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The Endoplasmic Reticulum Stress Transducer BBF2H7 Suppresses Apoptosis by Activating the ATF5-MCL1 Pathway in Growth Plate Cartilage*[S]

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Background: The endoplasmic reticulum stress transducer BBF2H7 is expressed in proliferating chondrocytes.
Results: Apoptosis is promoted in the cartilage of *Bbf2h7*$^{-/-}$ mice. BBF2H7 activates *Atf5* transcription, followed by apoptosis suppression.
Conclusion: BBF2H7 plays crucial roles in suppressing ER stress-induced apoptosis.
Significance: BBF2H7 acts as a bifunctional regulator to promote cartilage extracellular matrix protein secretion and inhibit ER stress-induced apoptosis.

BBF2H7 (box B-binding factor 2 human homolog on chromosome 7) is a basic leucine zipper transmembrane transcription factor that belongs to the cyclic AMP-responsive element-binding protein (CREB)/activating transcription factor (ATF) family. This novel endoplasmic reticulum (ER) stress transducer is localized in the ER and is cleaved in its transmembrane region in response to ER stress. BBF2H7 has been shown to be expressed in proliferating chondrocytes in cartilage during the development of long bones. The target of BBF2H7 is Sec23a, one of the coat protein complex II components. *Bbf2h7*-deficient (*Bbf2h7*$^{-/-}$) mice exhibit severe chondrodysplasia, with expansion of the rough ER in proliferating chondrocytes caused by impaired secretion of extracellular matrix (ECM) proteins. We observed a decrease in the number of proliferating chondrocytes in the cartilage of *Bbf2h7*$^{-/-}$ mice. TUNEL staining of the cartilage showed that apoptosis was promoted in *Bbf2h7*$^{-/-}$ chondrocytes. *Atf5* (activating transcription factor 5), another member of the CREB/ATF family and an antiapoptotic factor, was also found to be a target of BBF2H7 in chondrocytes. ATF5 activated the transcription of *Mcl1* (myeloid cell leukemia sequence 1), which belongs to the antiapoptotic B-cell leukemia/lymphoma 2 family, to suppress apoptosis. Finally, we found that the BBF2H7-ATF5-MCL1 pathway specifically suppressed ER stress-induced apoptosis in chondrocytes. Taken together, our findings indicate that BBF2H7 is activated in response to ER stress caused by synthesis of abundant ECM proteins and plays crucial roles as a bifunctional regulator to accelerate ECM protein secretion and suppress ER stress-induced apoptosis by activating the ATF5-MCL1 pathway during chondrogenesis.

The endoplasmic reticulum (ER)[3] is a cellular organelle that regulates the synthesis, folding, and posttranslational modifications of secreted proteins. Various cellular stress conditions lead to accumulation of unfolded or misfolded proteins in the ER. These conditions are collectively termed ER stress (1–3). The ER responds to these perturbations by activating the unfolded protein response, which transduces signals to enhance the capacity for protein folding (4–7), attenuate the synthesis of proteins (8–10), and facilitate the degradation of unfolded proteins though ER stress transducers (11–13). Excessive and/or prolonged ER stress causes cells to undergo ER stress-induced apoptosis (14–16). Recent advanced studies have revealed that ER stress also provides important signals for regulating cell differentiation and maturation or maintenance of cellular homeostasis (17–22). However, it remains unclear how cells avoid the apoptosis induced by ER stress, which is necessary for the maintenance of biological functions.

Previously, we identified BBF2H7 (box B-binding factor 2 human homolog on chromosome 7) as a novel ER stress transducer (23). BBF2H7 is a basic leucine zipper (bZIP) transcription factor that belongs to the cyclic AMP-responsive element (CRE)-binding protein (CREB)/activating transcription factor (ATF) family (23). Although BBF2H7 is localized to the ER membrane under normal conditions, it is cleaved at the trans-

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[3] The abbreviations used are: ER, endoplasmic reticulum; bZIP, basic leucine zipper; CRE, cAMP-responsive element; CREB, CRE-binding protein; ATF, activating transcription factor; ECM, extracellular matrix; E*n*, embryonic day *n*; Tg, thapsigargin; Tm, tunicamycin; STS, staurosporine; Etop, etoposide; c-CASP3, cleaved caspase 3.

membrane region in response to ER stress, and the processed N-terminal fragment of BBF2H7 containing the bZIP domain is translocated into the nucleus to promote the transcription of its target genes (23). BBF2H7 is preferentially expressed in proliferating chondrocytes in cartilage during the development of long bones (17). *Bbf2h7*-deficient (*Bbf2h7*$^{-/-}$) mice exhibit severe chondrodysplasia and die from suffocation shortly after birth because of an immature chest cavity (17). *Bbf2h7*$^{-/-}$ proliferating chondrocytes show an abnormally expanded rough ER containing aggregated extracellular matrix (ECM) proteins, such as type II collagen and cartilage oligomeric matrix protein, caused by decreased levels of *Sec23a*, which encodes a coat protein complex II component responsible for protein transport from the ER to the Golgi and is a BBF2H7 target gene in chondrocytes (17). The cartilage of *Bbf2h7*$^{-/-}$ mice also contains a decreased number of chondrocytes (17), indicating that *Bbf2h7*$^{-/-}$ chondrocytes are promoted toward apoptosis or attenuated proliferation during chondrogenesis. Although these findings suggest that BBF2H7 is involved in the apoptosis and/or proliferation of chondrocytes, the detailed relationships between BBF2H7 and these phenomena are still unknown.

Atf5 (activating transcription factor 5) is another member of the CREB/ATF family (24–27). The ability of ATF5 to regulate pathways that are integrated with the transduction cascades controlling apoptosis has been investigated in cell types from the lymphocytic lineage (28). When ATF5 is stably expressed in an IL-3-dependent cell line, cell apoptosis is suppressed through cytokine deprivation (29). Inhibition of endogenous ATF5 activity by the introduction of a dominant-negative form of ATF5 leads to apoptosis in asynchronously growing cells, even in the presence of growth factors (28–30). Interference with the ATF5 function results in glioma cell death in primary tumors (31, 32). The downstream effector of the ATF5-mediated survival pathway is *Mcl1* (myeloid cell leukemia sequence 1), which belongs the antiapoptotic B-cell leukemia/lymphoma 2 family and is a target gene of ATF5 in malignant glioma cells (33). These previous data indicate that ATF5 is an antiapoptotic factor that plays important roles in promoting survival and inhibiting apoptosis. Here, we show that BBF2H7 activated by ER stress directly up-regulates the ATF5-MCL1 antiapoptotic pathway to avoid ER stress-induced apoptosis caused by the production of abundant ECM proteins leading to an enhanced burden on the ER.

EXPERIMENTAL PROCEDURES

Generation of Bbf2h7$^{-/-}$ *Mice—Bbf2h7*$^{-/-}$ mice were previously established in our laboratory (17). In all studies comparing WT and *Bbf2h7*$^{-/-}$ mice, sex-matched littermates derived from the mating of *Bbf2h7*$^{+/-}$ mice were used. The experimental procedures and housing conditions for the animals were approved by the Committee of Animal Experimentation at Hiroshima University.

Histological Analysis—The limbs of embryonic day 18.5 (E18.5) mice were fixed overnight in 10% formalin neutral buffer solution (Wako). The samples were then decalcified in Morse solution, embedded in paraffin, and sectioned at 5-μm thickness. Hematoxylin-eosin (H&E) staining was performed according to standard protocols.

