XBP1u Is Involved in C2C12 Myoblast Differentiation via Accelerated Proteasomal Degradation of Id3

Satoko Hayashi1, Shotaro Sakata2, Shotaro Kawamura2, Yukako Tokutake1,† and Shinichi Yonekura1,2,3,*

1 Graduate School of Medicine, Science and Technology, Shinshu University, Nagano, Japan, 2 Graduate School of Science and Technology, Shinshu University, Nagano, Japan, 3 Department of Biomolecular Innovation, Institute for Biomedical Sciences, Shinshu University, Nagano, Japan

Myoblast differentiation is an ordered multistep process that includes withdrawal from the cell cycle, elongation, and fusion to form multinucleated myotubes. Id3, a member of the Id family, plays a crucial role in cell cycle exit and differentiation. However, in muscle cells after differentiation induction, the detailed mechanisms that diminish Id3 function and cause the cells to withdraw from the cell cycle are unknown. Induction of myoblast differentiation resulted in decreased expression of Id3 and increased expression of XBP1u, and XBP1u accelerated proteasomal degradation of Id3 in C2C12 cells. The expression levels of the cyclin-dependent kinase inhibitors p21, p27, and p57 were not increased after differentiation induction of XBP1-knockdown C2C12 cells. Moreover, knockdown of Id3 rescued myogenic differentiation of XBP1-knockdown C2C12 cells. Taken together, these findings provide evidence that XBP1u regulates cell cycle exit after myogenic differentiation induction through interactions with Id3. To the best of our knowledge, this is the first report of the involvement of XBP1u in myoblast differentiation. These results indicate that XBP1u may act as a “regulator” of myoblast differentiation under various physiological conditions.

Keywords: skeletal muscle differentiation, cell cycle exit, unfolded protein response, Id3, cyclin-dependent kinase inhibitor

INTRODUCTION

Myoblast differentiation is an ordered multistep process that includes withdrawal from the cell cycle and the expression of key myogenic factors leading to fusion into multinucleated myotubes (Stockdale, 1992). Progression through the cell cycle phases is dependent on consecutive activation and inhibition of phosphoproteins by cyclin-dependent kinases (CDKs) complexed with associated activatory cyclins (Harashima et al., 2013). Cyclin-dependent kinase inhibitors (CKIs) are negative cell cycle regulators (Vidal and Koff, 2000). Based on their sequence homology and specificity of action, CKIs are divided into two distinct families as follows: INK4 and Cip/Kip (Sherr and Roberts, 1999). Cip/Kip members, i.e., p21Cip (p21), p27Kip (p27), and p57Kip2 (p57) share a conserved N-terminal domain that mediates binding to cyclins and inhibit a broader spectrum of cyclin–CDK complexes (Harper et al., 1993; Poljak et al., 1994; Lee et al., 1995). CKIs inhibit CDK activity, and contribute to the cell cycle exit (Tane et al., 2014). In muscle cell differentiation, the CDK-cyclin complex is downregulated, while the expression of p21 is increased (Fujio et al., 1999;
differentiation. Degradation of XBP1s (Yoshida et al., 2006). However, relatively termination of UPR responses by mediating the proteasomal domain and can bind XBP1s. Therefore, XBP1u controls the transcription activation domain. XBP1u contains a degradation muscle development (Lazaro et al., 1997) is regulated by XBP1s. Furthermore, the expression of cyclin-dependent kinase 5 (CDK5), intimately involved in skeletal differentiation induction. Furthermore, the expression of cyclin- apoptosis and autophagy occurring in the first 24 h after myogenesis (Tokutake et al., 2019). XBP1s are involved in cell differentiation (Park et al., 2021). Indeed, our previous study demonstrated that XBP1s regulates the early stages of myogenesis (Tokutake et al., 2019). XBP1s are involved in apoptosis and autophagy occurring in the first 24 h after differentiation induction. Furthermore, the expression of cyclin-dependent kinase 5 (CDK5), intimately involved in skeletal muscle development (Lazaro et al., 1997) is regulated by XBP1s. However, XBP1u consists of a hydrophobic stretch and lacks the transcription activation domain. XBP1u contains a degradation domain and can bind XBP1s. Therefore, XBP1u controls the termination of UPR responses by mediating the proteasomal degradation of XBP1s (Yoshida et al., 2006). However, relatively few studies have investigated the role of XBP1u in cellular differentiation.

