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RESEARCH ARTICLE

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The aminopeptidase LAP3 suppression accelerates myogenic differentiation via the AKT-TFE3 pathway in C2C12 myoblasts

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Abstract

Skeletal muscle maintenance depends largely on muscle stem cells (satellite cells) that supply myoblasts required for muscle regeneration and growth. The ubiquitin–proteasome system is the major intracellular protein degradation pathway. We previously reported that proteasome dysfunction in skeletal muscle significantly impairs muscle growth and development. Furthermore, the inhibition of aminopeptidase, a proteolytic enzyme that removes amino acids from the termini of peptides derived from proteasomal proteolysis, impairs the proliferation and differentiation ability of C2C12 myoblasts. However, no evidence has been reported on the role of aminopeptidases with different substrate specificities on myogenesis. In this study, therefore, we investigated whether the knockdown of aminopeptidases in differentiating C2C12 myoblasts affects myogenesis. The knockdown of the X-prolyl aminopeptidase 1, aspartyl aminopeptidase, leucyl-cystinyl aminopeptidase, methionyl aminopeptidase 1, methionyl aminopeptidase 2, puromycine-sensitive aminopeptidase, and arginyl aminopeptidase like 1 gene in C2C12 myoblasts resulted in defective myogenic differentiation. Surprisingly, the knockdown of leucine aminopeptidase 3 (LAP3) in C2C12 myoblasts promoted myogenic differentiation. We also found that suppression of LAP3 expression in C2C12 myoblasts resulted in the inhibition of proteasomal proteolysis, decreased intracellular branched-chain amino acid levels, and enhanced mTORC2-mediated AKT phosphorylation (S473). Furthermore, phosphorylated AKT induced the translocation of TFE3 from the nucleus to the cytoplasm, promoting myogenic

differentiation through increased expression of myogenin. Overall, our study highlights the association of aminopeptidases with myogenic differentiation.

KEYWORDS
aminopeptidase, C2C12 myoblast, LAP3, myogenic differentiation, myogenin, TFE3

1 | INTRODUCTION

Skeletal muscle maintenance depends largely on muscle-specific stem cells, also called satellite cells, which supply myoblasts required for muscle regeneration and growth (Kuang et al., 2007). In skeletal muscle damage, satellite cells are activated, differentiate into proliferative myoblasts (Relaix & Zammit, 2012), and fuse with one another to form multinucleated myotubes that are integrated into the damaged segments of skeletal muscle tissue (Abmayr & Pavlath, 2012). Thus, because of its regenerative capacity, skeletal muscle is capable of recovering from injuries caused by mechanical overloads that frequently occur in high-intensity training and sports activities (Ambrosio et al., 2009).

The ubiquitin–proteasome system is the major intracellular protein degradation pathway (Collins & Goldberg, 2017; Hershko & Ciechanover, 1998). Proteasomal proteolysis is mediated by the 26S proteasome, an ATP-dependent protease complex found in both the cytoplasm and nucleus. It plays a key role in the regulation of several diverse cellular processes and functions by catalyzing the selective degradation of short-lived, as well as abnormal, proteins (Goldberg, 2003). Therefore, understanding the role of proteasomes in cells from different tissue types is indispensable to understanding the homeostatic regulation of cells. We previously reported that skeletal muscle-specific proteasome dysfunction significantly impaired muscle growth and development (Kitajima et al., 2014, 2020). We further demonstrated that a satellite cell-specific defect in proteasomes led to the loss of satellite cells, thereby critically compromising the regenerative capacity of skeletal muscle tissue after cardiotoxin-induced damage. Another study showed that primary myoblasts derived from proteasome-defective mice exhibited significantly impaired proliferative and differentiation capacities (Kitajima et al., 2018). These findings suggest that proteasomes are essential for myogenesis and the maintenance of skeletal muscle (Kitajima et al., 2020).

Through proteolysis, proteasomes generate oligopeptides composed of 2–25 amino acids in the cytoplasm (Hershko & Ciechanover, 1992). Most of these peptides are further cleaved by aminopeptidases into free amino acids (Saric et al., 2004). Aminopeptidases selectively hydrolyze amino acid residues from the N-terminus of peptides. Several aminopeptidases have been identified in mammalian cells, including myoblasts (Osana et al., 2022; Taylor, 1993). In a recent study, we found that the inhibition of intracellular aminopeptidases by bestatin methyl ester, a cell membrane permeable inhibitor, impairs the proliferation and differentiation ability of C2C12 myoblasts (Osana et al., 2020). Furthermore, the suppression of alanine aminopeptidase (Anpep) by small interfering RNA (siRNA) in C2C12 myoblasts decreased cell proliferative capacity and delayed myogenic differentiation (Osana et al., 2020). In addition, the knockdown of the puromycin-sensitive aminopeptidase (PSA) in C2C12 myoblasts resulted in abnormal cell cycles and the formation of spherical multinucleated myotubes, accompanied by aberrant cell polarity (Osana et al., 2021). These data suggest that proteasomal proteolysis, as well as peptide degradation by aminopeptidases, are essential for myogenesis. However, to the best of our knowledge, no evidence has been reported on the role of aminopeptidases with varieties of substrate specificities on myogenesis.

Therefore, we investigated whether the knockdown of the variety of aminopeptidases in differentiating C2C12 myoblasts affects myogenesis. In this study, we focused on intracellularly expressed aminopeptidases, including X-prolyl aminopeptidase 1 (APP1), aspartyl aminopeptidase (DNPEP), endoplasmic reticulum aminopeptidase 1 (ERAP1), leucine aminopeptidase 3 (LAP3), leucyl and cystinyl aminopeptidase (LNPEP), methionyl aminopeptidase 1 and 2 (METAP1 and METAP2, respectively), PSA, arginyl aminopeptidase (RNPEP), and arginyl aminopeptidase like 1 (RNPEPL1). In this study, we identified aminopeptidases involved in myogenic differentiation in myoblasts, with a particular focus on LAP3, which was found to promote myogenic differentiation when deprived of siRNA. Furthermore, we found that LAP3 is involved in the regulation of proteasomal proteolysis and the AKT-TFE3-myogenin pathway essential for myogenic differentiation. Our results provide new insights into the regulation of myogenic differentiation in C2C12 myoblasts by aminopeptidases.

2 | METHODS

2.1 | Cell culture

Mouse C2C12 myoblasts were cultured under standard conditions (37°C under a humidified atmosphere containing 5% CO_2) in a growth medium (GM; high-glucose Dulbecco's Modified Eagle Medium (DMEM; FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 100 mg/mL penicillin-streptomycin solution (Sigma-Aldrich Corporation). Myogenic differentiation was induced in a differentiation medium (DM; DMEM supplemented with 2% calf serum (Thermo Fisher Scientific) and 100 mg/mL penicillin-streptomycin). Cells were cultured in 6-, 12-, or 24-well plates, and used for each experiment. For cell counting, we prepared a 1:1 dilution of the cell suspension using 0.4% trypan blue solution (Nacalai Tesque). Cells were counted in four 1-mm^2 square areas using a hemocytometer to determine the

average number of cells. Akt1/2 kinase inhibitor (Abcam; Cas: 612847-09-3) were mixed at 1 μM with the C2C12 myoblasts under myogenic differentiation conditions.

2.2 | Enzymatic activity assay

Cells were disrupted with radioimmunoprecipitation assay buffer and transferred onto 96-well plates. The lysate was combined with 1.6 mM leucine aminopeptidase substrate (L-leucine p-nitroaniline hydrochloride; FUJIFILM Wako Pure Chemical Corporation) for 60 min at 37°C. Enzyme activity was measured using Multiskan Go Microplate Reader (Thermo Fisher Scientific) at 405 nm.

2.3 | RNA interference

siRNAs targeting each aminopeptidase gene were obtained from Sigma-Aldrich. The sequences of the siRNAs are shown in Table S1. A scramble sequence of siRNA was used as the negative control. For siRNA experiments, cells in each well were transfected with 20 nM of each aminopeptidase gene or scramble siRNA using lipofectamine RNAiMAX transfection reagent (Invitrogen) following the manufacturer's instructions.

2.4 | LAP3 overexpression by plasmid transfection

For LAP3 overexpression, C2C12 myoblasts were seeded on a 6-well plate the day before; when cells reached 60%–70% confluence, transfection of LAP3 overexpression vector (pCAG-mLAP3/3×FLAG-EGFP) and control vector (pCAG-EGFP/3×FLAG) purchased from VectorBuilder were carried out using Lipofectamine 3000 (Thermo Fisher Scientific) following the manufacturer's instructions. After 48 h of transfection, myoblasts-induced myogenic differentiation.

2.5 | Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen) and subsequently reverse-transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). RT-qPCR was performed using the StepOnePlus PCR System (Thermo Fisher Scientific) with SYBR Green Master Mix (Thermo Fisher Scientific). The primer sequences of the aminopeptidase genes are shown listed in Table S2.

