Molecules and Cells

NFI-C Is Required for Epiphyseal Chondrocyte Proliferation during Postnatal Cartilage Development

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Stringent regulation of the chondrocyte cell cycle is required for endochondral bone formation. During the longitudinal growth of long bones, mesenchymal stem cells condense and differentiate into chondrocytes. Epiphyseal chondrocytes sequentially differentiate to form growth-plate cartilage, which is subsequently replaced with bone. Although the importance of nuclear factor 1C (Nfic) in hard tissue formation has been extensively studied, knowledge regarding its biological roles and molecular mechanisms in this process remains insufficient. Herein, we demonstrated that Nfic deficiency affects femoral growth-plate formation. Chondrocyte proliferation was downregulated and the number of apoptotic cell was increased in the growth plates of *Nfic*$^{-/-}$ mice. Further, the expression of the cell cycle inhibitor p21 was upregulated in the primary chondrocytes of *Nfic*$^{-/-}$ mice, whereas that of cyclin D1 was downregulated. Our findings suggest that Nfic may contribute to postnatal chondrocyte proliferation by inhibiting p21 expression and by increasing the stability of cyclin D1 protein.

Keywords: cell cycle, chondrocyte, cyclin D1, nuclear factor 1-C, p21, proliferation, protein stability

INTRODUCTION

Mesenchymal stem cells differentiate into osteoblasts, odontoblasts, adipocytes, and chondrocytes. They have diverse biological functions during pre- and postnatal development and growth; these functions are cell specific (Archer and Francis-West, 2003). During the formation of long bone, primary ossification center appears within a cartilaginous scaffold at the future site of diaphysis as chondrocytes undergo hypertrophy. Secondary ossification center starts to form shortly after birth, compartmentalizing articular and growth-plate cartilage and establish epiphysis (Hall and Miyake, 2000; Kronenberg, 2003).

Chondrocytes in the epiphyseal growth plates differentiate to facilitate the longitudinal growth of long bones. Growth-plate chondrocytes are organized in parallel columns comprising resting, proliferative, prehypertrophic, and hypertrophic zones. When chondrocytes mature, a specialized cartilage matrix is deposited, which serves as a scaffold for blood vessels and osteoblasts to invade, leading to the accumulation of the bone matrix (Arsenault et al., 1988; Hunziker et al., 1999; Noonan et al., 1998). The formation of endochondral bone requires the stringent regulation of chondrocyte proliferation and differentiation. For example, skeletal anomalies,

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such as dwarfism, can occur when these two processes are not regulated (LuValle and Beier, 2000). Regulators of the cell cycle play important roles in endochondral bone formation. Therefore, it is essential to identify cell cycle genes and other factors, such as hormones and growth factors, that are involved in the regulation of cell cycle (Pines and Hurwitz, 1991; Shum and Nuckolls, 2002).

The nuclear factor I (NFI) family in vertebrates comprises NFI-A, NFI-B, NFI-C, and NFI-X, which bind as homo- or heterodimers to specific DNA sequences to regulate transcription (Gronostajski, 2000; Kruse and Sippel, 1994). Nfic functions as a key regulator during the postnatal development of hard tissues such as teeth and bones (Steele-Perkins et al., 2003). In *Nfic*$^{-/-}$ mice, Nfic deficiency severely inhibits the proliferation and differentiation of odontoblasts, leading to the formation of abnormal incisor dentin and short molar roots (Gronostajski, 2000; Lee et al., 2009; Oh et al., 2012; Park et al., 2007). Postnatal bone formation and maintenance are affected by Nfic deficiency as well. The transcription of regulators of the cell cycle such as p21 and cyclin D1 is controlled by Nfic (Lee et al., 2014). Although considerable research has been devoted to identifying the function of Nfic in the formation and maintenance of hard tissue, less attention has been paid to Nfic's role in cartilage.

The aim of the present research therefore was to investigate the cause of defective growth and formation of cartilage in *Nfic*$^{-/-}$ mice, primarily focusing on the regulation of the transcription of cell cycle regulators by Nfic.

MATERIALS AND METHODS

Reagents and antibodies

Recombinant human BMP-2 was purchased from R&D Systems. Antisera against NFI-C were produced as previously described (Lee et al., 2009). Rabbit cyclin D1 (#2922) and p-GSK3β (ser9, #9336) were purchased from Cell Signaling Technology (USA). Antibodies against PCNA (sc-400), p21 (sc-6246), Smurf1 (sc-25510), and Smurf2 (sc-25511) were purchased from Santa Cruz Biotechnology (USA).

Mice

Experiments involving mice were performed according to the guidelines of the Dental Research Institute guidelines and Institutional Animal Care and Use Committee of Seoul National University (SNU-111013-2). *Nfic*$^{-/-}$ mice were generated by deletion of the second exon of *Nfic* (Gronostajski, 2000). Homozygous *Nfic*$^{-/-}$ mice were obtained by crossing heterozygous *Nfic*$^{+/-}$ mice. The genotypes of the mice were determined as previously described (Steele-Perkins et al., 2003). As *Nfic*$^{-/-}$ mice have brittle teeth, a ground standard rodent chow was provided to all animals thrice a week beginning 3 days prior to weaning and continued for up to 6 weeks (Lee et al., 2014).

