JMJD5, a Jumonji C (JmjC) Domain-containing Protein, Negatively Regulates Osteoclastogenesis by Facilitating NFATc1 Protein Degradation

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Background: NFATc1 is a necessary and sufficient transcription factor for osteoclastogenesis.

Results: JMJD5 negatively regulates NFATc1 protein level through its hydroxylase activity.

Conclusion: JMJD5 is a novel osteoclastogenic repressor that induces the degradation of NFATc1 protein.

Significance: This study revealed a novel mechanism that regulates NFATc1 activity during osteoclastogenesis.

Osteoclastogenesis is a highly regulated process governed by diverse classes of regulators. Among them, nuclear factor of activated T-cells calcineurin-dependent 1 (NFATc1) is the primary osteoclastogenic transcription factor, and its expression is transcriptionally induced during early osteoclastogenesis by receptor activation of nuclear factor κB ligand (RANKL), an osteoclastogenic cytokine. Here, we report the novel enzymatic function of JMJD5, which regulates NFATc1 protein stability. Among the tested Jumonji C (JmjC) domain-containing proteins, decreased mRNA expression levels during osteoclastogenesis were found for JMJD5 in RAW264 cells stimulated by RANKL. To examine the functional role of JMJD5 in osteoclast differentiation, we established stable JMJD5 knockdown cells, and osteoclast formation was assessed. Down-regulated expression of JMJD5 led to accelerated osteoclast formation together with induction of several osteoclast-specific genes such as Ctsk and DC-STAMP, suggesting that JMJD5 is a negative regulator in osteoclast differentiation. Although JMJD5 was recently reported as a histone demethylase for histone H3K36me2, no histone demethylase activity was detected in JMJD5 in vitro or in living cells, even for other methylated histone residues. Instead, JMJD5 co-repressed transcriptional activity by destabilizing NFATc1 protein. Protein hydroxylase activity mediated by the JmjC domain in JMJD5 was required for the observed functions of JMJD5. JMJD5 induced the association of hydroxylated NFATc1 with the E3 ubiquitin ligase Von Hippel-Lindau tumor suppressor (VHL), thereby presumably facilitating proteasomal degradation of NFATc1 via ubiquitination. Taken together, the present study demonstrated that JMJD5 is a post-translational co-repressor for NFATc1 that attenuates osteoclastogenesis.

Mature osteoclasts are giant multinucleated cells that are responsible for bone resorption in bone remodeling and function in a regulated balance with bone-forming osteoblasts (1, 2). Commitment of hematopoietic stem cells to differentiate to osteoclasts is defined by two key factors, macrophage colony-stimulating factor and receptor activation of nuclear factor κB ligand (RANKL),3 factors that are produced by osteoblast lineage cells in bone tissues (3–6). During cell maturation, mononuclear precursor cells fuse to generate multinucleated osteoclasts. This multinucleation process is necessarily required for osteoclastic maturation to undergo efficient bone resorption (7, 8). In reorganizing genetic networks during osteoclastogenesis, diverse transcription factors play pivotal roles, and among them, nuclear factor of activated T-cells calcineurin-dependent 1 (NFATc1) is the necessary and sufficient transcription factor (9, 10). Osteoclastogenesis induced by RANKL is aborted in NFATc1−/− embryonic stem cells (9). Therefore, NFATc1 is considered to be an essential factor in osteoclastogenesis. Moreover, ectopic expression of NFATc1 in osteoclast precursor cells efficiently induces osteoclastogenesis even in the absence of RANKL stimulation (10). Supporting the importance of NFATc1 in osteoclastogenesis, NFATc1 is a transcriptional inducer of several osteoclast-specific genes.

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3 The abbreviations used are: RANKL, receptor activation of nuclear factor κB ligand; JmjC, Jumonji C; NFATc1, nuclear factor of activated T-cells calcineurin-dependent 1; RANK, receptor activation of nuclear factor κB; VHL, Von Hippel-Lindau tumor suppressor; DC-STAMP, dendritic cell-specific transmembrane protein; Acp5, acid phosphatase 5, tartrate-resistant; Ctsk, cathepsin K; FH1, factor inhibiting HIF1; JMJD6, Jumonji domain-6; HIF1, hypoxia-inducible factor 1; 2-OG, 2-oxoglutarate; RE, response element.
lates HIF1

histone proteins (24–27). FIH1 post-translationally hydroxylates 

main-6 (JMJD6), were characterized as hydroxylases for non-

hand, two JmjC domain-containing proteins, factor inhibiting

(H3K36), JHDM2 for H3K27, and so on (20–23). On the other

lases, including JHDM1 for the lysine 36 residue in histone H3

methylation modifiers, Jumonji C (JmjC) domain-

upstream in epigenetic regulation of transcription. Among var-

iation, and a histone methylation signature generally appears

has emerged in studies of the regulation of cellular differentia-

lational regulation.