2.6 | Western blot analysis

Total protein lysate was harvested using a radioimmunoprecipitation lysis buffer (ATTO Corporation), and the protein lysates of the nucleus and cytoplasm were collected using a nuclear extraction kit (Abcam) from the C2C12 myoblasts and myotubes. We used the bicinchoninic acid assay to determine protein concentrations. Next, we extracted the protein fractions using a reducing sample buffer containing 7% β-mercaptoethanol, a protease inhibitor, and a phosphatase inhibitor (ATTO Corporation). Protein extracts (10–20 μg per lane) were separated on a 5%–20% gradient sodium dodecyl sulfate-polyacrylamide gel and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane using the Trans-Blot Turbo System (Bio-Rad Laboratories, Inc.). The PVDF membrane was blocked with 3% bovine serum albumin (Sigma-Aldrich) in 1× Tris-buffered saline–1% Tween-20 (FUJIFILM Wako Pure Chemical Corporation) for 1 h at room temperature or Bullet Blocking Reagent (Nacalai Tesque) for 5 min at room temperature. The PVDF membrane was incubated with a primary antibody for 1 h at room temperature or overnight at 4°C with continuous shaking. The primary antibodies and dilution factors are presented in Table S3. After repeated washing, the PVDF membrane was incubated in 3% nonfat milk containing a horseradish peroxidase-conjugated secondary antibody for 1 h. The resultant bands were visualized using the ChemiDoc imaging system (Bio-Rad Laboratories) and ImageQuant LAS 4000 (GE Healthcare) enhanced chemiluminescence reagents (Cytiva). Finally, Coomassie brilliant blue (FUJIFILM Wako Pure Chemical Corporation) or Ponceau-S (Beacle) staining was performed following the manufacturer's instructions and used as a loading control. Densitometry was performed using Image Lab software (Bio-Rad Laboratories).

2.7 | Immunostaining

Immunocytochemistry of C2C12 myoblasts and myotubes was performed as described previously (Osana et al., 2021). Samples were incubated with primary antibodies at 4°C overnight following blocking and permeabilization with phosphate-buffered saline (PBS) containing 0.1% Triton X-100% and 5% goat serum for 1 h at room temperature. The immunocytochemistry of anti-myosin heavy chain (MyHC; 1:100; Developmental Studies Hybridoma Bank), anti-LAP3 (1:100; Santa Cruz Biotechnology), and nuclear were visualized using appropriate species-specific Alexa Fluor 488- or Alexa Fluor 555-conjugated secondary antibodies and Hoechst 33342 (Thermo Fisher Scientific). Samples were then examined under an Olympus fluorescence microscope (Olympus Corporation). The differentiation index, which is an index of the progression level of myogenic differentiation, was calculated by dividing the number of nuclei in myotubes (MyHC-positive elongated cells) by the total number of nuclear. Also, it was calculated for 3 fields/dish and evaluated on an average of 4 dishes. The lengths of myotubes were measured using ImageJ Fiji software, as previously described (Schneider et al., 2012). Also, it was calculated for 10–15 myotubes/dish and evaluated on an average of five dishes.

2.8 | Cell damage assay

Cell damage was measured using a Cytotoxicity LDH Assay Kit-WST (Dojindo Laboratories) following the manufacturer's instructions.

C2C12 myoblasts were cultured for 2 days after siRNA treatment in 12-well plates. The cultured supernatant (100 μL) was transferred to a new 96-well plate, to which 100 μL of the working solution was added. The plate was then incubated for 60 min. Finally, 50 μL of the stop solution was added to the wells, and the absorbance was measured at 490 nm using a Multiskan Go Microplate Reader (Thermo Fisher Scientific).

2.9 | RNA sequencing and bioinformatics analysis

Total RNA was measured using the Quantus Fluorometer and QuantiFluor RNA system (Promega Corporation). The quality of the total RNA was then checked using the 5200 Fragment Analyzer System and Agilent HS RNA Kit (Agilent Technologies). Libraries were prepared using the MGIEasy RNA Directional Library Prep Set (MGI Tech Co., Ltd.) according to the instruction manual. We measured the concentration of the prepared library solution using Synergy H 1 (Agilent Technologies) and QuantiFluor dsDNA System (Promega). The Fragment Analyzer and dsDNA 915 Reagent Kit (Agilent Technologies) were used to check the quality of the prepared library. Then, cyclized DNAs (DNBs) were synthesized using the prepared library and MGIEasy Circularization Kit (MGI Tech) according to the manufacturer's manual. DNBs were prepared using the DNBSEQ G-400RS High-throughput Sequencing Kit (MGI Tech) as described in the manufacturer's manual. Sequencing analysis of the DNBs was performed using DNBSEQ-G400 at 2 × 100 bp. The resulting FASTQ files were used in subsequent analysis.

After removing adapter sequences using cutadapt (ver. 1.9.1), sickle (ver. 1.33) was used to remove bases with a quality score <20 and paired reads with a quality score <40 bases. Using hisat2 (ver. 2.2.1), high-quality reads were mapped to reference sequences. Using featureCounts (ver. 2.0.0), reads mapped to the gene region of the reference sequence were counted, and transcripts per million (TPM) normalization was used to correct the total number of reads and gene lengths among the samples. Finally, after normalization using the DEGES normalization method in TCC (ver. 1.26.0), edgeR (ver. 3.28.1) was used to identify genes with variable expression.

2.10 | Measuring of BCAA level

Amino acid levels were quantified using a Branched Chain Amino Acid (BCAA) Assay Kit (Abcam) according to the manufacturer's instructions. Briefly, C2C12 myoblasts were cultured in a 6-well plate. Samples were washed with PBS and lysed in an assay buffer. Then, 50 μL of the reaction mixture was added to each well containing the samples, and the reaction was incubated for 30 min at room temperature. BCAA levels were quantified using colorimetric analysis on a Multiskan Go Microplate Reader (Thermo Fisher Scientific) at 450 nm and normalized by the amount of protein.

2.11 | Statistical analyses

Statistical analyses were performed using GraphPad Prism9 (Dotmatics, Inc.) Student's *t*-test was used to determine significant differences from a two-tailed distribution. For comparisons of more than two groups, a one-way analysis of variance for repeated measures was performed, followed by Dunnett's multiple comparisons tests.

3 | RESULTS

3.1 | Changes in the expression of intracellular aminopeptidase genes during myogenic differentiation

We examined the changes in gene expression of aminopeptidases during myogenic differentiation (Figure S1A). The gene expressions of myogenin and *MyHC* were significantly upregulated during myogenic differentiation (Figure S1B). No significant changes were observed in the expression of the *APP1* (Figure S1C). The expression of the *DNPEP* significantly increased on the third day after differentiation (Figure S1D). *ERAP1* significantly increased from day 1 after differentiation (Figure S1E). The expression of the *LAP3* did not differ significantly (Figure S1F), but, the expression of the *LNPEP* significantly increased from day 3 after differentiation (Figure S1G). The expression of the *METAP1* did not vary significantly (Figure S1H), whereas the expression of *METAP2* significantly increased from day 1 after differentiation (Figure S1I). Similarly, *PSA* significantly increased from day 3 after differentiation (Figure S1J). The expression of the *RNPEP*, *RNPEPL1*, and *b-actin* did not vary significantly (Figure S1K–M).

Next, we examined the changes in the protein expressions of intracellular aminopeptidases during myogenic differentiation by western blot analysis (Figure 1a,b). Similar to the mRNA expression, the protein expressions of myogenin and MyHC, as factors that induce myogenic differentiation, were significantly increased (Figure 1c). The expression of APP1 significantly increased 5 days after myogenic differentiation (Figure 1d), whereas that of DNPEP significantly decreased 3 days after myogenic differentiation (Figure 1e). The expression of ERAP1 and LAP3 were unchanged (Figure 1f,g), whereas that of LNPEP increased significantly from 3 days after myogenic differentiation (Figure 1h). The expression of METAP1 did not change significantly (Figure 1i). The expression of METAP2 significantly increased on the first day after myogenic differentiation but significantly decreased on the third day after myogenic differentiation (Figure 1j). The expression of PSA significantly increased from the third day after myogenic differentiation (Figure 1k). The expression of RNPEP significantly increased from the third day after myogenic differentiation (Figure 1l), whereas that of RNPEPL1 significantly decreased 1 day after myogenic differentiation (Figure 1m).