Skeletal Preparations—E18.5 mice were skinned, and muscles and organs were removed as possible. Mice were fixed with 99% ethanol (Wako) at room temperature. After staining of 0.15% Alcian blue (Sigma), they were cleared in 1% KOH (Wako) for 1 day. Then they were stained with 0.15% alizarin red S (Sigma).

TUNEL Staining—TUNEL assays were performed using the DeadEnd^{TM} fluorometric TUNEL system (Promega), according to the manufacturer's protocol. For cells, primary cultured chondrocytes prepared from E18.5 WT and *Bbf2h7*$^{-/-}$ mice were fixed in 4% PFA and then permeabilized in 0.1% sodium citrate and 0.1% Triton X-100. Cells were visualized under a fluorescence microscope. The number of positive cells was measured in five fields per well.

Immunohistochemistry—Limbs were fixed in 4% formalin and then decalcified with Morse's solution. Subsequently, the samples were dehydrated with ethanol, embedded in paraffin, and sectioned (5 μm). The sections were stained with antibody and visualized under a fluorescence microscope (BX51, Olympus) or a confocal microscope (FV1000D, Olympus). The following antibody and dilution were used: anti-cleaved caspase 3 (1:200; Promega).

In Situ Hybridization—In situ hybridization was performed using digoxigenin-labeled *Col2* (type II collagen) and *Col10* (type X collagen) antisense RNA (cRNA) probes (17). The *Col2* and *Col10* cRNA probes were obtained by *in vitro* transcription in the presence of digoxigenin-labeled dUTP using various cDNAs subcloned into the pGEM-Teasy vector (Promega) as templates. Limbs were fixed in 4% formalin and then decalcified with Morse's solution. Frozen sections (5 μm) were treated with 0.1% proteinase K for 5 min. After washing with PBS, the sections were refixed with 4% formalin for 20 min and treated with 0.1 M triethanolamine, 2.5% anhydrous acetic acid for 10 min. The sections were prehybridized for 1 h at 37 °C in hybridization buffer (0.01% dextran sulfate, 0.01 M Tris-HCl, pH 8.0, 0.05 M NaCl, 50% formamide, 0.2% sarcosyl, 1× Denhardt's solution, 0.5 mg/ml yeast tRNA, 0.2 mg/ml salmon testis DNA) and hybridized with the probes overnight at 55 °C. After washing with 4× saline sodium citrate buffer for 20 min at 60 °C followed by 2× saline sodium citrate buffer, 50% formamide for 30 min at 60 °C, the sections were treated with RNase A in RNase buffer (10 mM Tris-HCl, pH 7.4, 1 mM 0.5 M EDTA, pH 8.0, 0.5 M NaCl) for 30 min at 37 °C to remove non-hybridized probes. After RNase treatment, the sections were washed with 2× saline sodium citrate buffer, 50% formamide for 30 min at 60 °C and blocked with 1.5% blocking reagent in 100 mM Tris-HCl, pH 7.5, containing 150 mM NaCl for 60 min at room temperature. For detection of digoxigenin-labeled cRNA probes, an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Applied Science) was used at a dilution of 1:500, and color was developed by incubation with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate solution.

Cell Cultures and Treatment—Primary rib chondrocytes were cultured as described previously (17). Briefly, chondrocytes were isolated from E18.5 mouse rib cartilage using 0.2% collagenase D (Roche Applied Science) after adherent connective tissue had been removed by 0.2% trypsin (Sigma) pretreat-

ment. The isolated chondrocytes were maintained in α-MEM (Invitrogen) supplemented with 10% FCS. The medium was changed every 3 days and on the day of the assays to create identical conditions in each dish. Micromass cultures were performed as described previously (17). Briefly, mesenchymal cells were prepared from the limbs of E11.5 mice and digested with 0.1% trypsin and 0.1% collagenase D. Cells at 1×10^7 cells/ml were plated and maintained in α-MEM supplemented with 100 ng/ml bone morphogenic protein-2 (Sigma), 50 μg/ml ascorbic acid, and 5 nM β-glycerophosphate. The cells were treated with thapsigargin (Tg) (Wako), tunicamycin (Tm) (Sigma), staurosporine (STS) (Sigma), and etoposide (Etop) (Wako) for the indicated times. Adenovirus vectors expressing the mouse ATF5 were constructed using the AdenoX expression system (Clontech), according to the manufacturer's protocol. A recombinant adenovirus carrying the mouse BBF2H7 N terminus and Sec23a was generated previously (17).

Quantitative Real-time PCR—Total RNA was isolated using an RNeasy kit (Qiagen) according to the manufacturer's protocol. First-strand cDNA was synthesized in a 20-μl reaction volume using random primers (Takara) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative real-time PCR amplifications were performed using a Light Cycler 480 System II (Roche Applied Science) and Light Cycler SYBR Green I Master (Roche Applied Science). Each PCR amplification was performed using a specific primer set. The primer sequences were as follows: *Atf5* forward, 5′-CCGAATTCACAGCTCTCCTTCCTCTGG-3′; *Atf5* reverse, 5′-CCGAATTCGTGTGCTGGCAGGACTAG-3′; *Mcl1* forward, 5′-AGATCATCTCGCGCTACTTGC-3′; *Mcl1* reverse, 5′-AGGTCCTGTACGTGGAAGAACTC-3′; β-actin forward, 5′-TCCTCCCTGGAGAAGAGCTAC-3′; β-actin reverse, 5′-TCCTGCTTGCTGATCCACAT-3′. The primer set of *Bbf2h7* was generated previously (17).

Western Blotting—For Western blotting, proteins were extracted from primary rib chondrocytes using cell extraction buffer containing 10% SDS, 0.5 M EDTA (pH 8.0), 100 mM methionine, and a protease inhibitor mixture (MBL International). The lysates were incubated on ice for 60 min. After centrifugation at 16,000 × *g* for 15 min, the protein concentrations of the supernatants were determined using a microplate reader, model 680 (Bio-Rad). Protein-equivalent samples were separated by SDS-PAGE and then electroblotted onto PVDF membranes (Bio-Rad). The following antibodies and dilutions were used: anti-β-actin (1:2000; Millipore); anti-caspase 3 (1:1000; Cell Signaling Technology); anti-ATF5 (1:1000; Abnova); anti-rabbit IgG conjugated with alkaline phosphatase (1:2000; Sigma); anti-mouse IgG conjugated with alkaline phosphatase (1:2000; Enzo).

Cell Proliferation and Cell Counting Assay—For the cell proliferating analysis using WST-8 solution (Dojindo), a total of 1×10^4 cells/ml of primary cultured chondrocytes were plated in a 96-well microplate. 10 μl of WST-8 was added to each well at the indicated times, and the plate was incubated for 2 h at 37 °C. Absorbance was measured at 450 nm using a microplate reader (Bio-Rad). For the cell counting assay, cells were visualized under microscope and manually counted.