Microcomputed tomography (micro-cycle threshold [CT]) analysis, histology, and immunohistochemistry

The femurs of 6-week-old wild-type (WT) and *Nfic*$^{-/-}$ mice were removed, fixed in 4% paraformaldehyde at 4°C overnight, and subjected to micro-CT using a SkyScan scanner and associated software (SkyScan 1172; Bruker, Belgium). The isotopic resolution of the instrument is 10 μm. Decalcified femurs were embedded in paraffin and cut into sections 5-μm thick, stained using H&E and Alcian blue, and then analyzed using immunohistochemistry (IHC) as previously described (Lee et al., 2009).

Cell culture and transfection

ATDC5 cells (Riken Cell Bank, Japan) were cultured in DMEM/F12 supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5% fetal bovine serum (FBS). Primary chondrocytes were isolated from the growth plate of 6-week-old WT and *Nfic*$^{-/-}$ mice. Briefly, growth plates from distal femurs were dissected, washed in phosphate-buffered saline (PBS), and digested with 1% Collagenase Type II (Gibco BRL, USA) and 1.6% Dispase II (Gibco BRL) in Dulbecco's modified Eagle's medium: Nutrient mixture F-12 (DMEM/F12), for 1 h in an atmosphere containing 5% CO_2. The cells were cultured in DMEM/F12 supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5% FBS. Primary chondrocytes were incubated in the presence or absence of the proteasome inhibitor MG-132 at 5 μM (Sigma-Aldrich, USA) for 24 h. The pCH-Nfic expression plasmids were constructed as previously described (Lee et al., 2009), and the indicated expression plasmid (2 μg) was used to transiently transfect ATDC5 cells and primary chondrocytes in the presence of Lipofectamine Plus (Invitrogen, USA) according to the manufacturer's instructions.

MTT assays

The proliferation rates of primary chondrocytes and ATDC5 cells were evaluated using MTT assays. Cells were seeded and cultured in 96-well plates (3×10^3 cells/well). After washing with PBS, 50 μl of MTT (5 mg/ml) was added to each well, which as then incubated for 4 h at 37°C. After removing the MTT solution, the converted dye was dissolved in DMSO and the absorbance at 540 nm was measured using a microplate reader (Multiskan EX; Thermo Fisher Scientific, USA). Triplicate samples from two independent experiments were analyzed.

Terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick-end labeling (TUNEL) and DNA fragmentation assays

Apoptotic cells were identified using a TUNEL kit (Roche Biochemicals, Switzerland), according to the manufacturer's instructions. Enzymatically labeled cells were incubated with the substrate 3,3'-diaminobenzidine tetrahydrochloride to yield a colored reaction product. For negative controls, the enzyme solution was omitted from the normal TUNEL procedures. For positive controls, cells were treated with DNase I (0.7 μg ml^{-1}; Invitrogen) for 10 min before TUNEL processing. The endogenous peroxidase within the tissue sections was inactivated by a 10-min incubation in 3% hydrogen peroxide (H_2O_2) before enzymatic labeling. Visualization of TUNEL staining was achieved by incubation with 3,3'-diaminobenzidine (DAB) after enzymatic labeling, and the sections were counterstained with methyl green. DNA fragmentation assays were performed as previously described (Lee et al., 2012). Primary chondrocytes were collected 3 days after culturing commenced. Genomic DNA (gDNA) was isolated from

primary chondrocytes using a gDNA isolation kit (Cosmo Genetech, Korea) according to the manufacturer's instructions. The gDNA (200 ng) was electrophoresed through 2% agarose gels, stained with ethidium bromide, and visualized using ultraviolet light.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR analyses

Total RNA (2 μg) was reverse transcribed using 0.5 μg of Oligo d (T) and 1 μl (50 IU) of Superscript III (Invitrogen) in a 20-μl reaction mixture at 50°C for 1 h. The cDNAs were amplified using PCR. Primers specific for *Nfic*, *Cyclin D1*, *Mmp9*, *Mmp13*, and *Gapdh* were synthesized were used for real-time PCR (Table 1). Real-time PCR was performed using an Applied Biosystems 7500 Real-Time PCR System with SYBR

Table 1. Primers used for real-time PCR

Gene		Primer (5′-3′)
Nfic	Forward	GACCTGTACCTGGCCTACTTTG
	Reverse	CACACCTGACGTGACAAAGCTC
Mmp9	Forward	TGCCCATTTCGACGACGAC
	Reverse	GTGCAGGCCGAATAGGAGC
Mmp13	Forward	TCCCTGCCCCTTCCCTATGG
	Reverse	CCTCGGAGCCTGTCAACTGTGG
CyclinD1	Forward	CAC ACG GAC TAC AGG GGA GT
	Reverse	CACAGGAGCTGGTGTTCCAT
Gapdh	Forward	AGGTCGGTGTGAACGGATTTG
	Reverse	TGTAGACCATGTAGTTGAGGTCA