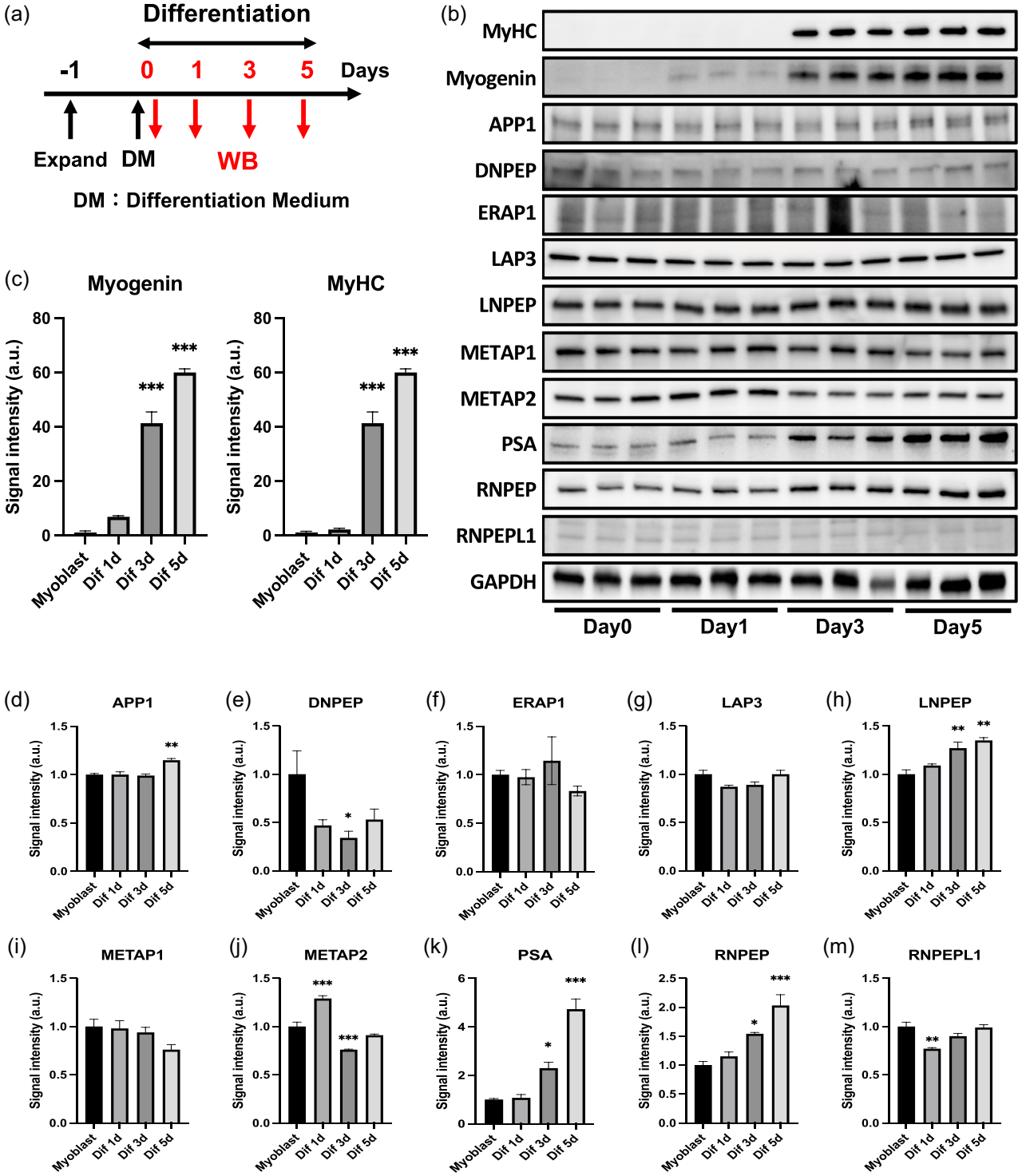

FIGURE 1 Changes in the expressions of intracellular aminopeptidases during myogenic differentiation. Changes in the expressions of aminopeptidases X-prolyl aminopeptidase 1 (APP1), aspartyl aminopeptidase (DNPEP), endoplasmic reticulum aminopeptidase 1 (ERAP1), leucine aminopeptidase 3 (LAP3), leucyl and cystinyl aminopeptidase (LNPEP), methionyl aminopeptidase 1 (METAP1), methionyl aminopeptidase 2 (METAP2), puromycin-sensitive aminopeptidase (PSA), arginyl aminopeptidase (RNPEP), and arginyl aminopeptidase like 1 (RNPEPL1) during myogenic differentiation. (a) Time course of treatment and analysis. (b–m) Western blot analysis of the levels of myosin heavy chain (MyHC), myogenin, APP1, DNPEP, ERAP1, LAP3, LNPEP, METAP1, METAP2, PSA, RNPEP, RNPEPL1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control during myogenic differentiation. Values are expressed as mean ± standard error of the mean (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Day 0 (myoblast); $n = 3$ per group).

3.2 | Impact of the knockdown of aminopeptidase genes on myogenic differentiation

We examined how the suppression of the expression of each intracellular aminopeptidase gene by siRNAs affects myogenic differentiation. The protein expression of each aminopeptidase was significantly decreased by siRNA-treated C2C12 myoblasts and myotubes (Figure S2A and S2B. The knockdown of ERAP1 or RNPEP in C2C12 myoblasts had no effect on myogenic differentiation (Figure 2c,i). In contrast, the knockdown of APP1, DNPEP, LNPEP, METAP1, METAP2, PSA, and RNPEPL1 in C2C12 myoblasts inhibited myogenic differentiation (Figure 2a, b, e–h, j). Surprisingly, the knockdown of LAP3 promoted myogenic differentiation (Figure 2d). Based on these results, we further investigated LAP3 and conducted further analysis to elucidate its molecular mechanism. Because LAP3 was the only aminopeptidase among the tested, which after siRNA inhibition resulted in the promotion of myogenic differentiation.

3.3 | Expression and localization changes of LAP3 during myogenic differentiation

We observed that the activity of leucine aminopeptidase was significantly decreased during myogenic differentiation (Figure 3a), whereas the protein level of LAP3 remained unchanged (Figure 1b,g). We then examined the localization of LAP3 in C2C12 myoblasts and myotubes 3 days after the induction of differentiation. In C2C12 myoblasts, the expression of LAP3 was mainly localized in the cytoplasm (Figure 3b, upper panel). In differentiating myotubes, LAP3 was mildly expressed both in the cytoplasm and nucleus (Figure 3b, lower panel). Additionally, LAP3 was also detected in the nuclei of C2C12 myotubes expressing GFP-tagged LAP3 (Figure 3c). Furthermore, in protein extraction of nuclear and cytoplasm fractions, LAP3 expression levels were found to be increased in the nuclear fraction of differentiated myotubes 3 days after myogenic differentiation (Figure 3d).

3.4 | LAP3 suppression induces myogenic differentiation with cell cycle dysregulation

Next, the effects of LAP3 suppression on myogenic differentiation and the changes in the expression of myogenic differentiation factors were examined. The lengths and differentiation indexes of LAP3-suppressed C2C12 myotubes significantly increased 3 days after the induction of myogenic differentiation (Figure 4a). In addition, myogenin expression significantly increased together with MyHC expression 2 days after the knockdown treatment and 1 day after the induction of differentiation in LAP3-knocked down C2C12 myoblasts (Figure 4b). In contrast, the expression of Myogenic Differentiation 1 (MyoD) was significantly decreased 1 day after the induction of differentiation in LAP3 knockdown C2C12 myoblasts (Figure S3). These results indicate that LAP3 suppression accelerated myogenesis and that LAP3 may be involved in the regulation of myogenin expression. To explore the effects of LAP3 in C2C12 myoblast proliferation, we performed cell enumeration and the evaluation of cell cycle-related protein expression in LAP3 knockdown C2C12 myoblasts. The increase in the number of cells was significantly suppressed in LAP3 knockdown C2C12 myoblasts 2–3 days after the knockdown treatment as compared to the control (Figure 4c). We performed a cell damage assay and analyzed the phosphorylation levels of retinoblastoma (Rb), cyclin D3, CDK4, P21, and cleaved caspase-3 (Cle-Cas3). Our results revealed a significant increase in cell damage (Figure 4d) and elevated levels of Rb phosphorylation and cyclin D3, CDK4, and Cle-Cas3 expression in LAP3-knockdown C2C12 myoblasts (Figure 4e). LAP3 knocked down C2C12 myoblasts underwent apoptosis and cell cycle withdrawal during myogenic differentiation.

3.5 | Effects of LAP3 overexpression on myogenic differentiation

To ascertain whether LAP3 serves as an inhibitory regulator of myogenic differentiation, we assessed the impact of LAP3 suppression on myogenic differentiation in growth media, rather than under differentiation conditions (Figure 5a). Surprisingly, we found that myogenic differentiation was enhanced in LAP3-knockdown C2C12 myoblasts cultured in GM (Figure 5b,c). To determine the effect of LAP3 overexpression on myogenic differentiation, we induced myogenic differentiation in C2C12 myoblasts overexpressing LAP3 (Figure 5d,e). However, contrary to our expectations, LAP3 overexpression had no effect on the ability of C2C12 myoblasts to undergo myogenic differentiation (Figure 5f).