Luciferase Assay—A reporter gene carrying a 4.0-kb promoter region of *Atf5* and the reporter construct mut CRE were kind gifts from Michael R. Green (University of Massachusetts Medical School). Primary chondrocytes were cultured for 4 days and then transfected with 0.2 μg of pGL3 basic reporter plasmid carrying the firefly luciferase gene (Promega) and 0.02 μg of reference plasmid pRL-SV40 carrying the *Renilla* luciferase gene under the control of the SV40 enhancer and promoter (Promega), together with 0.2 μg of an effector protein expression plasmid, using Lipofectamine 2000 reagent (Invitrogen). After 24 h, the luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega) and a GLOMAX Multi+ detection system (Promega), according to the manufacturer's protocol. The relative activity was defined as the ratio of firefly luciferase activity to *Renilla* luciferase activity.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed as described previously (17). The primers used for the mouse *Atf5* promoter were 5′-AGGTGGGACACACAGAACGAT-3′ (forward) and 5′-TGTGGTGGTCAGAGGAGATCG-3′ (reverse), yielding a 215-bp product. The following antibodies were used: anti-histone H3 (Santa Cruz Biotechnology, Inc.) and rabbit IgG (Sigma). An anti-BBF2H7 polyclonal antibody was generated previously (17).

Statistical Analysis—Statistical comparisons were made using the unpaired Student's *t* test. The difference between two samples was considered to be statistically significant for a *p* value of less than 0.05.

RESULTS

The Number of Chondrocytes Is Decreased in Bbf2h7$^{-/-}$ Mice—BBF2H7 contains a transmembrane domain, a transcription activation domain, and a bZIP domain at its N terminus (supplemental Fig. 1*A*). We previously reported that *Bbf2h7*$^{-/-}$ mice exhibit severe chondrodysplasia associated with short limbs, a protruding tongue, and a distended belly (supplemental Fig. 1*B*) (17). Skeletal preparations at E18.5 showed extreme hypoplasia of the craniofacial bones and a reduction in cartilage ECM proteins in the limbs (supplemental Fig. 1*C*) caused by a reduction in *Sec23a*, which is one of the target genes for BBF2H7 in chondrocytes and recruits other components of coat protein complex II vesicles, such as Sec13/31, followed by completion of the complex before transporting secretory proteins, including cartilage ECM proteins (17). H&E staining of *Bbf2h7*$^{-/-}$ cartilage sections showed a lack of the typical columnar structure in the proliferating zone with a decreased number of chondrocytes (supplemental Fig. 2). These data indicate that chondrogenesis and cartilage formation are disrupted by the reduced secretion of cartilage ECM proteins and the significantly decreased number of chondrocytes in *Bbf2h7*$^{-/-}$ mice. BBF2H7 may be involved in not only secretion of cartilage ECM proteins but also maintenance of the number of chondrocytes during chondrogenesis.

Apoptosis Is Promoted and Proliferation Is Inhibited in Bbf2h7$^{-/-}$ Cells—It is well known that prolonged ER stress causes cells to undergo ER stress-induced apoptosis (14–16). We reported that ER stress induced by the synthesis of abundant ECM proteins occurs during the differentiation of chon-

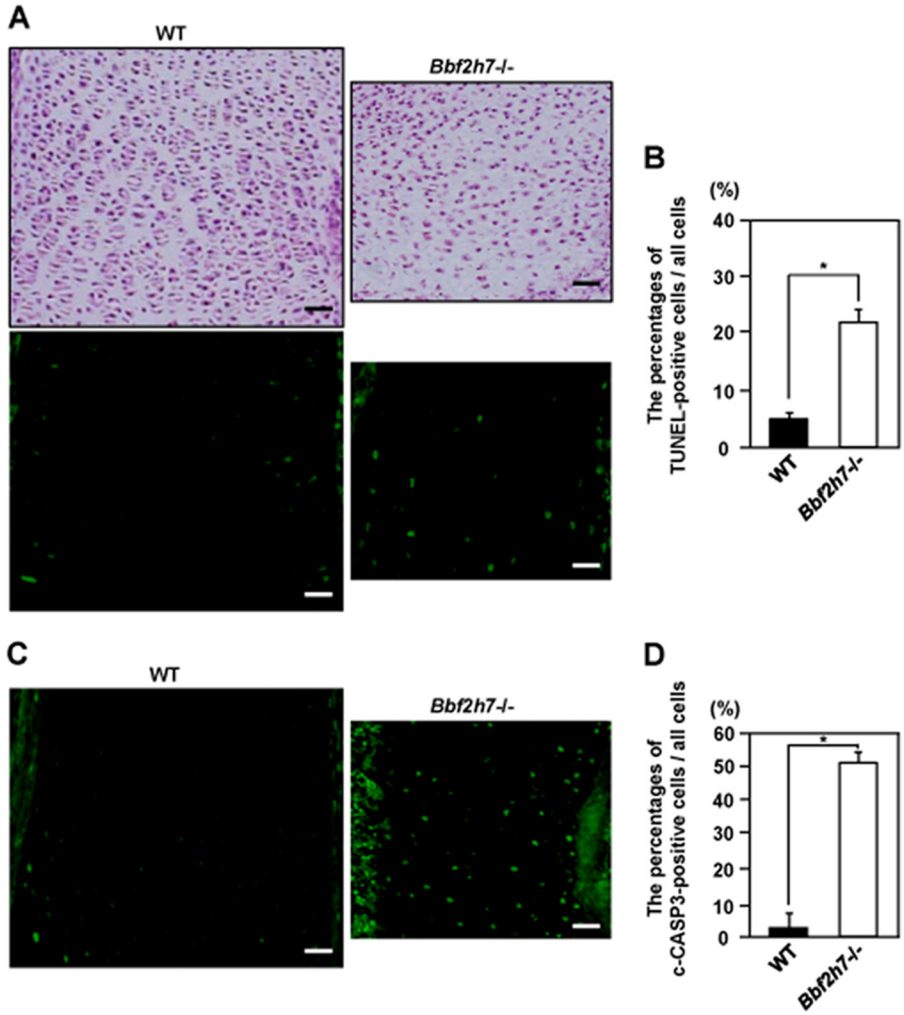

FIGURE 1. **Apoptosis is promoted in the cartilage of *Bbf2h7*$^{-/-}$ mice.** *A*, H&E staining (*top panels*) and TUNEL staining (*bottom panels*) of cartilage sections from the tibias of E18.5 WT and *Bbf2h7*$^{-/-}$ mice. *Bars*, 50 μm. *B*, numbers of TUNEL-positive proliferating chondrocytes in *A*. The data shown are means $\pm$ S.D. (*error bars*) ($n = 6$). *, $p < 0.05$, unpaired Student's *t* test. *C*, immunohistochemical analysis of cleaved caspase 3 (c-CASP3) in the tibias of E18.5 WT and *Bbf2h7*$^{-/-}$ mice. *Bars*, 50 μm. *D*, numbers of c-CASP3-positive proliferating chondrocytes in *C*. The data shown are means $\pm$ S.D. ($n = 4$). *, $p < 0.05$, unpaired Student's *t* test.