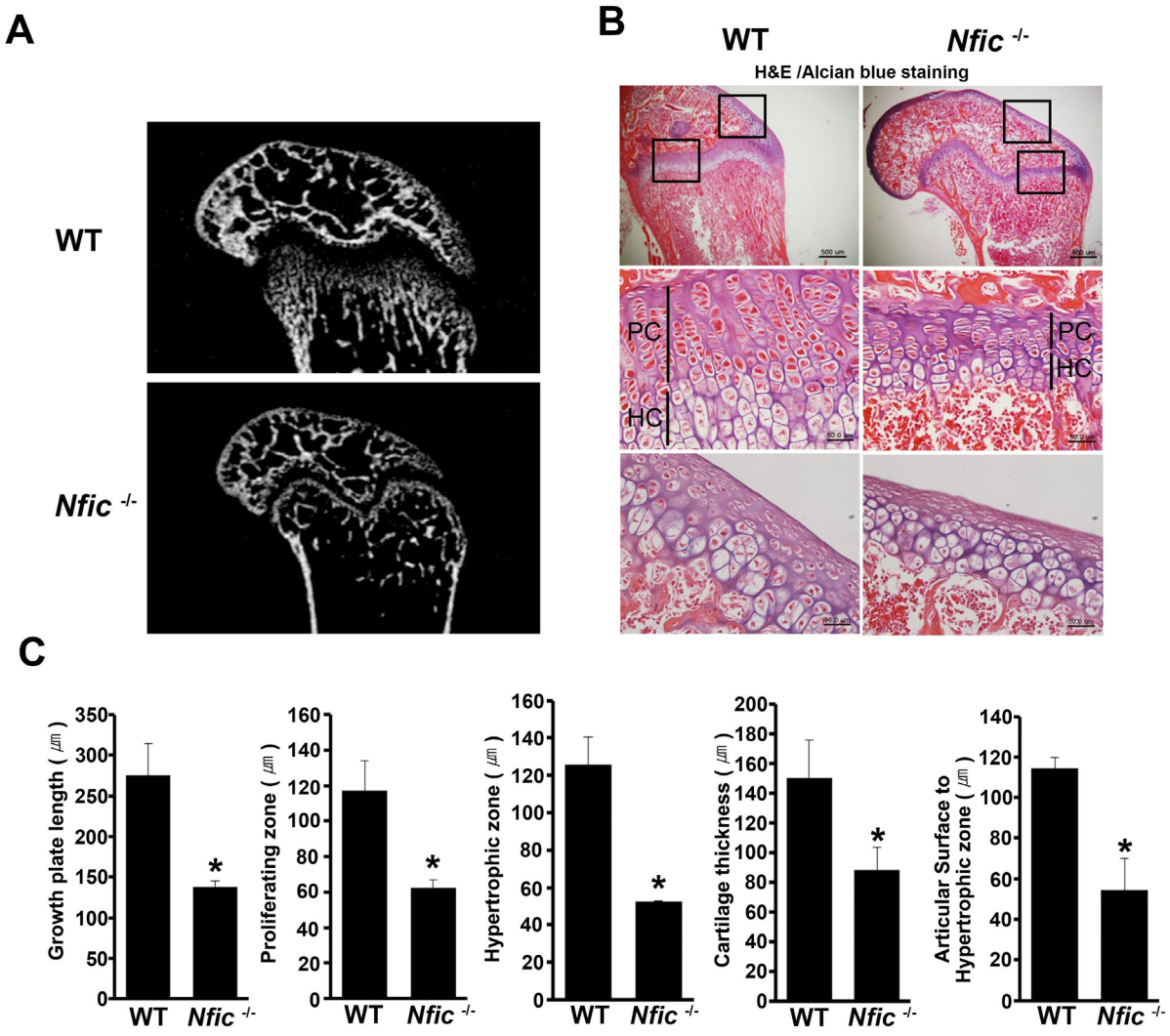

Fig. 1. Altered growth-plate morphology in *Nfic*$^{-/-}$ mice. (A) Representative micro-CT image of the distal femur. (B) Development of the growth plate in 6-week-old mice was analyzed using Alcian blue/H&E staining. Boxed areas in the upper panels show higher magnifications of the lower panels. PC, proliferating chondrocytes; HC, hypertrophic chondrocytes. Scale bars = 500 μm (top panels) and 50 μm (middle and bottom panels). (C) Histomorphometric analysis shows that the distance from the articular surface to the hypertrophic zone, growth-plate length, and the lengths of the proliferating zone and hypertrophic zones were significantly decreased in 6-week-old *Nfic*$^{-/-}$ mice compared with those of the WT (n = 3, $*P < 0.05$). Data represent the mean ± SD.