3.6 | Transcriptome analysis of LAP3 knockdown myoblasts

To explore the effects of LAP3 knockdown in C2C12 myoblasts, we performed RNA sequencing (RNA-seq) of C2C12 myoblasts subjected to knockdown treatment for 48 h. We identified 528 genes that were upregulated or downregulated based on q-values of <0.05 (Figures 6a and S4). Additionally, genes that were differentially expressed significantly were imported into the DAVID annotation tool (https://david.ncifcrf.gov/summary.jsp). We subsequently performed gene ontology (GO) term enrichment analysis (biological process [GO-BP], cellular component (GO-CC), and molecular function [GO-MF]) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. GO terms (e.g., muscle contraction) related to myoblast differentiation and muscle growth were identified from the GO analysis, which reflected the previous results of LAP3 suppression induced myogenic differentiation (Figure 6b). Also, the expression of *Myf5*, a gene related to muscle differentiation, was significantly decreased, whereas those of *Mef2a*, *Myod1*, *MYOG*, and *eMyHC* (MyHC-embryonic) were significantly increased, indicating an

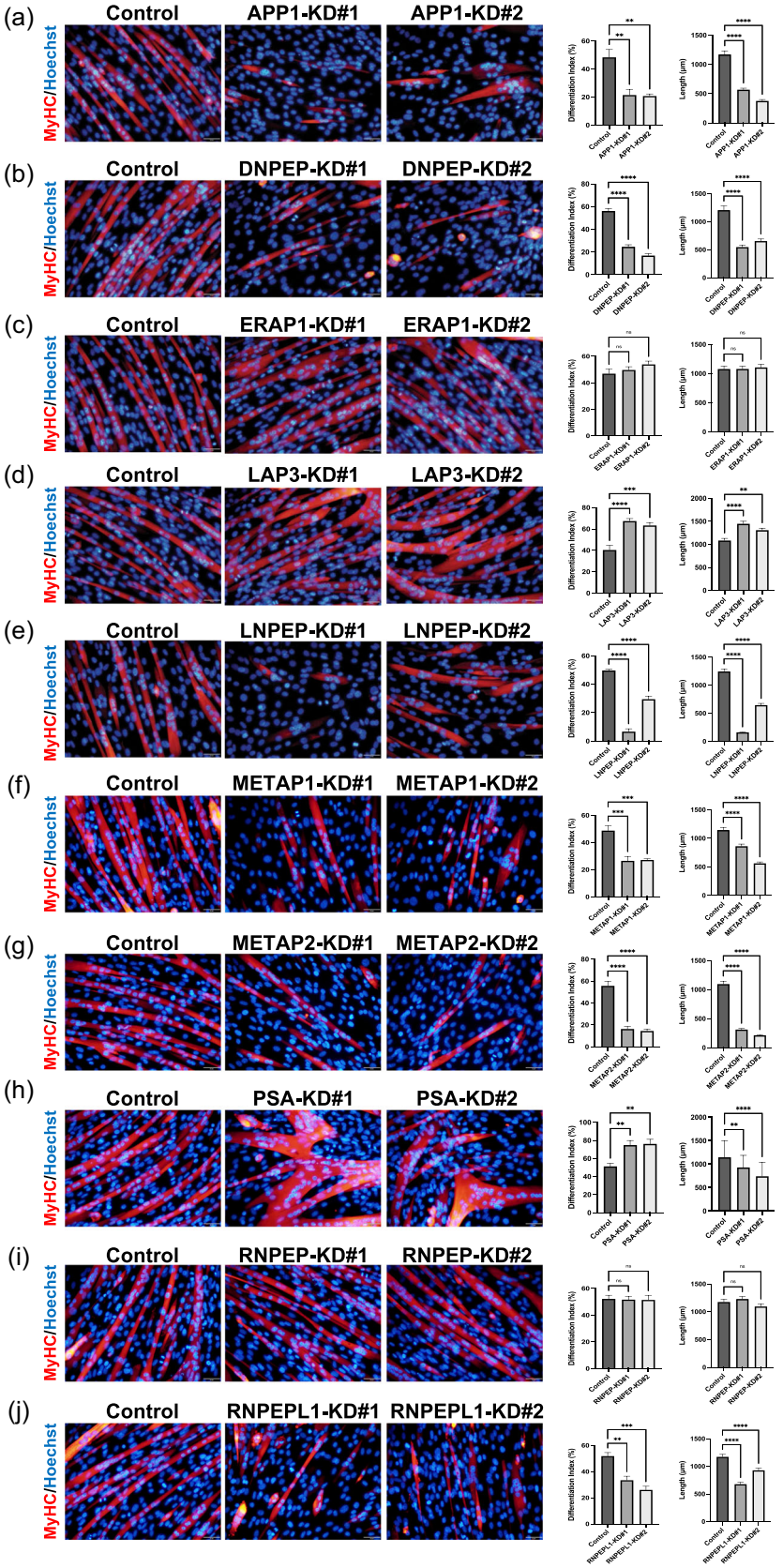

FIGURE 2 Impact of aminopeptidase gene knockdown on myogenic differentiation. Effects on myogenic differentiation of suppressing aminopeptidase gene expression (APP1-KD, DNPEP-KD, ERAP1-KD, LAP3-KD, LNPEP-KD, METAP1-KD, METAP2-KD, PSA-KD, RNPEP-KD, and RNPEPL1-KD) using two siRNA sequences (#1 and #2). (A–J) Immunocytochemistry for myosin heavy chain (MyHC, red) and nuclear marker (blue) in aminopeptidase-suppressed myotubes at 5 days after myogenic differentiation and evaluation of myogenic differentiation capacity using the differentiation index and myotube length measurement. Scale bar = 50 μm. Values are presented as mean ± standard error of the mean (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs. control; differentiation index, $n = 4$; length, $n = 5$). siRNA, small interfering RNA.

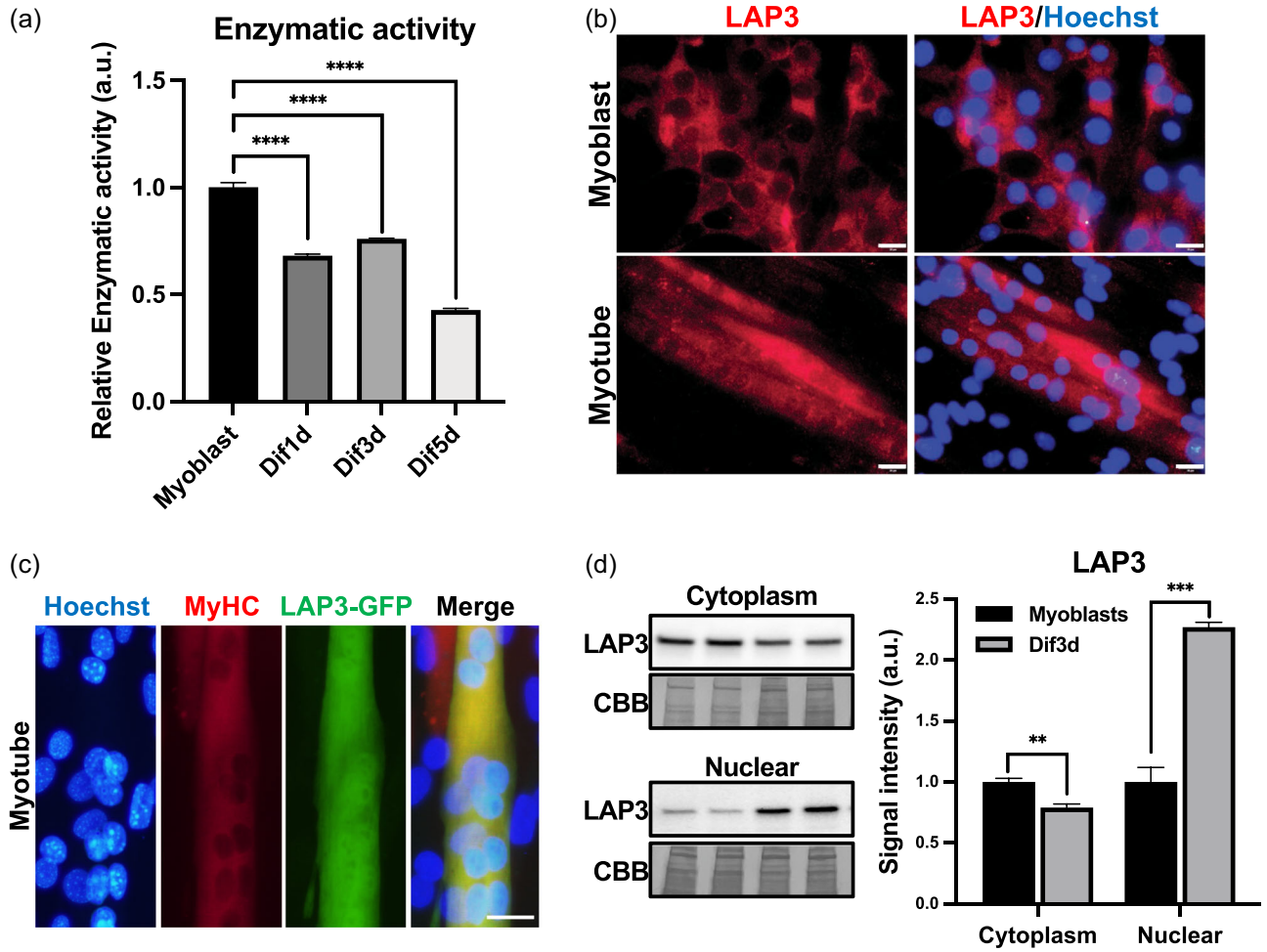

FIGURE 3 Expression and localization changes of LAP3 during myogenic differentiation. Expression changes and activity of LAP3 during myogenic differentiation. (a) Enzymatic activity of leucine aminopeptidase during myogenic differentiation. (b) Immunocytochemistry for LAP3 (red) and nuclear marker (blue) in C2C12 myoblasts and myotubes. Scale bar = 20 μm. (c) Evaluation of LAP3 localization through immunocytochemistry of myosin heavy chain (MyHC; red) and nuclear marker (blue) in LAP3-GFP-overexpressing C2C12 myotubes. Scale bar = 20 μm. (d) Evaluation of LAP3 expression levels in the cytoplasmic and nuclear fractions of C2C12 myoblasts and myotubes via western blot analysis. Values are expressed as the mean ± standard error of the mean (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs. myoblasts; $n = 3$ or 4).

enhanced myogenic differentiation (Figure 6b). To elucidate the signaling pathway by which LAP3 knockdown in myoblasts promoted myogenic differentiation, therefore, we focused on the PI3K-Akt signaling pathway (Figure 6c), which is widely known as a protein synthesis pathway and an essential pathway in the earlier stage of myogenic differentiation (Jiang et al., 1999).