drocytes (17). Furthermore, we showed that *Bbf2h7* knockdown cells are vulnerable to ER stress-induced apoptosis (23). Therefore, we hypothesized that the decreased number of chondrocytes in *Bbf2h7*$^{-/-}$ mice compared with WT mice is caused by the ER stress-induced apoptosis. To examine this possibility, we performed TUNEL staining of epiphyseal cartilage sections from WT and *Bbf2h7*$^{-/-}$ mice (Fig. 1*A*). In the proliferating zone of cartilage, the number of TUNEL-positive cells was higher in *Bbf2h7*$^{-/-}$ mice than in WT mice (Fig. 1, *A* and *B*). The number of cells positive for cleaved caspase 3, a critical executioner of apoptosis, was higher in *Bbf2h7*$^{-/-}$ mice than in WT mice (Fig. 1, *C* and *D*). Furthermore, the level of cleaved caspase 3 was increased in primary cultured *Bbf2h7*$^{-/-}$ chondrocytes treated with Tg (an ER stressor) compared with WT chondrocytes (Fig. 2, *A* and *B*), coinciding with the *in vivo* data. These findings indicate that *Bbf2h7*$^{-/-}$ chondrocytes were vulnerable to ER stress and that apoptosis was promoted compared with WT chondrocytes. We further investigated whether proliferation was inhibited in primary cultured *Bbf2h7*$^{-/-}$ chondrocytes. The data from cell counting (Fig. 2*C*) and WST-8 (Fig. 2*D*) assays showed that the number of *Bbf2h7*$^{-/-}$ chondrocytes was lower than that of WT chondrocytes. These findings suggest that inhibition of proliferation as well as progression of apoptosis was observed in *Bbf2h7*$^{-/-}$ chondrocytes.

The ATF5-MCL1 Pathway Is Inhibited in Bbf2h7$^{-/-}$ *Chondrocytes*—Previously, it was reported that BBF2H7 can activate the transcription of *Atf5* in malignant glioma (33). ATF5 is an antiapoptotic factor that plays an important role in promoting the survival of various types of cells (28, 29, 34). *Atf5* may be one of the direct target genes of BBF2H7 in chondrocytes, and it is possible that BBF2H7 is involved in the inhibition of apoptosis through up-regulation of *Atf5*. Therefore, we examined the expression levels of *Atf5* during chondrocyte differentiation using micromass cultures of mesenchymal cells. *Atf5* was transiently up-regulated during the differentiation of mesenchymal cells into chondrocytes, synchronizing with the up-regulation of *Bbf2h7* (Fig. 3*A*). However, the expression of *Atf5* was markedly inhibited in *Bbf2h7*$^{-/-}$ cells (Fig. 3*A*). Because BBF2H7 is activated in response to ER stress, *Atf5*

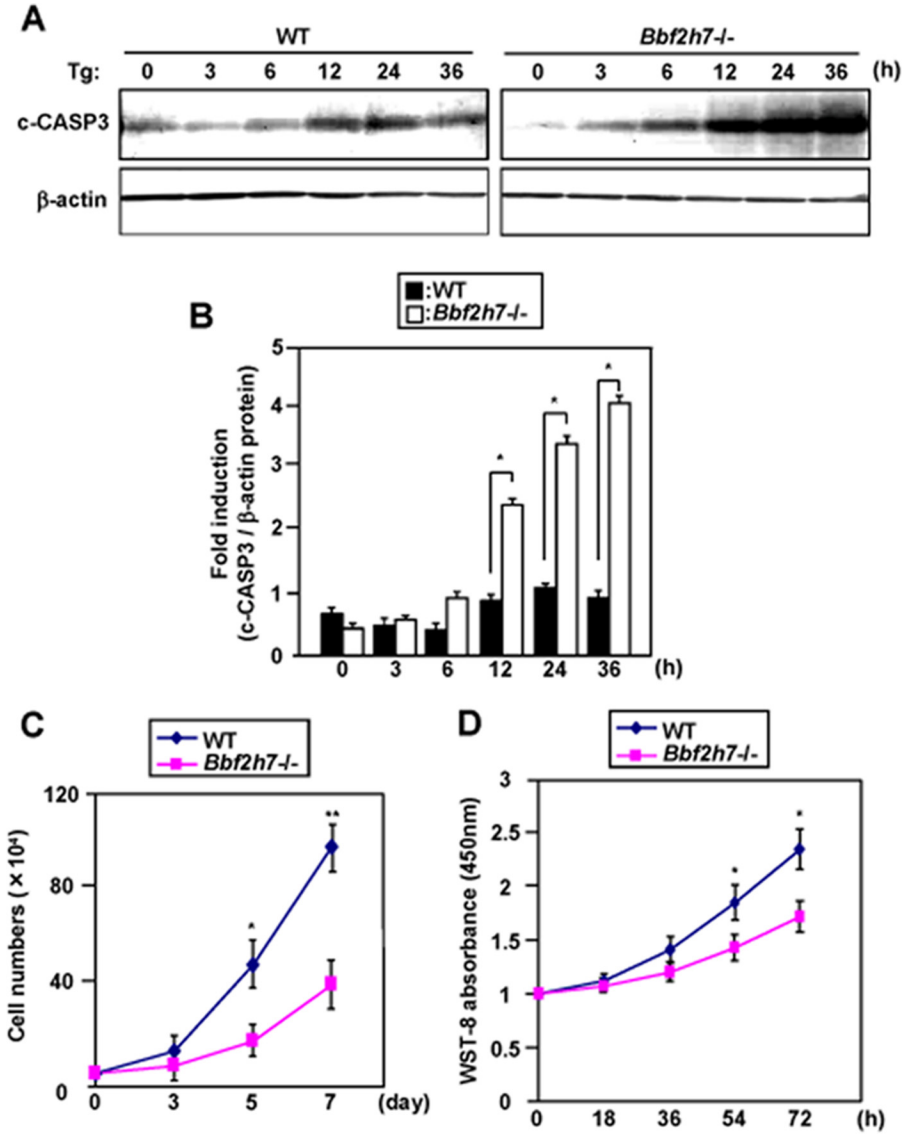

FIGURE 2. **Apoptosis is promoted and proliferation is inhibited in *Bbf2h7*$^{-/-}$ cells.** *A*, Western blotting of c-CASP3 in primary cultured chondrocytes. Cells were treated with Tg (1 μM) for the indicated times. *B*, quantitative analysis of c-CASP3 expression in *A*. The data are means ± S.D. (*error bars*) ($n = 3$). *, $p < 0.05$, unpaired Student's *t* test. *C* and *D*, cell counting (*C*) and WST-8 (*D*) assays using primary cultured chondrocytes. *Bbf2h7*$^{-/-}$ chondrocytes show inhibited proliferation compared with WT chondrocytes. The data are means ± S.D. ($n = 4$). *, $p < 0.05$; **, $p < 0.01$, unpaired Student's *t* test.

could be induced in chondrocytes exposed to ER stress if *Atf5* is a direct target of BBF2H7 in chondrocytes. Indeed, we found that *Atf5* was induced in WT chondrocytes during ER stress (Fig. 3*C*). In contrast, the induction of *Atf5* was significantly attenuated in *Bbf2h7*$^{-/-}$ chondrocytes (Fig. 3*C*). Infection of *Bbf2h7*$^{-/-}$ chondrocytes with an adenovirus expressing the BBF2H7 N terminus resulted in significant up-regulation of *Atf5* (Fig. 3*C*). These findings support the idea that *Atf5* may be a direct target of BBF2H7 in chondrocytes. It has been reported that ATF5 promotes cell survival by activating the transcription of *Mcl1*, an antiapoptotic Bcl2 family member (33). We investigated the expression levels of *Mcl1* during the differentiation of mesenchymal cells into chondrocytes. There were no differences in the expression patterns between WT and *Bbf2h7*$^{-/-}$ cells (Fig. 3*A*). However, when micromass-cultured cells were treated with Tg, the up-regulation of *Mcl1* was inhibited in *Bbf2h7*$^{-/-}$ cells (Fig. 3*B*). The expression of *Mcl1* was lower in primary cultured *Bbf2h7*$^{-/-}$ chondrocytes than in WT chondrocytes during ER stress (Fig. 3*D*). In *Bbf2h7*$^{-/-}$ chondrocytes, *Mcl1* expression was up-regulated by overexpression of the BBF2H7 N terminus (Fig. 3*D*). Furthermore, we generated adenoviruses expressing ATF5 to examine whether MCL1 was up-regulated by ATF5 in chondrocytes (Fig. 3*E*). We found that *Mcl1* expression was significantly up-regulated by overexpression of ATF5 (Fig. 3*F*). Thus, we concluded that ATF5 activated by BBF2H7 up-regulates MCL1 expression in chondrocytes. However, it is possible that *Mcl1* is mainly regulated by ATF5 and that its expression may be affected by other regulators.