Therefore, the aim of this study was to investigate the potential role of XBP1u in Id3-mediated cell exit and inhibition of C2C12 myogenic differentiation. The results showed that Id3 expression was decreased, while XBP1u expression was increased after induction of muscle differentiation and XBP1u accelerated the degradation of Id3 via the proteasomes of C2C12 cells. Knockdown of Id3 rescued myogenic differentiation of XBP1-knockdown C2C12 cells. These finding suggest that XBP1u plays an important role in switching from the undifferentiated to differentiated state by targeting Id3 for degradation.

**MATERIALS AND METHODS**

**Reagents**

Dulbecco's modified Eagle medium (DMEM) was purchased from Invitrogen (Grand Island, NY, United States). Fetal bovine serum (FBS) was purchased from EQUITECH-BIO (Cotton Gin Lane, TX, United States). Horse serum was obtained from Thermo Scientific (Waltham, MA, United States). Precast, 4-20% Mini-PROTEAN TGX gels and Polyvinylidene fluoride (PVDF) membranes were obtained from Bio-Rad (Hercules, CA, United States). MG132, Cycloheximide (CHX), Id3 siRNA, and non-targeting control siRNA were purchased from Sigma-Aldrich (Saint Louis, MO, United States). All other compounds were purchased from Nacalai Tesque (Kyoto, Japan).

**Antibodies**

For immunoblotting analysis, the following antibodies were used: anti-Myosin 4 (MF20) monoclonal antibody (Thermo Scientific), anti-Flag (catalog number: PM020) rabbit polyclonal antibody (Aldrich), anti-p21 (catalog number: sc-6246), anti-cyclin D1 (catalog number: sc-8396) mouse monoclonal antibody (Santa Cruz Biotechnology), anti-α-tubulin (catalog number: PM054), antimouse or anti-rabbit IgG HRP-linked whole Ab (GE Healthcare, Chicago, IL, United States).

**Plasmids**

The XBP1u expression plasmid was generated using standard DNA techniques. Mouse cDNA encoding Id3 (GenBank accession number NM_008321) was amplified from total RNA of skeletal muscle tissue by RT-PCR using a sense primer (5′-TCCCTCTCTATCTCTACTCCAAC-3′) and an antisense primer (5′-AGTCCTCGAGGTCCAGC-3′). Flag-Id3 was produced by cloning into a pFLAG-CMV expression vector (Sigma-Aldrich). The nucleotide sequences of PCR products and construct were verified by sequencing. The sequence was analyzed using the basic local alignment search tool (BLAST).

**Cell Culture**

C2C12 mouse myoblast cell line (DS Pharma Biomedical, Osaka, Japan) and previously generated XBP1-knockdown cell lines
FIGURE 1 | XBP1u expression is increased after differentiation induction. (A) XBP1s and XBP1u protein expression levels in C2C12 cells during differentiation. C2C12 cells were treated with the proteasome inhibitor MG132 (20 µM) for 2 h prior to lysate preparation. Protein levels of each XBP1 isoform and were detected by western blot analysis. *Non-specific band. Left panel: Representative images of three independent experiments are shown. Middle panel: Quantification of XBP1 isoform expression obtained from three independent experiments and normalized to α-tubulin as represented in the bar graph. The results are presented as the mean ± SEM (n = 3). Student’s t-test. ‡p < 0.05 vs. 0 h of each group. Right panel: Protein level of myosin heavy-chain (MHC) was detected via western blot analysis. Representative images of three independent experiments are shown. (B) Relative expression of XBP1s, XBP1u, Myogenin, and Myh1 mRNA were (Continued)
were cultured as described previously (Tokutake et al., 2019). Briefly, the cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum under an atmosphere of 5% CO₂/95% air at 37°C. Primary mouse myoblasts were isolated from 4 week-old C57bl/6J mice (Japan SLC, Hamamatsu, Japan). Undifferentiated myoblasts were maintained in growth medium consisting of DMEM supplemented with 2% horse serum. To assess degradation of Id3 by XBP1u, C2C12 cells were co-transfected with p cDNA3.1(-)-XBP1u and a Flag-Id3 construct using Lipofectamine 2000 reagent (Invitrogen Corporation, Carlsbad, CA, United States) in accordance with the manufacturer’s protocol. At 24 h post transfection, the cells were immunostained using an anti-muscle heavy-chain antibody (green). Nuclei were stained with DAPI (blue). Representative fluorescent images of the primary mouse myoblasts treated with control siRNA or XBP1 siRNA in differentiation medium for 72 h. Scale bar = 400 µm. The images below show larger magnification views of boxed regions. Scale bar = 50 µm. Right panel: The fusion index was calculated. The data are representative of four independent experiments. Ten views were analyzed for each experiment. Mann–Whitney U test. ***p < 0.001 vs. the control group.