3.7 | Suppression of LAP3 expression in C2C12 myoblasts enhanced AKT phosphorylation and regulated myogenin expression via the TFE3 translocation

From the results of transcriptome analysis, we evaluated the phosphorylation level of the AKT in C2C12 myoblasts after suppressing *LAP3* expression (Figure 7a). We found that the phosphorylation level of AKT(S473), but not AKT(T308), significantly increased (Figures 7b and S5A). Next, we investigated the role of AKT phosphorylation in the regulation of myogenin expression in C2C12 myoblasts by determining whether AKT inhibition suppresses the upregulation of myogenin expression in LAP3 knockdown C2C12 myoblasts. We confirmed that the phosphorylation level of AKT (S473) was significantly reduced by AKT inhibitors. The expression of myogenin was also markedly suppressed under AKT inhibition (Figures 7c and S5B). A previous study reported that transcription factor E3 (TFE3) played a regulatory role in C2C12 myoblast differentiation and that the transcriptional suppression of myogenin expression may be a part of the mechanism of action (Iwasaki et al., 2012; Naka et al., 2013). Another study found that AKT phosphorylates TFE3 and suppresses its nuclear translocation (Palmieri et al., 2017). Therefore, we hypothesized that the increased expression of myogenin in LAP3 knockdown C2C12 myoblasts is induced by the suppression of the nuclear translocation of TFE3 by AKT-induced phosphorylation. We examined the expression levels of

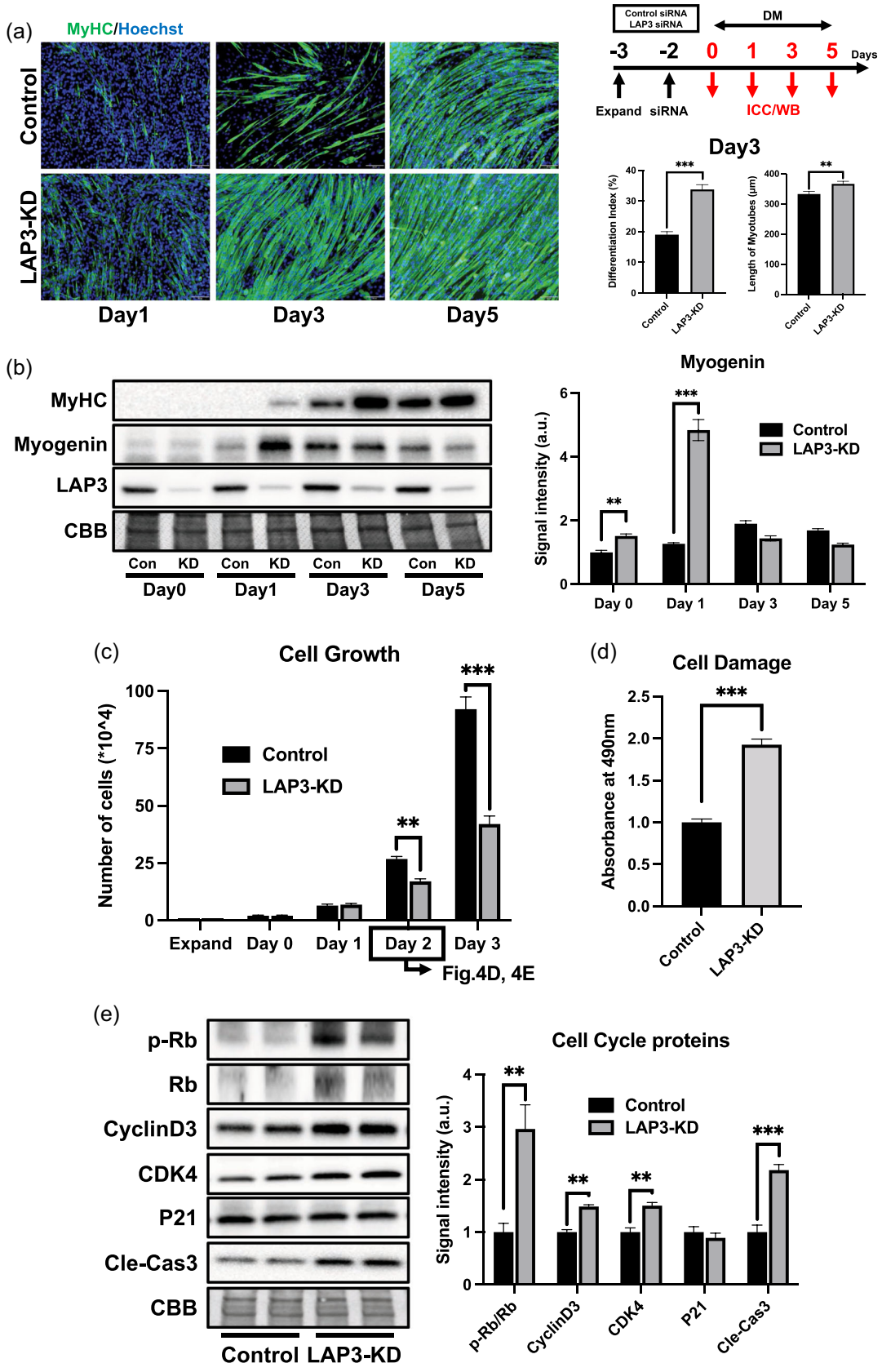

FIGURE 4 (See caption on next page).

TFE3 in the nuclear and cytoplasmic fractions in LAP3 knockdown C2C12 myoblasts and found that the levels of phosphorylated AKT and myogenin in the cytoplasm fraction were increased in LAP3 knocked down C2C12 myoblasts (Figure S6). Furthermore, as expected, while the level of TFE3 expression in the cytoplasm fraction increased, that in the nucleus fraction decreased (Figure 7d). These results suggest that the promotion of myogenic differentiation in LAP3 knocked down C2C12 myoblasts may be due to the suppression of the nuclear translocation of TFE3 by upregulated AKT phosphorylation, resulting in increased myogenin expression. These results suggest that myogenin expression is regulated and, in fact, increased by AKT phosphorylation in LAP3 knocked down C2C12 myoblasts.

3.8 | Effects of LAP3 expression suppression on the ubiquitin–proteasome system

The phosphorylation of AKT(S473) is considered a major factor in cell metabolism. Therefore, to gain an indepth understanding of the molecular mechanism linking LAP3 and the AKT pathway, we investigated the metabolic conditions, including changes in the expression of proteasome-related proteins and intracellular amino acid levels. We found that the expression of Atrogin1 (also known as MAFbx1) and MuRF1 (also known as TRIM63), which are E3 ubiquitin ligases, was significantly decreased in LAP3-knockdown C2C12 myoblasts (Figures 8a and S7A), whereas that of ubiquitin was significantly increased (Figures 8a and S7A). Furthermore, in LAP3-knockdown C2C12 myoblasts, branched-chain amino acid (BCAA) levels were significantly decreased (Figures 8b and S7B). These findings suggest that intracellular BCAA supply may be mediated by protein and peptide degradation through proteasomes and LAP3 activities.

4 | DISCUSSION

We evaluated the effects on myogenic differentiation of the suppression of aminopeptidase gene expression in C2C12 myoblasts and investigated the role of aminopeptidases in myogenic differentiation of C2C12 myoblasts. Unlike the results observed with the suppression of the other aminopeptidases in which myogenic differentiation was inhibited, the suppression of LAP3, which selectively cleaves leucine residues from the N-terminus of several peptide substrates (Tsujimoto et al., 2008), promoted myogenic differentiation of C2C12 myoblasts. Therefore, we further investigated the role of LAP3.

In the present study, LAP3 knockdown C2C12 myoblasts showed significantly increased myogenin expression, and significantly decreased MyoD expression, suggesting that LAP3 knockdown promoted myogenic differentiation in C2C12 myoblasts through the regulation of myogenin expression. In this study, it was observed that the amount of LAP3 did not change during myogenic differentiation. There was, however, a striking difference in the localization of LAP3. Under undifferentiated conditions, LAP3 showed a predominant cytoplasmic localization, whereas when differentiation was induced and myotubes are formed LAP3 localized both in the cytoplasm and in the nuclei of C2C12. Additionally, leucine aminopeptidase activity was found to be decreased during myogenic differentiation. These findings suggest that LAP3 is regulating myogenin expression through the changes in its subcellular localization and the reduction of its activity during myogenic differentiation. Conversely, a recent study in sheep embryonic myoblasts found that suppression of LAP3 expression inhibited myogenic differentiation (Ge et al., 2022), indicating that the regulatory mechanism of LAP3 may vary across species. It is generally accepted that the differentiation ability of C2C12 myoblasts has distinct characteristics from primary myoblasts and satellite cells (Grabowska et al., 2011). Further experiments using primary cultures are warranted to investigate these cell-type-specific differences in LAP3 function.