We previously identified *Sec23a* as one of the target genes of BBF2H7 in cartilage (17). Therefore, we examined whether Sec23a expression affects *Atf5* expression by infecting adenoviruses expressing Sec23a or ATF5 into *Bbf2h7*$^{-/-}$ chondrocytes. As shown in Fig. 3, *G* and *H*, Sec23a overexpression did not affect ATF5 expression, and ATF5 overexpression did not

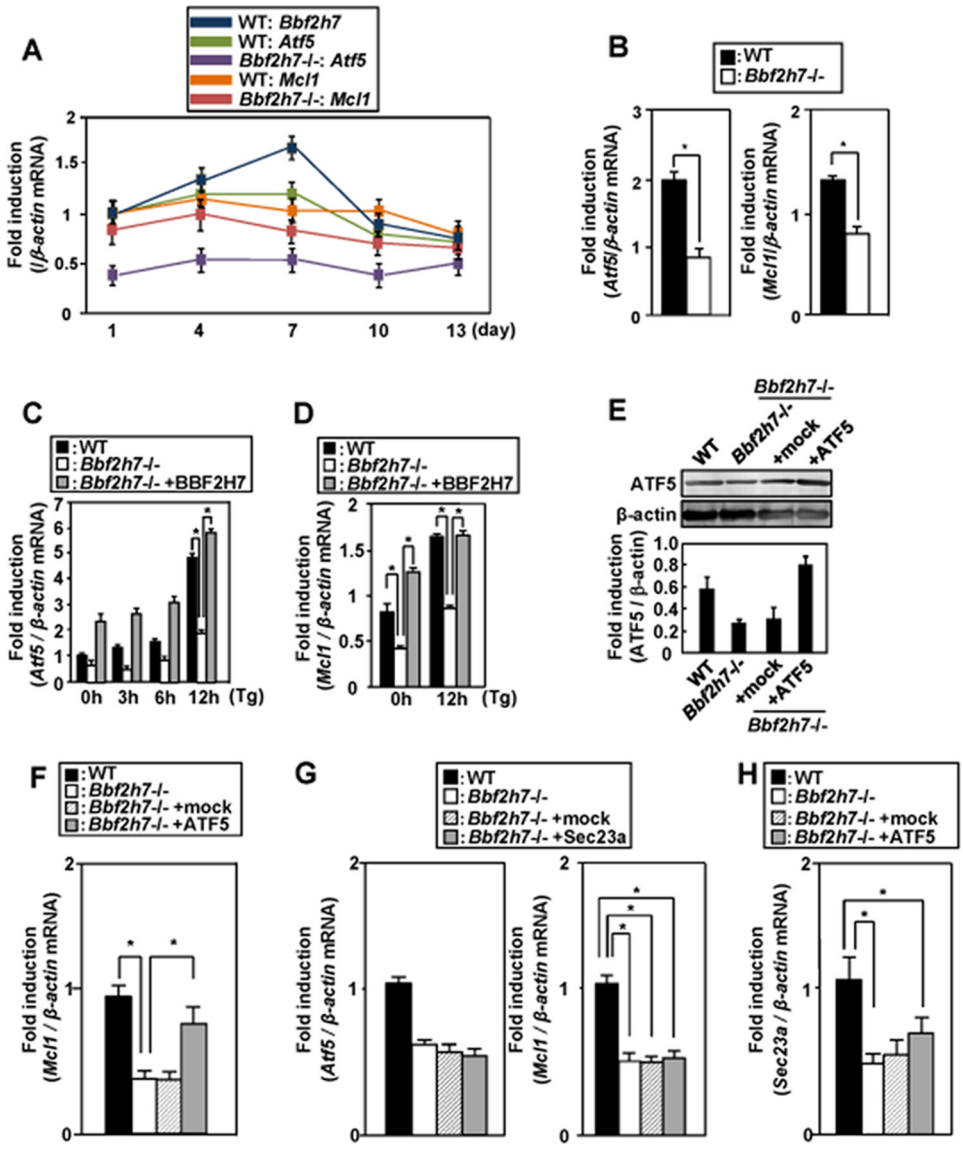

FIGURE 3. **BBF2H7 is involved in the up-regulation of *Atf5* and *Mcl1*.** *A*, real-time PCR analyses of *Bbf2h7*, *Atf5*, and *Mcl1* in mesenchymal cells prepared from WT and *Bbf2h7*$^{-/-}$ mice and maintained as micromass cultures for 1, 4, 7, 10, and 13 days. The data are means ± S.D. (*error bars*) ($n = 3$). The WT expression level/β-actin level of each gene at 1 day was set as 1.0 on the *y* axis. *B*, real-time PCR analyses of *Atf5* (*left*) and *Mcl1* (*right*) in mesenchymal cells prepared from WT and *Bbf2h7*$^{-/-}$ mice and maintained as micromass cultures for 4 days. The cells were treated with Tg (1 μM) for 12 h. The data are means ± S.D. ($n = 3$). *, $p < 0.05$, unpaired Student's *t* test. *C*, real-time PCR analysis of *Atf5* using primary cultured chondrocytes. The cells were treated with Tg (1 μM) for the indicated times. The data are means ± S.D. ($n = 3$). *, $p < 0.05$, unpaired Student's *t* test. *D*, real-time PCR analysis of *Mcl1* using primary cultured chondrocytes. The cells were treated with Tg (1 μM) for the indicated times. The data are means ± S.D. ($n = 3$). *, $p < 0.05$, unpaired Student's *t* test. *E*, Western blotting analysis of ATF5 (*top*) and quantitative analysis of ATF5 expression in the *top panel* (*bottom*) using primary cultured chondrocytes. Although ATF5 expression was down-regulated in *Bbf2h7*$^{-/-}$ cells, that was induced by the infection of adenovirus expressing ATF5. *mock*, empty vector. *F*, real-time PCR analyses of *Mcl1* in primary cultured chondrocytes. The data are means ± S.D. ($n = 3$). *, $p < 0.05$, unpaired Student's *t* test. *G*, real-time PCR analyses of *Atf5* (*left*) and *Mcl1* (*right*) using primary cultured chondrocytes. *Atf5* and *Mcl1* expression are not affected by overexpression of Sec23a. The data are means ± S.D. ($n = 3$). *, $p < 0.05$, unpaired Student's *t* test. *H*, real-time PCR analysis of *Sec23a* using primary cultured chondrocytes. *Sec23a* expression is not affected by overexpression of ATF5. The data are means ± S.D. ($n = 3$). *, $p < 0.05$, unpaired Student's *t* test.

affect Sec23a expression. We concluded that the ATF5-mediated pathway is independent of the Sec23a-mediated pathway.