For the knockdown experiment, XBP1u-knockdown cells or primary mouse myoblasts were seeded in growth medium at a confluence of 40–50% in the wells of a 6-well plate and incubated overnight at 37°C. The next day, the cells were transfected with either small interfering RNA (siRNA) against Id3 or control siRNA using Lipofectamine 2000 reagent in accordance with the manufacturer’s protocol. For induction of myogenic differentiation, at 6 h post transfection, the cells were cultured in differentiation medium.

**RNA Extraction and Quantitative Real-Time PCR**

Total RNA was isolated from C2C12 cells, XBP1u-knockdown cells or primary mouse myoblasts using the TRIzol reagent (Invitrogen) following the manufacturer’s instructions. The concentration of total isolated RNA was determined by optical density measurements at 260 nm and its purity was measured at a wavelength ratio of 260/280 nm (1.85–2.0 was the acceptable value range) using a spectrophotometer (NanoDrop One Spectrophotometer, Thermo Scientific). The cDNA was synthesized from total RNA using a qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). Quantitative real-time PCR was
FIGURE 3 | XBP1u proteins were co-immunoprecipitated with Id3 and ectopic XBP1u decreased the level of ectopic Id3 in C2C12 cells. (A) Co-immunoprecipitation of ectopic XBP1u with ectopic Flag-Id3 from cell lysates. C2C12 cells were co-transfected with pcDNA3.1-XBP1u and Flag-Id3 expression plasmids, then treated with MG132 (20 µM) for 6 h before lysate preparation. The lysates were immunoprecipitated with anti-Flag antibodies and, as a negative control, with rabbit IgG. The immunoprecipitates were subjected to western blot analysis with antibodies against Id3 or FLAG. Aliquots of the lysates (input) were similarly subjected to western blot analysis. Representative images of three independent experiments are shown.

(B) Ectopic XBP1u decreased the levels of ectopic Id3 proteins in C2C12 cells in a dose-dependent manner. C2C12 cells in 3.5-cm dishes were transfected with 2 µg of Flag-Id3 expression plasmids and, if indicated, with pcDNA3.1-XBP1u. After 36 h of incubation, the cells were lysed in radioimmunoprecipitation assay buffer, and the levels of Id3 and α-tubulin (internal control) were determined by western blot analysis. Left panel: Representative images of three independent experiments are shown. Right panel: Quantification of Id3 expression obtained from three independent experiments and normalized to α-tubulin as represented in the bar graph. The results are presented as the mean ± SEM (n = 3), Tukey–Kramer test. Means with different letters are significantly different, p < 0.05.

(C) Xbp1u accelerated proteosomal degradation of ectopic Id3-Flag in C2C12 cells. C2C12 cell were co-transfected with pcDNA3.1-XBP1u and Flag-Id3 expression plasmids. After 24 h, the cultures were digested with trypsin/EDTA, pooled, replated, and incubated for a further 24 h. Then, the cells were treated with CHX (100 µg/ml) with or without MG132 (20 µM) and incubated for the indicated times. The cells were lysed and the levels of Id3-Flag and α-tubulin (internal control) were determined by western blot analysis. Representative images of three independent experiments are shown.