Myogenic differentiation is induced by cell cycle withdrawal in myoblasts. In LAP3 knockdown C2C12 myoblasts, the expression of cell cycle-promoting molecules, such as Rb, cyclin D3, and CDK4, was increased, but a significant growth inhibition accompanying apoptotic cell death was observed. Previous studies have reported that Rb is required in myogenic differentiation and activates the myogenic transcriptional program (Endo & Goto, 1992; Zappia et al., 2019). Cyclin D3, which promotes the cell cycle from the G1 phase to the S phase, has also been suggested to contribute to promoting

FIGURE 4 Suppression of LAP3 induces myogenic differentiation with cell cycle dysregulation. Effect of LAP3 knockdown (LAP3-KD) on myogenic differentiation and cell proliferation capacity in C2C12 myoblasts. (a) Immunocytochemistry for myosin heavy chain (MyHC, green) and nuclear marker (blue) in LAP3-suppressed myotubes at 3 days after myogenic differentiation and evaluation of myogenic differentiation capacity using the differentiation index and length measurement. Scale bar = 100 μm. (b) Evaluation of time-dependent changes in myogenic differentiation-related factors (MyHC and Myogenin) and LAP3 during myogenic differentiation by western blot analysis in LAP3-knocked down C2C12 myoblasts. (c) Evaluation of cell growth at 0, 1, 2, and 3 days after knockdown of LAP3 in C2C12 myoblasts. (d) Assessment of the cytotoxicity levels in C2C12 myoblasts after LAP3 knockdown for 2 days by cell damage assay. (e) Changes in the expression of cell cycle-related molecules (Ser807/811-phosphorylated retinoblastoma [p-Rb], Rb, cyclin D3, CDK4, P21, cleaved caspase-3 [Cle-Cas3]) in C2C12 myoblasts evaluated by western blot analysis after LAP3 knockdown for 2 days. (**$p < 0.01$, ***$p < 0.001$, vs. control; western blot analysis, $n = 3$; differentiation index, $n = 4$; length, $n = 50–70$ myotubes).

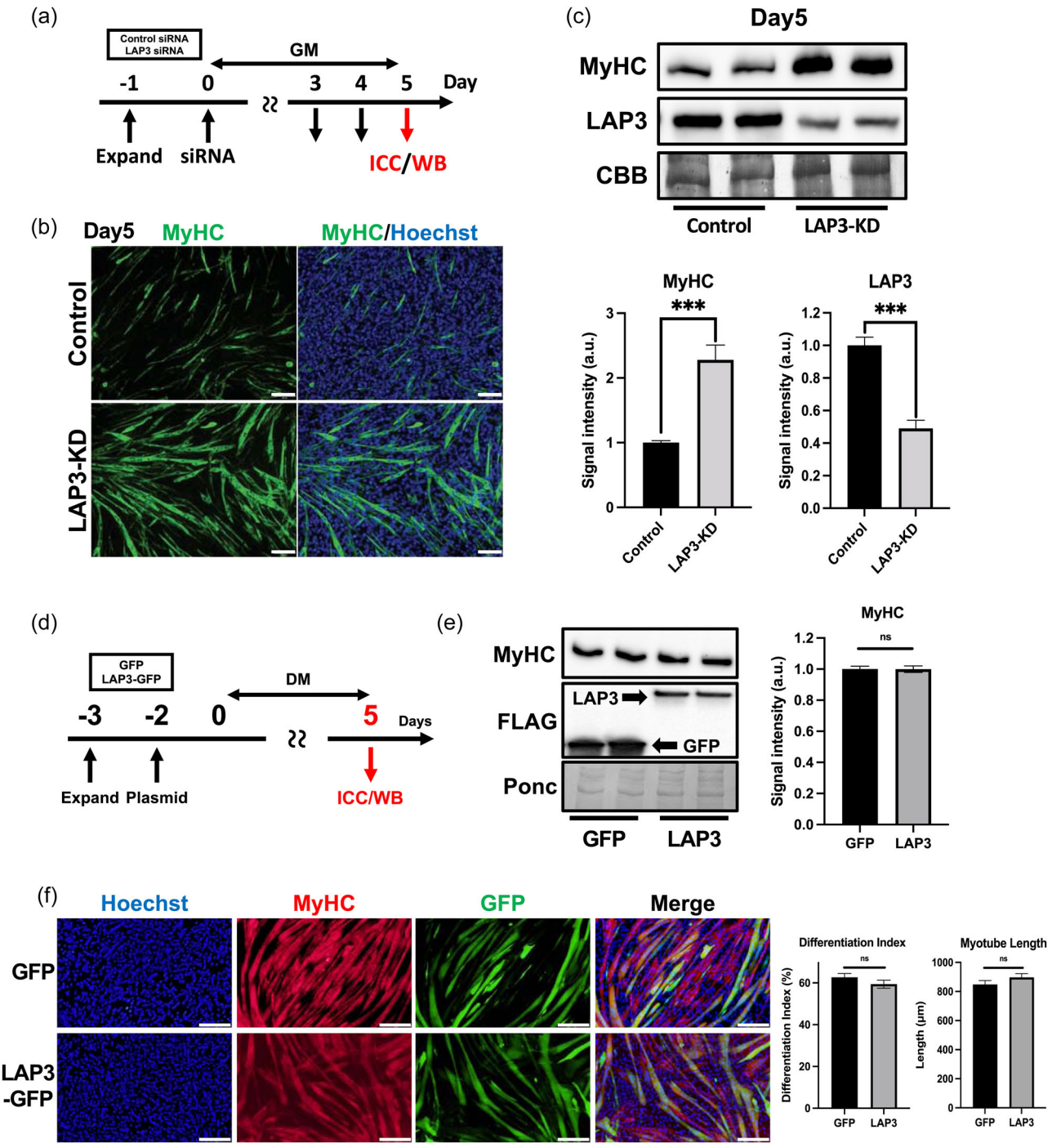

FIGURE 5 Effects of LAP3 overexpression on myogenic differentiation. Evaluation of LAP3 as a regulator of myogenic differentiation through suppression or overexpression of LAP3 in C2C12 myoblasts. (a) Time course of treatment and analysis of the effect of LAP3 knockdown in C2C12 myoblasts on myogenic differentiation under growth medium conditions. (b) Immunocytochemistry for myosin heavy chain (MyHC; green) and nuclear marker (blue) in LAP3-suppressed myotubes at 5 days of culture in growth media. Scale bar = 100 μm. (c) Expression changes of MyHC and LAP3 based on western blot analysis in LAP3-suppressed myotubes at 5 days of culture in growth media. (d) Time course of processing and analysis of the effect of LAP3 overexpression on myogenic differentiation in C2C12 myoblasts. (e) Evaluation of MyHC expression via western blot analysis in LAP3-overexpressing myotubes. (f) Immunocytochemistry for MyHC (green) and nuclear marker (blue) in LAP3-overexpressed myotubes at 5 days after myogenic differentiation, and evaluation of myogenic differentiation capacity using the differentiation index and length measurement. Scale bar = 100 μm. Values are expressed as the mean ± standard error of the mean (***$p < 0.001$ vs. control or GFP; western blot analysis, $n = 3$; differentiation index, $n = 3$; length, $n = 90$ myotubes).