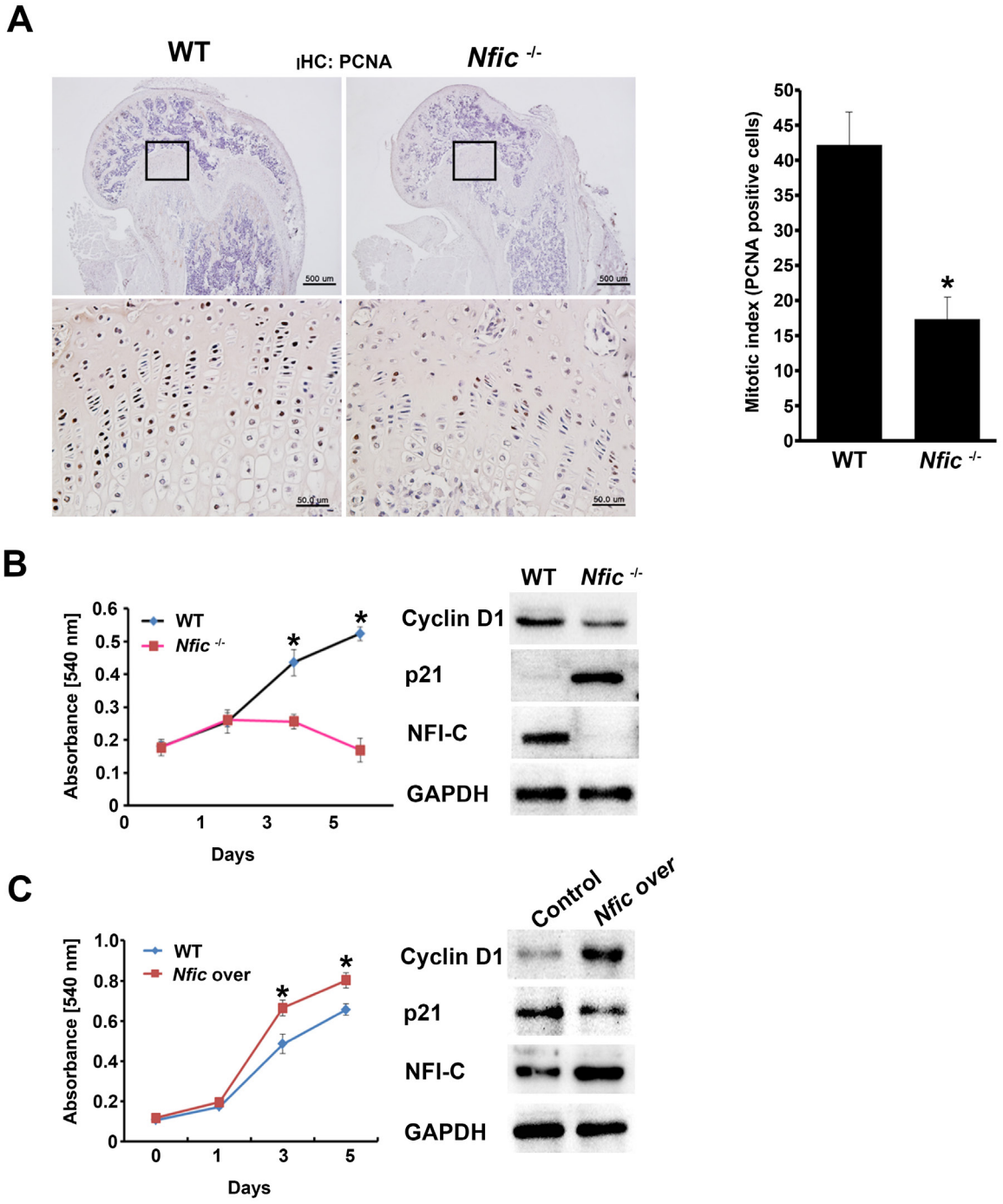

Fig. 2. Decreased proliferation of chondrocytes in the growth plates of *Nfic*$^{-/-}$ mice. (A) Analysis of PCNA expression in the femurs of WT and *Nfic*$^{-/-}$ mice aged 6 weeks. Quantification of PCNA-positive cells in the growth plate of femurs of WT and *Nfic*$^{-/-}$ mice. Scale bars = 500 μm (top panels) and 50 μm (bottom panels). (B) Primary chondrocytes isolated from the growth plate of the femurs of WT and *Nfic*$^{-/-}$ mice. Cell proliferation was measured using MTT assays on days 0, 1, 3, and 5 (left panel). The expression of cyclin D1, p21, and NFI-C were examined in ATDC5 cells by western blot analysis (right panel). (C) ATDC5 cells were transfected with an Nfic expression plasmid and cultured for 48 h. Cell proliferation was analyzed using an MTT assay (left panel). Western blot analysis of the expression of cyclin D1, p21, and NFI-C (right panel). Data represent the mean ± SD (n = 3, $*P < 0.05$).

GREEN PCR Master Mix (Applied Biosystems, USA) according to the manufacturer's instructions. PCR was performed as follows: 94°C for 1 min, 95°C for 15 s, and 60°C for 34 s, 40 cycles. All reactions were performed in triplicate, and the quantities of amplicons were normalized to those of *Gapdh*. Relative differences in PCR data were calculated using the

comparative cycle threshold CT method.

Western blot analysis

Western blot analyses were performed as previously described (Lee et al., 2009). Briefly, proteins (30 μg) were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, electrophoretically transferred onto a nitrocellulose membrane (Schleicher & Schuell BioScience, USA), and incubated with specific antibodies. Immunocomplexes were detected using an enhanced chemiluminescence system (GE Healthcare, USA).

Statistical analysis

All quantitative data are presented as the mean ± SD. The significance of differences was evaluated using the Student *t*-test ($*P < 0.05$). Statistical analysis was performed using SPSS Statistics for Windows (ver. 17.0; SPSS Inc., USA).

RESULTS

Decreased femoral growth-plate length in Nfic-deficient mice

To investigate the role of Nfic in chondrocyte proliferation and differentiation, we initially examined the effects of Nfic deficiency on developing femoral growth plates. *Nfic*$^{-/-}$ mice had narrower growth plates compared with those of WT mice (Fig. 1A, Supplementary Fig. S1), and femur length was slightly shorter in *Nfic*$^{-/-}$ mice (Supplementary Fig. S2). Histological and IHC analyses revealed decreased formation of growth cartilage and reduced thickness of the proliferating and hypertrophic zones (Fig. 1B). Histomorphometric analysis revealed a 49% decrease in growth-plate thickness, a 51% decrease in the length of the proliferating zone, a 44% decrease in the length of the hypertrophic zone, and a 60% decrease in cartilage thickness of *Nfic*$^{-/-}$ vs WT mice (Fig. 1C). There was no significant difference in prenatal cartilage formation (Supplementary Fig. S3). These results suggest that Nfic deficiency caused abnormal postnatal proliferation and hypertrophy of chondrocytes, leading to reduced thickness of the epiphyseal cartilage and decreased growth.