performed using SYBR Premix Ex Taq TM II (TaKaRa Bio Inc., Shiga, Japan). Relative expression was normalized to TATA-binding protein (Tbp) or Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) gene expression. The following primers were used: Tbp F: 5’-cattctcaacctcgtacctgc-3’, R: 5’-CAGCCAAAGATCAGGTGATACA-3’; Gapdh F: 5’-TTGTGATGGGTGTAACAGAGG-3’, R: 5’-CATGACACCTCACAATGCAA-3’; Xbp1s F: 5’-TCCATGACGAGG-3’, R: 5’-TGAGAACCAGGA-3’; Tbp F: 5’-CAGCCAAAGATCAGGTGATACA-3’, R: 5’-CATGACACCTCACAATGCAA-3’; Xbp1u F: 5’-TGAGAACCAGGA-3’, R: 5’-TCCATGACGAGG-3’; Cca3 F: 5’-TTGTGATGGGTGTAACAGAGG-3’, R: 5’-CATGACACCTCACAATGCAA-3’; Myogenin F: 5’-TCAAGTTCTCTGTTGAGGACAT-3’, R: 5’-TCAGCTAATTCCCTCGGTG-3’; Myh1 F: 5’-AGAGCCAAAGAGAGCACCTGCTGACG-3’, R: 5’-CTCTCGTGAGAGGACAT-3’; p21/Cdkn1a F: 5’-GCAGACAGAGAGCAGATCCTG-3’, R: 5’-GAGGATGACGAGGTATTCCCTAG-3’; p27/Cdkn1b F: 5’-CTCGTGTAAGGTAGTGTGTC-3’, R: 5’-CCAGGCGCATGAGTTCGTTG-3’; p57/Cdkn1c F: 5’-GCAGAAAGAGGACATGTCG-3’, R: 5’-CCAAGTGAGAGGACATGTCG-3’; Myh1 F: 5’-AGAGCCAAAGAGAGCACCTGCTGACG-3’, R: 5’-CTCTCGTGAGAGGACAT-3’; Myh1 F: 5’-TCAAGTTCTCTGTTGAGGACAT-3’, R: 5’-TCAGCTAATTCCCTCGGTG-3’; Myh1 F: 5’-AGAGCCAAAGAGAGCACCTGCTGACG-3’, R: 5’-CTCTCGTGAGAGGACAT-3’; Relative transcript expression was calculated by the 2−ΔΔCt method (Pfaffl, 2001) and represented as relative values to control or 0 h.
**Co-immunoprecipitation and Immunoblotting**

The cells were harvested and lysed in RIPA lysis buffer [50 mM Tris–HCl (pH 7.4) containing 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and 1 × protease inhibitor cocktail (Nacalai Tesque)] to prepare protein extracts. For coimmunoprecipitation, a Pierce Crosslink magnetic IP and Co-IP kit (Thermo Scientific) was used to capture Id3-Flag-binding cellular proteins by co-immunoprecipitation (co-IP) according to the manufacturer’s instruction. The cell extracts or co-IP samples were size-fractionated using SDS-PAGE and protein bands were subsequently transferred to PVDF membranes. Membranes were incubated with primary followed by secondary antibodies in blocking buffer. Labeled proteins were visualized using the ECL Prime Western Blotting Detection Reagent kit (GE Healthcare); images were captured using an Image Quant LAS 500 (GE Healthcare) and analyzed with ImageJ software from the NIH.

**EdU Proliferation Assay**

At 0, 12, 24, and 48 h after differentiation stimuli, cell proliferation was detected using incorporation of 5-ethynyl-2′-deoxyuridine (EdU) with the Click-iT EdU Cell Proliferation Assay Kit (Invitrogen). Briefly, cells were incubated with 10 μM EdU for 1 h before fixation, permeabilization, and EdU staining, which were carried out according to the kit’s protocol. The cells were incubated in a DAPI solution for 5 min. Fluorescence photographs were taken on EVOS FL Auto (Life Technologies; Carlsbad, CA, United States). Quantification of proliferation nuclei was accomplished by performing counts for DAPI and EdU. Nuclei were counted manually using digital photography and Adobe Photoshop software.

**Immunocytochemistry and Myotube Quantification**

Immunocytochemistry was performed as described previously (Tokutake et al., 2015). Briefly, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS, and blocked with 10% goat serum in PBS with 0.01% Triton-X 100. Then, the cells were incubated with mouse anti-Myosin 4 (MF20) monoclonal antibody (Thermo Scientific) (1:50) for 2 h at room temperature. The cells were then incubated with Alexa-Fluor 488-conjugated goat anti-mouse antibody (Thermo Scientific) for 1 h. The cell nuclei were stained with DAPI (Thermo Scientific) and observed under the EVOS FL Auto (Thermo Scientific). The differentiation potential of the myoblasts, known as the fusion index, was evaluated as a percentage of the number of nuclei contained within MF20-positive myotubes per total number of nuclei. At least 500 nuclei from ten random fields were counted for group (n = 3).