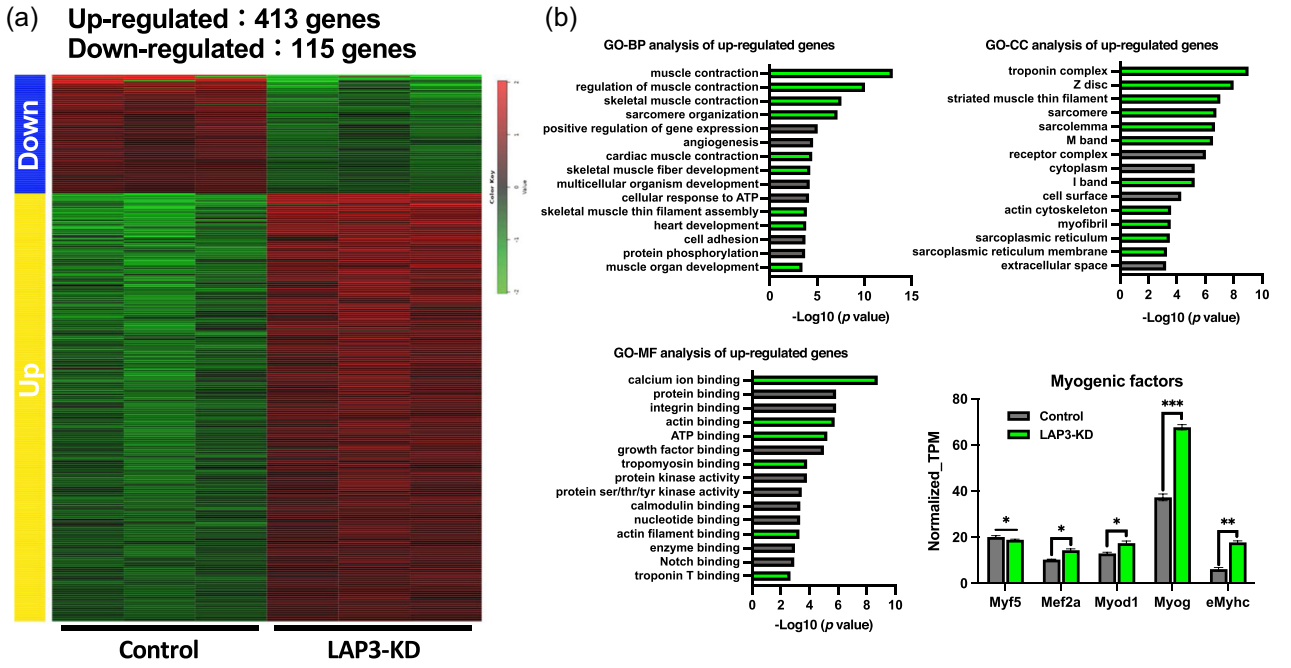

FIGURE 6 Transcriptome analysis of LAP3 knockdown C2C12 myoblasts. Verification of the effect of suppressing LAP3 expression on comprehensive gene expression in C2C12 myoblasts by RNA-Seq analysis. (a) Heat map showing the expression changes of genes (412 upregulated and 117 downregulated with q-values of <0.05) in LAP3 knockdown C2C12 myoblasts. (b) Gene Ontology (GO) term-enrichment analysis (Biological Process [GO-BP], Cellular Component [GO-CC], Molecular Function [GO-MF]) of LAP3 knockdown C2C12 myoblasts. The green graph shows a group of genes related to myogenesis. (c) The data show the top 15 out of 41 gene categories resulting from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of LAP3 knockdown C2C12 myoblasts. Values are expressed as mean ± standard error of the mean (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, vs. control; $n = 3$).

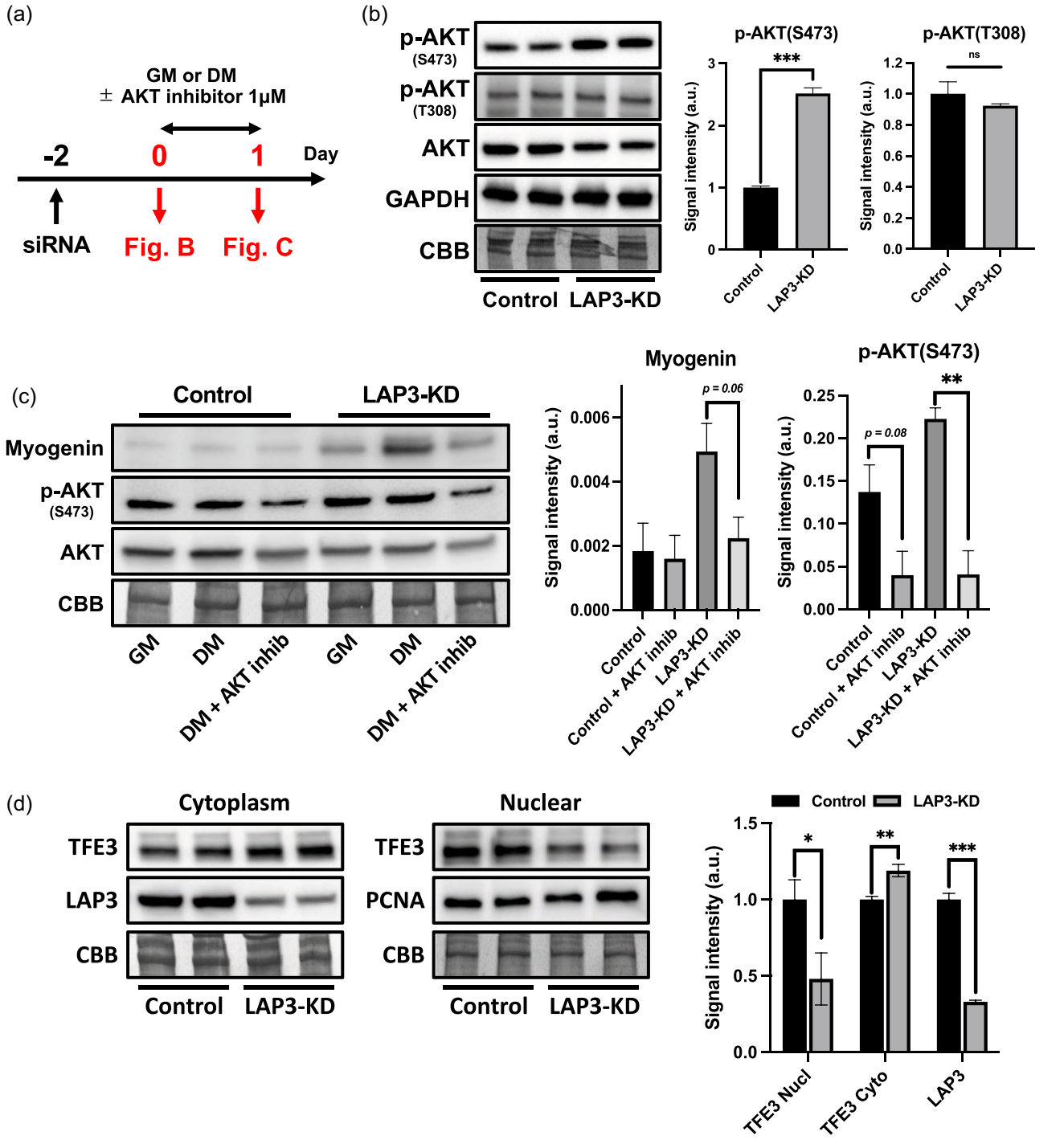

FIGURE 7 Suppression of LAP3 expression in C2C12 myoblasts enhanced AKT phosphorylation and regulated myogenin expression via the TFE3 translocation. Effects of the suppression of LAP3 in C2C12 myoblasts on the AKT Pathway. (a) Time course of treatment and analysis to assess the effect of LAP3 knockdown in C2C12 myoblasts on AKT phosphorylation and myogenin expression. (b) Evaluation of Ser473 and Thr308 phosphorylation level of AKT (p-AKT) by western blot analysis in LAP3 knockdown C2C12 myoblasts. (c) Changes in p-AKT levels and myogenin expression in LAP3 knockdown C2C12 myoblasts caused by AKT inhibitors. (d) Changes in the TFE3 expression levels in the cytoplasm and nucleus as evaluated by western blot analysis in LAP3 knockdown C2C12 myoblasts. Values are expressed as mean ± standard error of the mean (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, vs. control; $n = 3$).

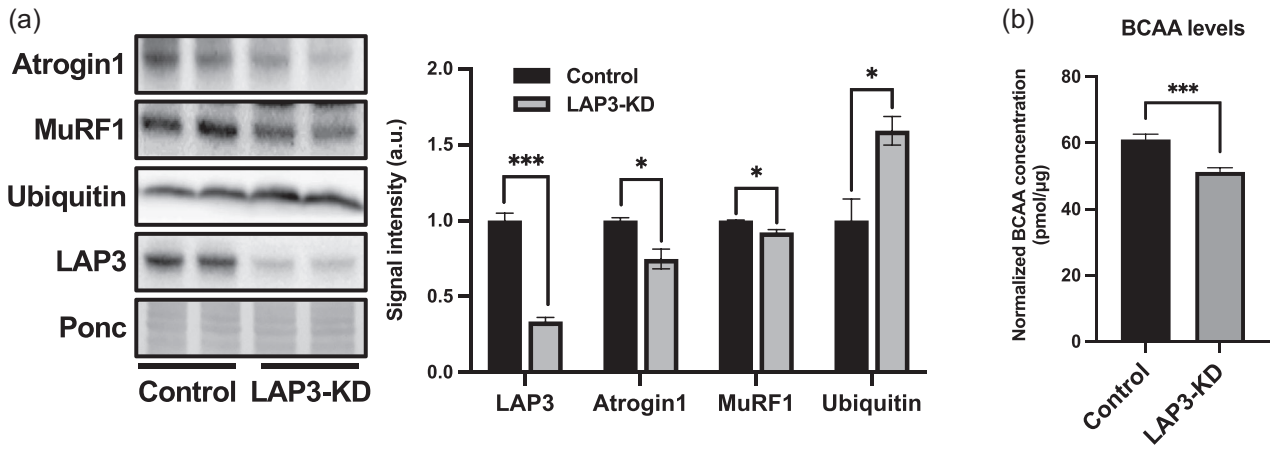

FIGURE 8 Effects of LAP3 expression suppression on the ubiquitin–proteasome system. Effects of suppressing of LAP3 expression in C2C12 myoblasts on the proteasome and intracellular amino acid levels. (a) Evaluation of the expression level of Atrogin1, MuRF1, and ubiquitin through western blot analysis in LAP3-knockdown C2C12 myoblasts. (b) Assessment of the intracellular amino acid (BCAA) levels in LAP3-knockdown C2C12 myoblasts. Values are expressed as the mean ± standard error of the mean (*$p < 0.05$, ***$p < 0.001$ vs. control; western blot analysis, $n = 3$; amino acid assay, $n = 4$).