Downregulated chondrocyte proliferation in the femoral growth plate of Nfic-deficient mice

The changes in lengths of the proliferating and hypertrophic zones in growth-plate cartilage suggest that Nfic may be required for chondrocyte proliferation. To answer this question, PCNA levels were determined as an indicator of cell proliferation in the distal femurs of *Nfic*$^{-/-}$ mice. The number of PCNA-positive cells of the epiphyseal cartilage tissues of *Nfic*$^{-/-}$ mice decreased by ≥50% compared with those of WT mice (Fig. 2A). To further ascertain whether such defects in growth-plate cartilage were caused by Nfic deficiency, the proliferation of chondrocytes was analyzed. The proliferation of WT chondrocytes was indicated by the increased absorbance; however, *Nfic*$^{-/-}$ chondrocytes did not exhibit any significant increase in absorbance until day 5.

The levels of p21 and cyclin D1 were measured to further characterize the effects of Nfic deficiency on chondrocyte proliferation. The levels of p21 and cyclin D1 increased and decreased, respectively, suggesting that Nfic deficiency induced the growth arrest of chondrocytes (Fig. 2B). The effects of Nfic overexpression on the chondrocyte cell cycle was examined using the mouse chondroblast cell line ATDC. Ectopic expression of Nfic increased chondrocyte proliferation, while decreasing p21 and increasing cyclin D1 levels, indicating that Nfic promoted the progression of the cell cycle (Fig. 2C). These data suggest that Nfic may control the chondrocyte cell cycle by regulating the expression of p21.

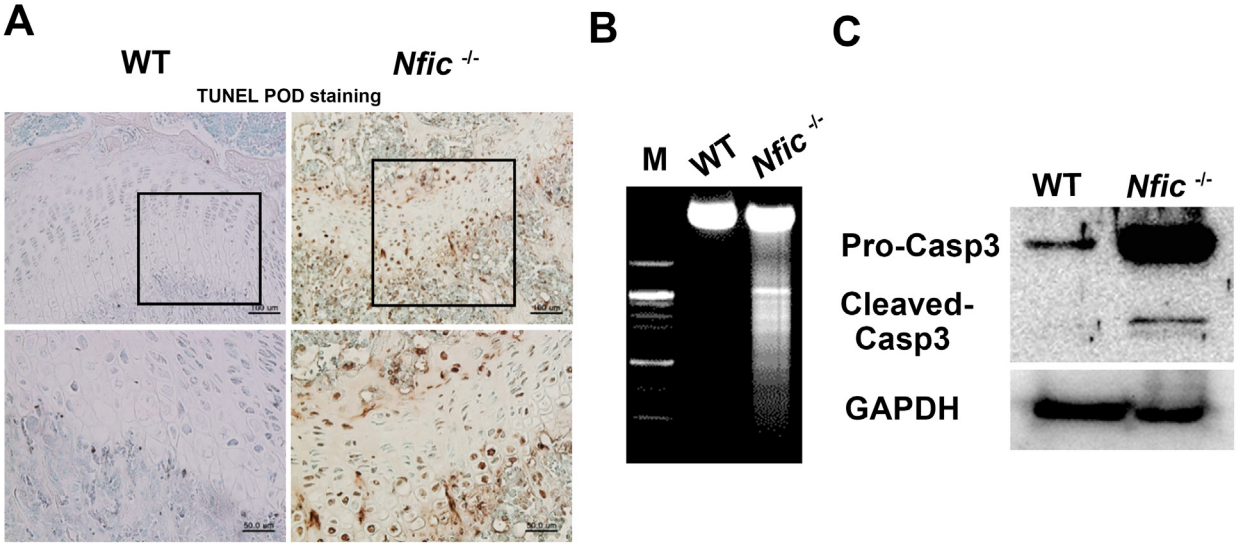

Fig. 3. Increased apoptosis of growth-plate chondrocytes in *Nfic*$^{-/-}$ mice. (A) TUNEL POD analysis of apoptosis of growth-plate chondrocytes in femurs of 6-week-old WT and *Nfic*$^{-/-}$ mice. Boxed areas in the upper panels are shown at higher magnifications in the lower panels. Scale bars = 100 μm (top panels) and 50 μm (bottom panels). (B) DNA fragmentation analysis of cell death. Primary chondrocytes were isolated from the femur growth plates of WT and *Nfic*$^{-/-}$ mice. M, DNA size marker. (C) Western blot analysis of the expression pro-caspase-3 and cleaved caspase-3 in primary chondrocyte lysates of WT and *Nfic*$^{-/-}$ mice.