**Statistical Analysis**

All data are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments. Comparisons between two samples were conducted using the Student’s t-test or Mann–Whitney U test, while comparisons of multiple groups were performed using analysis of variance followed by the post hoc Tukey–Kramer’s honestly significant difference test. A probability (p) value of < 0.05 was considered statistically significant.

**RESULTS**

**XBP1u Expression Was Increased After Differentiation Induction of C2C12 Cells**

First, the protein expression levels of XBP1s and XBP1u in differentiated cells were measured by western blot analysis. As shown in Figure 1A, XBP1u expression was significantly increased at 12 h after differentiation induction, whereas XBP1s expression was significantly decreased at 24 h after differentiation induction and remained low. The mRNA expression levels of XBP1s and XBP1u were also assessed by quantitative real-time polymerase chain reaction (RT-qPCR). As shown in Figure 1B, mRNA expression of XBP1u was significantly increased after differentiation induction, whereas XBP1s mRNA expression was decreased. We confirmed that protein expression of myosin heavy-chain (MHC) and mRNA expression of Myogenin and Myh1 increased 48 h after differentiation (Figures 1A,B). Consistent with our previous report, myotube formation in previously generated XBP1-knockdown C2C12 cell lines that stably expressed XBP1 shRNA (XBP1-knockdown cells), was inhibited 5 days post differentiation induction (Figure 1C). Our previous study has also showed that the expression of myogenesis related genes (MyoD, Myogenin, Mrf4, and Mef2c) was significantly repressed with differentiation in XBP1-knockdown cells (Tokutake et al., 2019). To confirm the role of XBP1 in other type of myoblast, XBP1 was knocked down in primary-cultured mouse myoblasts by siRNA. RT-qPCR data confirmed that Xbp1 expression in the knockdown cells was significantly lower compared with that in control cells. XBP1 silencing impaired myogenic differentiation of primary-cultured mouse myoblasts. The fusion index (average number of mononuclei/MyHC + cells) of knockdown cells was significantly lower than that in control cells (Figure 1D).

**Id3 Expression Was Maintained at a Higher Level After Differentiation Induction of XBP1- Knockdown Cells**

Since Id3 is an important factor of early stage myogenic differentiation, Id3 protein expression was monitored in the early differentiation stage. As shown in Figure 2, XBP1 depletion maintained high Id3 expression after differentiation induction, although Id3 expression rapidly diminished in control cells. These results suggest that maintenance of Id3
expression is associated with differentiation inhibition of XBP1-knockdown cells.

**XBP1u Accelerated Proteasomal Degradation of Id3 in C2C12 Cells**

XBP1u is a negative mediator of XBP1s, ATF6, and Foxo1 by targeting these molecules for proteasomal degradation (Yoshida et al., 2006, 2009; Zhao et al., 2013). Therefore, to determine whether XBP1u interacts with Id3, co-expression of XBP1u and Id3 was induced in C2C12 cells. Co-immunoprecipitation analysis revealed that XBP1u was physically bound to Id3 in transfected cells (Figure 3A). As shown in Figure 3B, there was an ectopic XBP1u dose-dependent decrease in Id3 expression. Degradation of Id3 is reported to occur through the ubiquitin-proteasome pathway.
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FIGURE 5 | Loss of Id3 rescues XBP1-knockdown-mediated inhibition of myogenic differentiation. (A) C2C12 cells were transfected with Id3 siRNA or control siRNA. The mRNA level of Id3 was determined by RT-qPCR and normalized to Gapdh. The results are presented as the mean ± SEM of three independent determinations. Student’s t-test. *p < 0.05 vs. the control group. (B) XBP1-knockdown cells were transfected with Id3 siRNA or control siRNA prior to differentiation induction. Cells were immunostained with anti-MHC antibody (green) and DAPI (blue) on day 3 post differentiation. Scale bars = 400 µm. The images below show larger magnification views of boxed regions. Scale bar = 50 µm. (C) The fusion index was calculated. KD indicates “knockdown.” The data are representative of four independent experiments. Ten views were analyzed for each experiment. Mann–Whitney U test. **p < 0.001 vs. the control group, ‡p < 0.05 CL siRNA vs. Id3 siRNA.