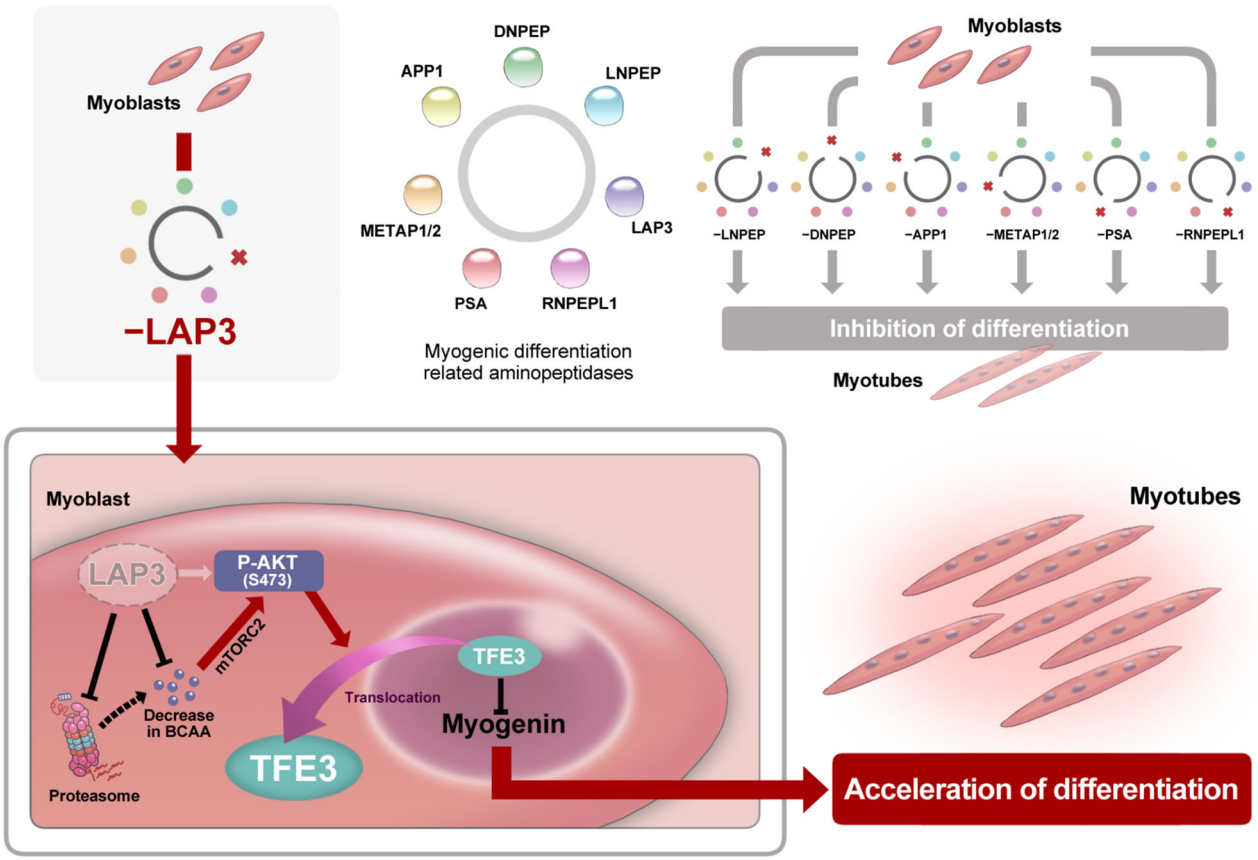

FIGURE 9 Schematic illustration of the effect of aminopeptidase gene knockdown on myogenic differentiation. We identified APP1, DNPEP, LAP3, LNPEP, METAP1, METAP2, PSA, and RNPEPL1 as the aminopeptidases that related to the myogenic differentiation of C2C12 myoblasts. Suppression of LAP3 expression in C2C12 myoblasts resulted in the inhibition of proteasomal proteolysis and a decrease in intracellular amino acid levels (especially BCAA). Additionally, it enhanced mTORC2-mediated AKT phosphorylation (S473). Furthermore, AKT induced translocation of TFE3 from the nucleus to the cytoplasm, promoting myogenic differentiation through increased expression of myogenin.

myogenesis, unlike cyclin D1 (Gurung & Parnaik, 2012). In addition, the increased expression of Cle-Cas3 and concomitant apoptotic cell death are known to occur during myogenic differentiation (Fernando et al., 2002). Together with the previous observation it is reasonable that LAP3 knocked down C2C12 myoblasts exhibited accelerated myogenic differentiation rather than enhanced cell cycle progression. In fact, LAP3 knocked down C2C12 myoblasts-induced myogenic differentiation even when cultured in a growth medium without providing conventional induction of myogenic differentiation, suggesting that LAP3 may function as a switching enzyme for myogenic differentiation. However, overexpressed LAP3 in C2C12 myoblasts did not affect myogenic differentiation; therefore, further analysis of the regulatory mechanism underlying myogenic differentiation by LAP3 is needed. It is possible that GFP-tagged LAP3 may not have effectively translocated into nuclei or may have some interference in the interaction with TFE3.

We performed pathway analysis to clarify the regulatory mechanism of myogenic differentiation by LAP3 and found that the calcium signaling pathway was the prominent pathway. Skeletal muscle contraction is achieved by the release of calcium contained in the sarcoplasmic reticulum into the cytosol (Hernández-Ochoa et al., 2015), considering that the expression of calcium-related molecules will be more relevant in the later phase of differentiation or maturation of myotubes. Therefore, we focused on the AKT pathway, which is widely known as a protein synthesis pathway and an essential pathway in the earlier stage of myogenic differentiation (Jiang et al., 1999), but not the calcium signaling pathway. In the present study, it was observed that the phosphorylation level of AKT(S473) was enhanced, whereas that of AKT(T308) remained unchanged in LAP3-knockdown C2C12 myoblasts. The phosphorylation of AKT(S473) is known to be regulated by mTORC2 (Szwed et al., 2021), it has been reported that mTORC2 is activated by intracellular metabolic abnormalities (Moloughney et al., 2016; Szwed et al., 2021). In a previous study, we demonstrated that Bestatin-methyl-ester, a cell membrane-permeable aminopeptidase inhibitor, reduced the amount of amino acids in C2C12 myoblasts (Osana et al., 2020). Therefore, we hypothesized that LAP3 is involved in the maintenance of intracellular metabolism through peptide degradation. In this study, the protein expression levels of Atrogin1 and MuRF1, which are E3 ligases associated with the proteasome, and intracellular BCAA levels were significantly decreased in LAP3-knockdown C2C12 myoblasts. These findings suggest that LAP3 plays a role in the regulation of proteolysis by proteasomes and could potentially contribute to amino acid supply through peptide degradation. Based on these results, we propose that LAP3 may be involved in the regulation of the AKT pathway through proteasome-mediated proteolysis and regulation of intracellular amino acid levels. To the best of our knowledge, this is the first report in which LAP3 is involved in the AKT pathway responsible for myogenic differentiation.

We further investigated the regulatory mechanism of myogenin expression by AKT. Previous studies have reported that TFE3 plays a regulatory role in the transcriptional repression of myogenin during myoblast differentiation (Iwasaki et al., 2012; Naka et al., 2013). In addition, transcriptional repression by TFE3 is regulated by the nuclear translocation of TFE3, and the phosphorylation of TFE3 by AKT suppresses nuclear translocation (Palmieri et al., 2017). We hypothesized that this pathway is also involved in LAP3 knockdown C2C12 myoblasts and examined the localization of TFE3. We found that TFE3 expression in the nuclear was decreased, whereas that in the cytoplasm was increased, indicating that the transcriptional repression of myogenin by TFE3 may be alleviated by upregulating the level of AKT phosphorylation, which initiated the process of myogenic differentiation in LAP3 knockdown C2C12 myoblasts. A literature search on BioGRID (https://thebiogrid.org/) revealed that TFE3 and LAP3 could directly interact (Villegas et al., 2019), thus suggesting that AKT may be a regulator of interaction between TFE3 and LAP3. However, it is untested the interactions with TFE3 and LAP3 in the present study, so further studies are needed.

In conclusion, our findings reveal that suppression of LAP3 expression in C2C12 myoblasts results in the inhibition of proteasomal proteolysis and a decrease in the levels of intracellular amino acids, particularly BCAAs, along with enhanced mTORC2-mediated AKT phosphorylation (S473). Translocation of TFE3 from the nucleus to the cytoplasm may have promoted myogenic differentiation through the upregulation of myogenin (Figure 9). These results provide compelling evidence that LAP3 serves as a key regulator of myogenic differentiation in C2C12 myoblasts. How LAP3 activity with its localization is physiologically regulated will be further investigated in the context of amino acid metabolism.

AUTHOR CONTRIBUTIONS

S. Osana designed the experiments, performed the experiments, interpreted the data, assembled the input data, and wrote the manuscript. Y. Kitajima, N. Suzuki, and Y. Kano interpreted the data. H. Takada, T. Ayaka, and K. Murayama performed the experiments. R. Nagatomi interpreted the data, and assembled the input data. All authors discussed the results and implications and commented on the manuscript.

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CONFLICT OF INTERESTS STATEMENT

The authors declare that there is no conflict of interest.

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SUPPORTING INFORMATION

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