Atf5 Is a Direct Target of BBF2H7 in Chondrocytes—To determine whether BBF2H7 acts on the *Atf5* promoter region and activates the transcription of *Atf5* in chondrocytes, we performed reporter assays with a reporter gene carrying the 4.0-kb promoter region of *Atf5* (4.0kb-Luc) (Fig. 4*A*). In *Bbf2h7*$^{-/-}$ chondrocytes transfected with the 4.0kb-Luc construct, the reporter activities were dramatically reduced (Fig. 4*B*). Furthermore, the reporter activities were significantly induced by expression of the BBF2H7 N terminus into *Bbf2h7*$^{-/-}$ chondrocytes (Fig. 4*B*). The *Atf5* promoter includes a CRE sequence (−632 to −639 bp), to which BBF2H7 can bind (32). Next, we performed promoter assays using the reporter construct mut CRE, which has a mutated CRE sequence (Fig. 4*A*). In chondrocytes transfected with the construct, the reporter activities were markedly reduced (Fig. 4*B*). Furthermore, we performed ChIP assays and detected a high level of BBF2H7 binding to the endogenous *Atf5* promoter in chondrocytes expressing the BBF2H7 N terminus (Fig. 4, *C–E*). These findings indicate that

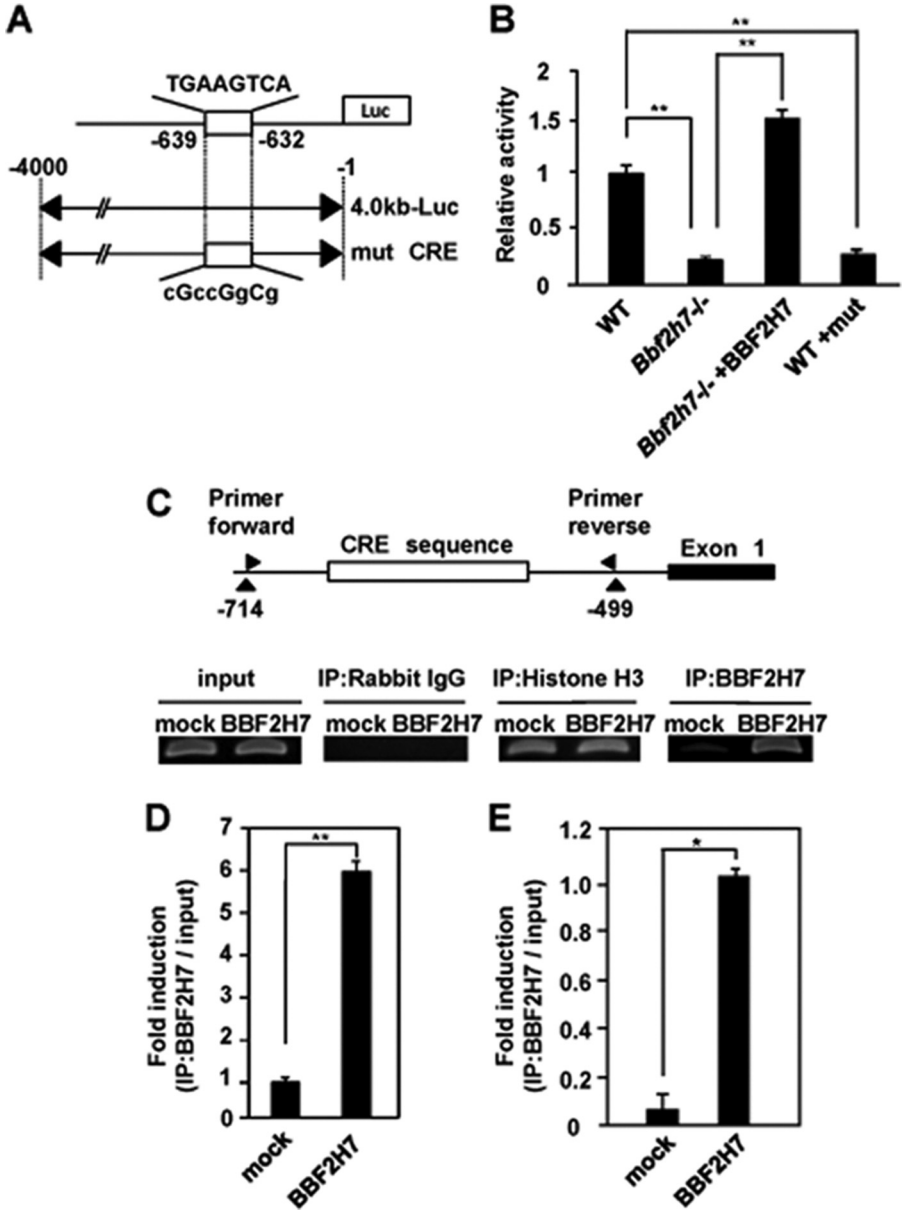

FIGURE 4. **Atf5 is a direct target of BBF2H7.** *A*, scheme of the *Atf5* promoter region and reporter constructs. The 4.0-kb Luc construct consists of the 4-kb *Atf5* promoter region. The mut CRE reporter construct has a mutation in the CRE sequence (shown as *lowercase letters*). *Luc*, luciferase gene. *B*, reporter assays using primary cultured chondrocytes. A vector expressing the BBF2H7 N terminus was cotransfected with each reporter construct. The data are means ± S.D. (*error bars*) ($n = 3$). **, $p < 0.01$, unpaired Student's *t* test. *C*, The *top panel* shows a schematic representation of the *Atf5* promoter and the annealing sites of the primer set used in the ChIP assays. The *bottom panel* shows the results of PCR amplification of the *Atf5* promoter region containing the CRE sequence (−632 to −639). Chondrocytes were infected with an adenovirus for overexpression of the BBF2H7 N terminus. *mock*, empty vector. *D*, quantitative analysis of immunoprecipitation with an anti-BBF2H7 antibody in *C*. The data are means ± S.D. ($n = 3$). **, $p < 0.01$, unpaired Student's *t* test. The immunoprecipitation/BBF2H7 (*IP:BBF2H7*) level/input level of mock was set as 1.0 on the *y* axis. *E*, real-time PCR amplification of the *Atf5* promoter region containing the CRE sequence (−632 to −639). Chondrocytes were infected with an adenovirus for overexpression of the BBF2H7 N terminus, followed by immunoprecipitation with an anti-BBF2H7 antibody.

BBF2H7 directly acts on the CRE sequence within the *Atf5* promoter and facilitates its transcription in chondrocytes.