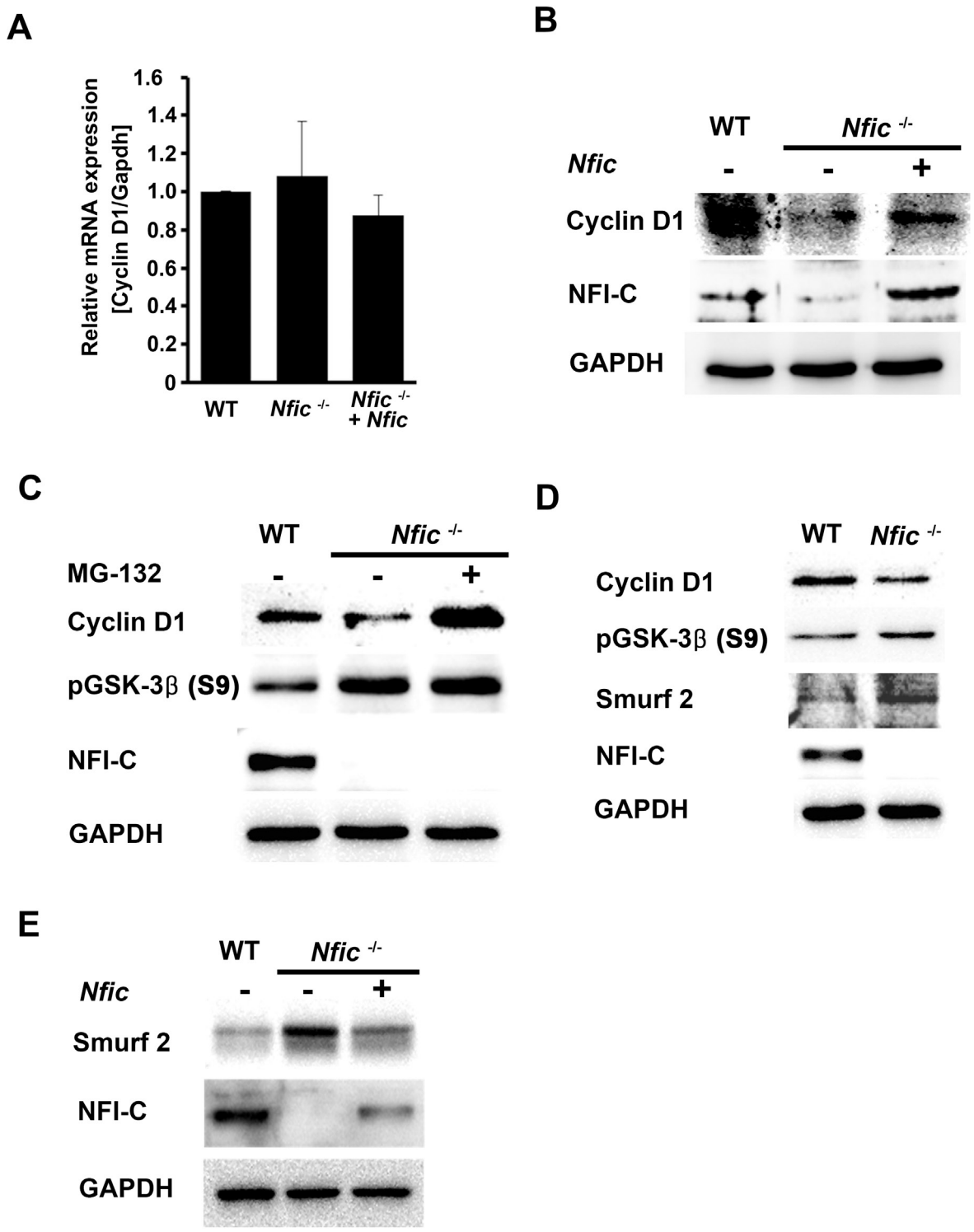

Fig. 4. Nfic is required for the regulation of cyclin D1 protein stability in chondrocytes. (A) Real-time PCR analysis of the expression of cyclin D1 in primary WT and *Nfic*$^{-/-}$ chondrocytes. Primary chondrocytes were transfected with the Nfic expression plasmid for 48 h. (B) Primary chondrocytes were transfected with the Nfic expression plasmid for 48 h. Western blot analysis of the expression of cyclin D1 and NFI-C. (C) Primary chondrocytes were incubated in the presence or absence of the proteasome inhibitor MG-132 (5 μM) for 24 h. Western blot analysis of the expression of cyclin D1, pGSK-3β, and NFI-C. (D) Western blot analysis of the expression of cyclin D1, pGSK-3β, and Smurf2. (E) Western blot analysis of Smurf2 expression. Primary chondrocytes were transfected with the Nfic-expressing plasmid for 48 h.

Increased chondrocyte apoptosis in the femoral growth plates of Nfic-deficient mice

We next determined if the decrease in overall length of the growth plate was caused by cell death. Apoptotic cells detected using the TUNEL POD assay were mainly present in the hypertrophic zone of *Nfic*$^{-/-}$ mice (Fig. 3A). DNA fragmentation assays revealed increased apoptosis of chondrocytes isolated from *Nfic*$^{-/-}$ vs WT mice (Fig. 3B). Activation of the effector caspase-3 is required for the activities of intrinsic and extrinsic apoptosis pathways. Western blot analysis detected upregulation of the levels of the precursor and active forms of caspase-3 in *Nfic*-deficient chondrocytes vs those of the WT (Fig. 3C). These results suggest that Nfic deficiency may induce caspase-dependent apoptosis of hypertrophic chondrocytes in growth plates.