FIGURE 6 | Schematic illustration of conclusion. XBP1u physically bound to ID3 accelerated proteasomal degradation of Id3, a novel regulator of the CDK inhibitor. XBP1u regulated cell cycle exit after differentiation induction via interactions with Id3 and promoted myogenic differentiation.

Bounpheng et al., 1999). Western blot analysis revealed that in C2C12 cells, in which protein synthesis was blocked by CHX, the degradation of Id3 was inhibited by MG132, an agent that blocks protein degradation by proteasomes (Figure 3C). These results indicate that XBP1u accelerated proteasomal degradation of Id3 in C2C12 cells.

XBP1-Knockdown Exhibited Abnormal Proliferation and CDK Inhibitor Expression After Differentiation Induction

Next, the proliferation of XBP1-knockdown cells after differentiation induction was examined. The EdU incorporation assay showed that XBP1-knockdown cells maintained the ability to proliferate after differentiation induction, while proliferation of control cells was significantly reduced (Figure 4A). In order to further investigate the mechanism of XBP1-knockdown to alter the cell cycle after differentiation induction, we identified key cell cycle regulatory genes. The mRNA expression levels of the CDK inhibitors p21, p27, and p57 were significantly increased after differentiation induction of control cells (Figure 4B). On the other hand, the expressions levels were unchanged
in XBP1-knockdown cells. The mRNA expression level of cyclin D1 was decreased after differentiation induction in both control and XBP1-knockdown cells (Figure 4B). Western blot analysis was performed to measure p21 and cyclin D1 protein expression levels to confirm that XBP1-knockdown alters the cell cycle after differentiation induction. As shown in Figure 4C, XBP1-knockdown altered p21 protein expression after differentiation induction, but not cyclin D1 expression. These results indicate that XBP1-knockdown altered the expression of the CDK inhibitor.

**Loss of Id3 Rescues XBP1-Knockdown-Mediated Inhibition of Myogenic Differentiation**

Finally, the effect of Id3 silencing on myogenic differentiation in XBP1-knockdown cells was investigated. XBP1-knockdown cells were treated with Id3 siRNA prior to differentiation induction. RT-qPCR confirmed the knockdown of Id3 (Figure 5A). The fusion index of XBP1-knockdown cells was partially rescued by knockdown of Id3 (Figure 5B).

**DISCUSSION**

The intracellular mechanisms underlying myoblast differentiation and cell cycle withdrawal remain unclear. It is well known that Id3 is involved in both myoblast proliferation and differentiation. The results of this study demonstrated that XBP1u, a UPR-related molecule, plays a role in Id3-mediated myoblast differentiation.

XBP1 is a major regulator of the UPR and mediates adaptation to ER stress. XBP1s is a key transcriptional factor that regulates the transcription of genes involved in the UPR (Frakes and Dillin, 2017). Additionally, XBP1s contributes to the differentiation of various cell types (Iwakoshi et al., 2003; Sha et al., 2009; Tohmonda et al., 2011; Tsuchiya et al., 2017). Indeed, our previous study demonstrated that XBP1-knockdown remarkably suppressed C2C12 myoblast differentiation and the expression of CDK5 (cyclin-dependent kinase 5), which is associated with myogenic cell differentiation and patterning and regulated by XBP1s (Philpott et al., 1997; Tokutake et al., 2019). In this study, however, expression of XBP1u, but not XBP1s, was increased after differentiation induction of C2C12 cells (Figure 1A). This is the first report of increased XBP1u expression after differentiation induction. However, further studies are needed to identify the mechanisms underlying the regulation of XBP1u expression after differentiation induction.

XBP1u has no transcriptional activity (Calfon et al., 2002) and undergoes rapid proteasomal degradation (Tirosh et al., 2006). Although relatively short-lived, XBP1u has a degradation domain and acts as a negative regulator of the UPR by targeting XBP1s and activates ATF6 for degradation (Yoshida et al., 2006, 2009). Therefore, XBP1u is thought to act as a regulator involved in fine-tuning of the UPR. Moreover, XBP1u affects autophagy by interacting with the transcription factor FOXO1 (Zhao et al., 2013). In addition, XBP1u physically bound to ID3 accelerated the proteasomal degradation of Id3 (Figure 3). Id3, which is expressed at a higher level after differentiation induction of XBP1-knockdown cells (Figure 2), prevents skeletal muscle differentiation (Melnikova and Christy, 1996). Taken together, these results suggest that XBP1u degrades Id3 after differentiation induction of C2C12 myoblasts. Moreover, the results suggest that XBP1u plays an unexpectedly important role as a regulator, at least in response to differentiation induction.

