle regulation and regeneration. MAFbx is strongly regulated at an early stage of muscle wasting, even before muscle weight loss is detectable, and its expression is maintained during muscle atrophy. This suggests a role of these proteins in both initiation and maintenance of the accelerated proteolysis (27, 28). Our data strongly suggested that overexpression of MAFbx would be required to suppress all MyoD functions inhibiting the formation of new myofibers. Thus, direct inhibition of MAFbx expression may prove beneficial in reducing the muscle wasting associated with cachexia in cancer patients as well as with other disorders.

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**REFERENCES**

Interactions between MAFbx and MyoD in Muscle Cells

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Supplementary data

Supplementary data Figure 1
Multiple alignments of the amino acid sequences of the mammalian MAFbx proteins family showing high conservation of the predicted putative domains.

Supplementary data Figure 2

Phosphorylation and acetylation are not implicated in the binding of MyoD with MAFbx.
A, MAFbx colocalizes in nucleus as MyoD. 10T1/2 cells were cotransfected with mammalian expression plasmids encoding GFP-MAFbx (green) and MyoD. Twenty-four hours later, the transfected cells were fixed and immunostained with anti-MyoD antibodies (red) and Hoechst 33258 (blue) and examined by indirect immunofluorescence microscopy.
B, 10T1/2 cells were cotransfected with expression vectors encoding Flag-tagged MAFbx (lane 1), Skp2 (lane 2) or FBX03 (lane 3) together with MyoD. Cells were harvested 24 hours after transfection and total cell lysates were immunoprecipitated with anti-Flag antibodies, then F-box proteins and MyoD were detected by western blotting using anti–Flag (M2) and anti-MyoD (C-20) antibodies respectively (right panel). Portions of the cells lysates corresponding to 10% of the input were subjected to immunoblot analysis (left panel).
C, MyoD but not Myf-5 binds to MAFbx. 10T1/2 cells were cotransfected with expression vectors encoding HA-tagged MyoD, HA-tagged Myf-5 or empty vector together with Flag-tagged MAFbx. Cells were harvested 24 hr after transfection. Whole cell extracts (WCE) were directly probed with HA and/or Flag antibodies or immunoprecipitated with anti-Flag antibodies prior to western blotting with HA and Flag antibodies (IP).
D, 10T1/2 cells were cotransfected with expression plasmids: pCMV-Flag-MAFbx alone (lane 2) or together with pEMSV-MyoD-wt (lane 1), pEMSV-MyoD S200D, (lane 3), pEMSV-MyoD S5A/S200A (lane 4) and pEMSV-MyoD K>R (lane 5). Lysates were directly probed with anti-MyoD and anti-Flag antibodies respectively (WCE) or immunoprecipitated with anti-Flag antibodies and then MAFbx and MyoD proteins were detected by western blotting.
Supplementary data Figure 3.
Characterization of MAFbx binding domain in MyoD.
A, 10T1/2 cells were cotransfected with each indicated HA-tagged MyoD mutant together with expression plasmid encoding Flag-MAFbx. Cells were lysed and aliquots of total cell lysates (input) processed for western blot analysis with anti-HA and anti-Flag antibodies (right panel, lanes 1-6). Portions of cell lysates were immunoprecipitated with anti-HA antibodies (12CA5). Immunoprecipitates were then analyzed by western blot with anti-Flag antibodies (lower panel). Note that anti-HA antibodies failed to immunoprecipitate Flag–MAFbx (lane 1).

B/ Identification of sequences in MAFbx required for interaction with MyoD.
10T1/2 cells were transfected with each indicated expression plasmid, then cells were lysed and aliquots (input) processed for western blot analysis with anti-Flag and anti-MyoD antibodies (left panels, lanes 1-9) or for GST-pull down with glutathione–agarose beads). Beads were processed further by immunoblotting with anti-Flag and anti-MyoD antibodies respectively (right panels, lanes 1-9).
Supplementary data  Figure 2