The BBF2H7-ATF5-MCL1 Pathway Inhibits ER Stress-induced Apoptosis—It has been reported that ATF5 contributes to the inhibition of apoptosis (28, 29). Apoptosis is induced via two main routes involving either mitochondria (internal pathway) (34) or activation of death receptors (external pathway) (35, 36). Both pathways converge to activate caspases as the final executioners of cell death (37, 38). In addition, it has been revealed that apoptosis is induced by the ER stress response (14–16). Because BBF2H7 is activated in response to ER stress, the BBF2H7-ATF5-MCL1 pathway could specifically inhibit ER stress-induced apoptosis. To examine this possibility, we performed TUNEL staining using primary cultured WT and *Bbf2h7*$^{-/-}$ chondrocytes treated with various agents (Fig. 5, *A–D*). The numbers of cells for TUNEL-positive staining did not differ between WT and *Bbf2h7*$^{-/-}$ chondrocytes treated with STS (Fig. 5*A*) or Etop (Fig. 5*B*), which do not induce ER stress. In *Bbf2h7*$^{-/-}$ chondrocytes, the numbers of cells for TUNEL-positive staining were significantly higher than those

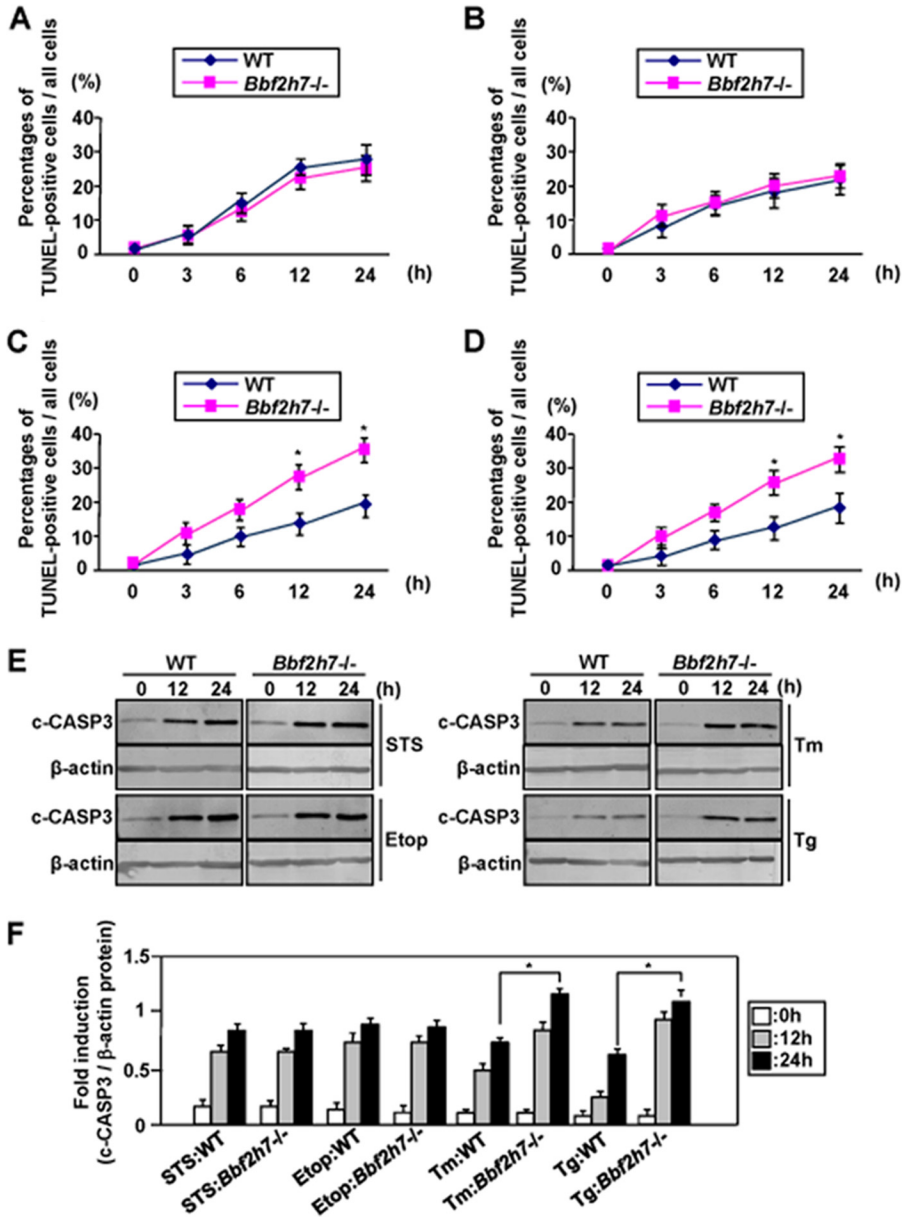

FIGURE 5. ***Bbf2h7*$^{-/-}$ chondrocytes are vulnerable to ER stress-induced apoptosis.** *A–D*, TUNEL staining in primary cultured WT and *Bbf2h7*$^{-/-}$ chondrocytes treated with STS (100 nM) (*A*), Etop (100 nM) (*B*), Tm (3 μg/ml) (*C*), or Tg (1 μM) (*D*) for the indicated times. The data are means ± S.D. (*error bars*) ($n = 3$). *, $p < 0.05$, unpaired Student's *t* test. *E*, Western blotting of c-CASP3 in primary cultured WT and *Bbf2h7*$^{-/-}$ chondrocytes. The cells were treated with STS, Etop, Tm, or Tg at the above concentrations for the indicated times. *F*, quantitative analysis of c-CASP3 expression in *E*. The data are means ± S.D. ($n = 3$). *, $p < 0.05$, unpaired Student's *t* test.

of WT chondrocytes after treatment with Tm (Fig. 5*C*) or Tg (Fig. 5*D*), which are ER stressors. Furthermore, cleaved caspase 3 was increased in primary cultured *Bbf2h7*$^{-/-}$ chondrocytes treated with the ER stressors, but not STS or Etop, compared with WT chondrocytes (Fig. 5, *E* and *F*). These data indicate that *Bbf2h7*$^{-/-}$ chondrocytes are vulnerable to ER stress-induced apoptosis. Finally, we examined whether the ATF5-MCL1 pathway inhibits the ER stress-induced apoptosis in *Bbf2h7*$^{-/-}$ chondrocytes. When adenoviruses expressing ATF5 were infected into *Bbf2h7*$^{-/-}$ chondrocytes, the number of TUNEL-positive cells and the cleaved caspase 3 levels were decreased compared with non-treated *Bbf2h7*$^{-/-}$ chondrocytes (Fig. 6, *A–C*). These findings indicate that BBF2H7-ATF5-MCL1 signaling specifically inhibits ER stress-induced apoptosis. Taken together, our data suggest that BBF2H7 plays an essential role in the inhibition of apoptosis induced by ER stress during chondrocyte differentiation (Fig. 6*D*).