Cell cycle arrest is critical for muscle differentiation. Exit of the cell cycle is accomplished by the down-regulation of cyclins, with the exception of cyclin D3 (Skapek et al., 1995), and induction of the CDKIs p21, p57, and p27, which inhibit a wide range of CDKs essential for cell cycle progression (Guo et al., 1995; Halevy et al., 1995; Sherr and Roberts, 1999). In the present study, abnormal proliferation and the expression levels of CDKIs p21, p27, and p57 were unchanged after differentiation induction of XBP1-knockdown cells (Figure 4). Also, XBP1-knockdown myoblasts exhibited abnormal proliferation (Supplementary Figure 1). A recent study indicated that XBP1u downregulated p21 expression (Huang et al., 2017). Moreover, Id3 is a novel regulator of CDKIs, which could lead to decreased expression of p21 accompanied by proliferation (Mueller et al., 2002). Silencing of Id3 primarily attenuated p21 and p27 expression (Sharma et al., 2012), although Id3 appears to be involved in the control of the steady-state level of p27 at the G1/S boundary (Chassot et al., 2007). Further, Id3 potently repressed expression of the p57 (Lee et al., 2011). Therefore, the abnormal proliferation of XBP1-knockdown cells may be due to the maintenance of Id3 expression.

Finally, the loss of Id3 rescued XBP1-knockdown-mediated inhibition of myogenic differentiation (Figure 5). Therefore, abnormal cell cycling after differentiation induction may be a factor in abnormal differentiation of XBP1-knockdown cells. However, only partial phenotype rescue was observed, as XBP1s is also involved in muscle differentiation. The results of our previous study implied that XBP1s is necessary for myogenic cell adaptation and viability upon differentiation induction (Tokutake et al., 2019). Also, cell cycle exit during osteogenic differentiation of mesenchymal stem cells is reportedly mediated by Xbp1s-induced upregulation of p21 and p27 (Zhang et al., 2020). Considering that Xbp1s is involved in the expression of genes related to the cell cycle and cell adaptation as a transcription factor, and that XBP1u regulates the expression of other protein, including XBP1s and Id3 via its degradation domain, it is expected that XBP1s and XBP1u regulate the muscle differentiation process in a coordinated and complex manner. Further studies investigating the sequential expression regulation and role of XBP1s and XBP1u will contribute to a better understanding of the mechanism underlying myoblast differentiation, especially in the initial stage of differentiation.

**CONCLUSION**

These results indicate that XBP1u regulates cell cycle exit after differentiation induction via interactions with Id3 in C2C12 cells.
(Figure 6). Moreover, this XBP1u-Id3 interaction is necessary for C2C12 myoblast differentiation. To the best of our knowledge, this is the report of the involvement of XBP1u in myoblast differentiation. However, the functions of XBP1u remain largely unknown, although it has been suggested that XBP1u may acts as a “regulator” of myoblast differentiation under various physiological conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SH and SY contributed to the experimental design. SH, SS, SK, and YT conducted the experiments and analyzed the data. SH and YT drafted the manuscript. SY supervised the project and directed to the corresponding author/s.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.796190/full#supplementary-material

Supplementary Figure 1 | (A) EdU (green) and DAPI (blue) staining of undifferentiated XBP1 KD or mock cells (left). Quantification of the percentage of EdU-positive nuclei. The results are presented as the mean ± SEM of three independent experiments. Student’s t test. ***p < 0.001 vs. the control group. (B) Cell growth curves of XBP1 KD and mock cells. Results are means ± SEM for three independent determinations. Student’s t test. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. the control group.

Supplementary Table 1 | The effects of XBP1 on cell cycle progression. The data are presented as the mean ± SEM of three independent experiments. Student’s t test. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. the control group.

Supplementary Table 2 | The effects of XBP1 on terminal differentiation. The data are presented as the mean ± SEM of three independent experiments. Student’s t test. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. the control group.
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