Nfic regulates the stability of cyclin D1 protein

To determine whether the overexpression of Nfic restored the levels of cyclin D1 in *Nfic*$^{-/-}$ mice to those of WT, primary chondrocytes were transfected with Nfic expression vectors. The levels of the mRNA encoding cyclin D1 did not significantly differ among WT, *Nfic*$^{-/-}$ chondrocytes, and transfected *Nfic*$^{-/-}$ chondrocytes (Fig. 4A). In contrast, cyclin D1 protein levels in transfected *Nfic*$^{-/-}$ chondrocytes were restored to those of WT (Fig. 4B). The discrepancy in the relative levels of mRNA and protein suggests that Nfic may regulate the protein stability of cyclin D1. To address this possibility, primary chondrocytes were incubated with the proteasome inhibitor MG-132 and subjected to western blot analysis. Cyclin D1 levels in primary chondrocytes from *Nfic*$^{-/-}$-deficient mice were upregulated upon MG-132 treatment, indicating that cyclin D1 levels in primary chondrocytes from the growth-plate cartilage of *Nfic*$^{-/-}$-mice were restored to those of WT by inhibiting proteasomal degradation (Fig. 4C). To gain further insight into the mechanism, we analyzed the expression of other proteins that may participate in the degradation of cyclin D1.

Glycogen synthase kinase-3 (GSK3) regulates cyclin D1 expression at the transcriptional and post-translational levels. Post-translational regulation is achieved via direct phosphorylation of cyclin D1, which is subsequently degraded (Diehl et al., 1997; 1998; Reya and Clevers, 2005). We found that the expression of pGSK-3β, the inactive form of GSK-3β, was upregulated in primary chondrocytes of *Nfic*$^{-/-}$ mice vs those of WT. This result indicates that cyclin D1 protein expression was not likely regulated in a GSK-3β-dependent manner in *Nfic*$^{-/-}$ mice (Fig. 4C). Another candidate regulatory molecule called Smurf is a HECT-type E3 ubiquitin ligase that mediates Nfic degradation in odontoblasts. When ATDC5 cells were transfected with Nfic expression vectors, Smurf2 mRNA expression was downregulated (Supplementary Fig. S4). In *Nfic*$^{-/-}$ chondrocytes, Smurf1 (data not shown) and Smurf2 protein levels were upregulated compared with those of WT (Fig. 4D). This upregulation was inhibited by transfecting Nfic-expressing plasmids in *Nfic*$^{-/-}$ chondrocytes (Fig. 4E). These findings indicate that Nfic may regulate cyclin D1 expression in chondrocytes via inhibition of proteasomal degradation, possibly through a Smurf2-mediated mechanism.

DISCUSSION

In this study, we showed that postnatal deficiency of Nfic affects the development of the femoral growth plate. The reduced thickness of the proliferating and hypertrophic zones in *Nfic*$^{-/-}$ mice led us to analyze the expression of cell cycle regulators such as P21 and cyclin D1 to determine the function of Nfic in the formation and homeostasis of growth-plate cartilage. We show here that the levels of the mRNA encoding cyclin D1 were unaffected in primary chondrocytes isolated from *Nfic*$^{-/-}$ mice compared with those of WT mice. However, the protein levels of cyclin D1 in the former were significantly downregulated compared with those of WT. Moreover, ectopic expression of Nfic in *Nfic*$^{-/-}$ chondroyctes restored its levels to those of WT. These results suggest that the discrepancy in the levels of Nfic mRNA and protein was associated with regulation of the protein stability of cyclin D1 via Nfic.

The cell cycle is mainly regulated by cyclin-dependent kinases (CDK) in association with their partner proteins, the cyclins. During the G1–S phases of the cell cycle, decisions are made to proceed through the cell cycle and proliferate or to exit and arrest the cell cycle (Baldin et al., 1993; Ewen et al., 1993; Sherr, 1993). Cyclin D1 forms a complex with CDK4 or CDK6 to mediate the transition from G1 to S phase (Morgan, 1997; Sherr, 1995; Takahashi-Yanaga and Sasaguni, 2008). Moreover, cyclin D1 is specifically expressed in proliferating chondrocytes and is required for their proliferation. Further, cyclin D1-knockout mice exhibit a shortened growth-plate proliferative zone (Fantl et al., 1995; Yang et al., 2003; Zhang et al., 2009).

Cyclin D1 protein expression is regulated via a ubiquitin-dependent mechanism. Phosphorylated cyclin D1 is translocated from the nucleus to the cytoplasm where it undergoes degradation by the 26S proteasome. Evidence indicates that phosphorylation of cyclin D1 is mediated by GSK-3β, although other studies demonstrate that the degradation of cyclin D1 occurs in a GSK-3β-independent manner (Alao, 2007; Diehl et al., 1998; Takahashi-Yanaga and Sasaguri, 2008). For example, lithocholic acid hydroxyamide simultaneously up-regulates GSK-3β phosphorylation and down-regulates cyclin D1 protein expression in HCT116 cells (Magiera et al., 2017). These studies support our hypothesis that the stability of cyclin D1 is not regulated in a GSK-3β dependent manner in primary chondrocytes of *Nfic*$^{-/-}$ mice.

Regulation of the stability of cyclin D1 may be mediated instead via Smad ubiquitin regulatory factors (Smurfs). Smurfs are C2-WW-HECT-domain E3 ubiquitin ligases that regulate the components of the TGF-β and BMP signal transduction pathways as well as others (Izzi and Attisano, 2004; Waterfield, 1991). Smurf1 negatively regulates the proliferation and differentiation of MSCs into osteoblasts. Upregulation of cyclin D1 expression occurs in Smurf1$^{-/-}$ MSCs (Zhao et al., 2010), and Smurf2 causes an osteoarthritis-like phenotype when overexpressed (Huang et al., 2016; Xing et al., 2010). In odontoblasts, Smurf mediates the ubiquitination and degradation of Nfic by forming an Nfic-Smad2/3-Smurf complex (Lee et al., 2011). Here we found that the upregulated expression of Smurf1 (data not shown) and Smurf2 in

Nfic$^{-/-}$ chondrocytes suggests interactions between Nfic and Smurfs that regulate the proliferation of chondrocytes. Future research is therefore required to confirm whether Smurfs directly regulate the expression of cyclin D1. Similarly, SCF E3 ubiquitin ligases such as Fbx4 and Skp2 are suggested to regulate the stability of cyclin D1; these ligases may interact with Nfic and these interactions need to be investigated (Ganiatsas et al., 2001; Lin et al., 2006; Russell et al., 1999).