DISCUSSION

We previously demonstrated that BBF2H7 is activated in response to ER stress and promotes chondrocyte differentiation and cartilage formation through regulation of cartilage ECM protein secretion by activating the transcription of *Sec23a*, a coat protein complex II component (17). Although ER stress activated by the synthesis of abundant ECM proteins is essential for chondrogenesis, the mechanisms for how cells prevent ER stress-induced apoptosis have remained unclear. In this study, we found that ATF5, another direct target of

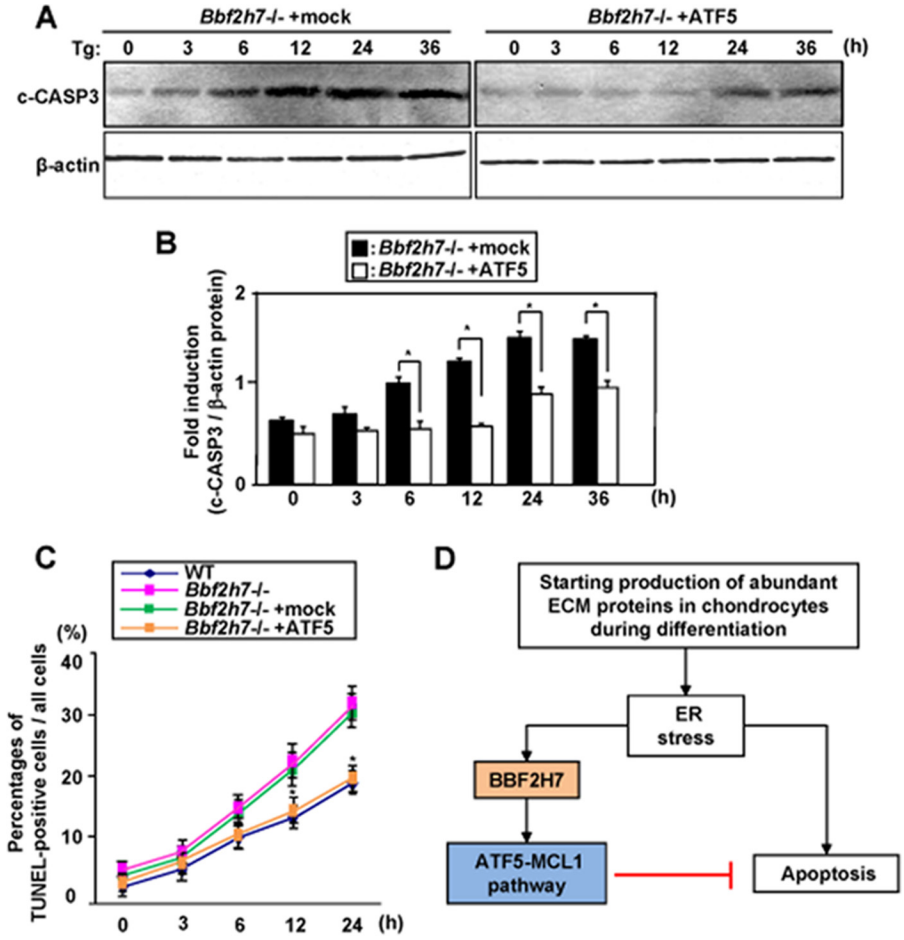

FIGURE 6. **The BBF2H7-ATF5-MCL1 pathway inhibits ER stress-induced apoptosis.** *A*, Western blotting of c-CASP3 in primary cultured chondrocytes. The cells were treated with Tg (1 μM) for the indicated times. *mock*, empty vector. *B*, quantitative analysis of c-CASP3 expression in *A*. The data are means ± S.D. (*error bars*) ($n = 3$). *, $p < 0.05$, unpaired Student's *t* test. *C*, TUNEL staining in primary cultured WT and *Bbf2h7*$^{-/-}$ chondrocytes treated with Tg (1 μM) for the indicated times. The introduction of ATF5 in *Bbf2h7*$^{-/-}$ chondrocytes inhibits ER stress-induced apoptosis. The data are means ± S.D. ($n = 3$). *, $p < 0.05$, unpaired Student's *t* test. *D*, proposed model for the BBF2H7-mediated signaling pathway in the secretion of cartilage ECM proteins and inhibition of ER stress-induced apoptosis during chondrocyte differentiation.

BBF2H7, inhibits ER stress-induced apoptosis and maintains the number of chondrocytes against ER stress. Therefore, BBF2H7 controls two essential mechanisms, namely 1) acceleration of ECM protein secretion followed by promotion of cartilage formation and 2) avoidance of ER stress-induced apoptosis caused by abundant cartilage ECM protein synthesis by activating the transcription of *Atf5*. In this context, our study strongly suggests that BBF2H7 possesses a critical ability to act as a bifunctional regulator activated by ER stress during chondrogenesis. Additionally, *Bbf2h7*$^{-/-}$ chondrocyte proliferation was inhibited compared with WT chondrocytes. To maintain the number of chondrocytes, BBF2H7 may activate the transcription of proliferation-related genes as well as antiapoptotic factors.

We found that *Atf5* is a target gene of BBF2H7 in chondrocytes. This conclusion is supported by the following facts: 1) *Atf5* was significantly down-regulated in primary cultured chondrocytes from *Bbf2h7*$^{-/-}$ mice; 2) exogenous expression of the BBF2H7 N terminus, the active form of BBF2H7, up-regulated *Atf5* expression; 3) the promoter activities of the *Atf5* gene were facilitated by BBF2H7; 4) a CRE sequence, to which BBF2H7 can bind, exists in the promoter region of *Atf5*; 5) the *Atf5* promoter activities were drastically reduced by a mutation in the CRE sequence; and 6) BBF2H7 directly bound to the *Atf5* promoter region. These findings and those in a previous report indicate that ATF5 activates the transcription of *Mcl1* to suppress apoptosis (32). However, in micromass cultures of mesenchymal cells, the expression patterns of *Mcl1* showed no significant differences between WT and *Bbf2h7*$^{-/-}$ cells. We revealed that *Mcl1* is mainly regulated by the BBF2H7-ATF5 pathway in chondrocytes. However, *Mcl1* could be regulated by other factors as well as ATF5, or ATF5 could suppress the apoptosis to induce the expression of not only *Mcl1* but also other target genes. The expression levels of various antiapoptotic factors should be precisely analyzed to elucidate the detailed mechanisms underlying the suppression of ER stress-induced apoptosis regulated by BBF2H7, ATF5, and MCL1.

The process of apoptosis is controlled by diverse cell signaling pathways from mitochondria; death receptors, such as FAS and TNF receptor; and the ER (39–42). We previously demonstrated that chondrocytes have an increased ER burden caused by the synthesis and secretion of large amounts of ECM proteins during differentiation (17). It is inevitable that ER stress is augmented in various secretory cells, including chondrocytes,

osteoblasts, goblet cells, and astrocytes (17–22). These cells may activate specific pathways to avoid apoptosis induced by ER stress. However, the mechanism for how such ER stress activates only unfolded protein response signaling, which is essential for cell differentiation, and does not cause ER stress-induced apoptosis remains unclear. Our study has revealed that BBF2H7 plays a crucial role in suppressing ER stress-induced apoptosis caused by the synthesis of abundant ECM proteins by directly activating the ATF5-MCL1 antiapoptotic pathway. It is possible that the BBF2H7-ATF5-MCL1 pathway may also be activated during the differentiation of various other secretory cells.

We previously reported that *Bbf2h7*$^{-/-}$ mice exhibit severe chondrodysplasia caused by decreased secretion of ECM proteins (17). It has also been reported that one of the etiologies of chondrodysplasia, such as thanatophoric dysplasia, is an increase in the number of apoptotic chondrocytes caused by the accumulation of mutated FGF receptor 3 in the ER lumen followed by extension of ER stress (43, 44). It is possible that the chondrodysplasia observed in *Bbf2h7*$^{-/-}$ mice causes not only the decrease in ECM proteins in the extracellular region but also the decrease in the number of chondrocytes through ER stress-induced apoptosis, similar to thanatophoric dysplasia. Further advanced studies, such as precise morphological analyses, are necessary to clarify the association between BBF2H7 and the pathogenesis of chondrodysplasia.

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