In joint cartilage, TGF-β1 promotes matrix synthesis and prevents terminal differentiation of chondrocytes, and the expression of TGF-β1 is regulated in a temporospatial manner (Liu, 2006; Ouellet et al., 2006). Interactions between TGF-β and Nfic may stringently regulate the expression of TGF-β during cartilage formation. Upregulated expression of p21 in hypertrophic chondrocytes suggests that p21 functions in the exit from the cell cycle and in the differentiation of chondrocytes (Stewart et al., 1997). p21$^{CIP1/Waf1}$ (p21) is one of the inhibitors of CDK (INK4) family that are capable of inactivating the cyclin-CDK complexes (Roussel, 1999). Interestingly, TGF-β and Nfic perform opposing functions in the regulation of p21 expression, and TGF-β induces the inhibition of cell proliferation by up-regulating the expression of p21 (Stewart et al., 1997). Moreover, Nfic inhibits the expression of p21 (Liu, 2006; Ouellet et al., 2006). These findings suggest that the crosstalk between Nfic and TGF-β may regulate the proliferation and differentiation of chondrocytes via the regulation of p21 expression.

Degradation and remodeling of the cartilage matrix is essential during the formation of endochondral bone. Matrix metalloproteinase (MMP) family members cleave the major components of the cartilage ECM (Birkedal-Hansen et al., 1993; Werb, 1997). Endochondral bone formation in mice deficient in MMP9 and MMP13 is severely impaired and is associated with decreased ECM remodeling and an expanded hypertrophic zone in the growth plate (Stickens et al., 2004; Vu et al., 1998). Here we show that in *Nfic*$^{-/-}$ mice the levels of MMP9 and MMP13 were upregulated at the transcriptional and translational levels in the growth plate (Supplementary Fig. S5). Further, a markedly shrunken hypertrophic zone morphology is consistent with previous observations. These findings suggest that Nfic deficiency may lead to aberrant MMP expression and cause excess degradation of the cartilage ECM, resulting in defective cartilage formation in the growth plate. Among the transcription factors that regulate MMP expressions, hypoxia-inducible factor 2 alpha (HIF-2α) shares an overlapping transcription factor binding site with NFI-C near the promoter region (Smythies et al., 2019). This raises an intriguing possibility that the interaction between HIF-2α and NFI-C plays a role during cartilage development.

In summary, our results indicate that Nfic is essential for the postnatal proliferation of chondrocytes and cartilage formation in the growth plate cartilage. Nfic modulated the expression of cell cycle regulators such as p21 and cyclin D1 (Fig. 5). The stability of cyclin D1 protein was regulated via Nfic, and Smurfs may be involved. Further studies are warranted to identify the detailed mechanisms underlying the regulation of the protein stability of cyclin D1 by Nfic through Smurfs. Moreover, other factors and signaling pathways may interact with Nfic during cartilage formation, for example, SCF E3 ubiquitin ligases and the TGF-β signal transduction pathway as well as with MMP9, and MMP13. Further studies are required to confirm this hypothesis. We believe that it is therefore reasonable to conclude that therapy targeting Nfic may contribute to the prevention and treatment of disorders caused by damage to cartilage and the associated growth defects.

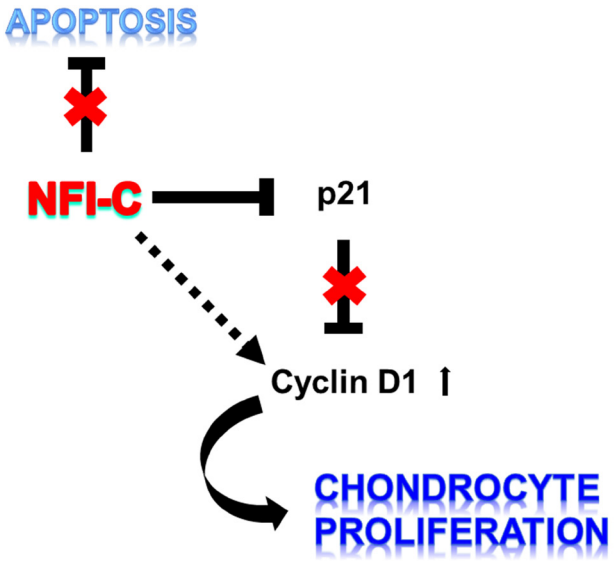

Fig. 5. Nfic regulates chondrocyte proliferation by modulating the expression of p21 and cyclin D1. Schematic showing the function of Nfic during chondrocyte proliferation. Nfic regulates cyclin D1 expression by inhibiting p21 expression and modulating the protein stability of cyclin D1.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS

D.S.L. designed the model and the computational framework and analysed the data. S.Y.R. analyzed the data and wrote the manuscript with input from all authors. H.C. developed the theoretical framework and analyzed the data. J.C.P. supervised the project. All authors discussed the results and commented on the manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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