stages of osteoclast differentiation by Cbl protein-mediated

ing to its robust induction (16). On the other hand, it was

recently reported that NFATc1 protein is degraded during late

of RNA splicing factors, suggesting its specific roles in the reg-

quences of all cDNAs were verified by automated sequenc-

For the NFATc1-response element (RE) reporter plasmid, the 

Acp5 promoter (−485 to +525) was amplified from genomic DNA of RAW264 cells and cloned into the upstream

region of the luciferase gene derived from pGL4.10 (luc2) vec-

ector (Promega). Oligonucleotide short hairpin RNA (shRNA)

targeting mouse JMJD5 (shRNA 1; 5′-GGAAGGAATTACATCCGAGTTG-3′) was inserted into the pSUPER-Retro vec-

tor (OligoEngine) according to the manufacturer’s instructions.

Cell Culture and Transfection—HEK293T cells were main-

in Dulbecco’s modified Eagle medium (Wako) supplemented

with 10% fetal bovine serum (JHR Biosciences) and 1%

medium (Invitrogen) and a control oligo-

sequences of all cDNAs were verified by automated sequenc-

In this study, we show that one of the epigenetic regulators, a

JmjC domain-containing protein, JMJD5, negatively regulates

osteoosteoclastogenesis by reducing the stability of NFATc1 protein via its hydroxylase activity. Moreover, JMJD5 was found to

duce the association of NFATc1 with E3 ubiquitin ligase Von

Hippel-Lindau tumor suppressor (VHL) in HEK293T cells. Thus, the present study demonstrates that the enzymatic func-
tion of a JmjC domain-containing protein acts as a post-trans-
lational co-repressor of a primary transcription factor during 

osteoosteoclastogenesis.

EXPERIMENTAL PROCEDURES

Plasmids—Full-length cDNA of mouse JMJD5 was obtained by RT-PCR from total RNA of mouse heart and cloned into the 

pcDNA3-FLAG or the pcDNA3.1-Myc mammalian expression plasmids (Invitrogen). To generate an expression plasmid of 

ZsGreen-tagged JMJD5, cDNA for ZsGreen protein was amplified from pZsGreen1-DR plasmid (Clontech) by PCR. Ampli-
fied cDNA was subcloned into the C terminus of the previously generated pcDNA3-FLAG-JMJD5 plasmid. The point mutant 

FLAG-JMJD5-K334A plasmid was generated from the wild-

type plasmid by site-directed mutagenesis (28). GST-fused 

JMJD5 and mutant JMJD5-K334A expression plasmids were 

generated by subcloning into the pGEX-4T-1 plasmid. Full-
lengt

cDNAs of mouse NFATc1α and NFATc1β were obtained by RT-PCR from mouse spleen and cloned into the 

pcDNA3-FLAG or the pcDNA3.1-Myc mammalian expression vector. To generate GST-fused truncated NFATc1α con-

structs, amino acids 1–300, 301–596, and 589–827 were par-
tially amplified by PCR from pcDNA-FLAG-NFATc1α plasmid 

and then subcloned into the pGEX-4T-1 plasmid. A full-length

cDNA of mouse FIH1 was obtained by RT-PCR from mouse 

tests and cloned into the pGEX-4T-1 plasmid. A full-length 
cDNA of mouse p105 was obtained by RT-PCR from total RNA 
of mouse spleen and cloned into the pGEX-4T-1 plasmid. For 

the GST-fused p105-ARD expression plasmid, amino acids 

537–809 were partially amplified by PCR from a full-length 

plasmid and then subcloned into the pGEX-4T-1 plasmid. 

Sequences of all cDNAs were verified by automated sequenc-

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targeting mouse JMJD5 (shRNA 1; 5′-GGAAGGAATTA-

CATCCGAGTTG-3′) was inserted into the pSUPER-Retro vec-

tor (OligoEngine) according to the manufacturer’s instructions.

Cell Culture and Transfection—HEK293T cells were main-

in Dulbecco’s modified Eagle medium (Wako) supplemented 

with 10% fetal bovine serum (JHR Biosciences) and 1%

antibiotic-antimycotic solution (Invitrogen), and 1% minimum 

medium (Invitrogen) and a control oligo-

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10^4 cells/well and were treated with 235 ng/ml GST-RANKL (Oriental Yeast Co., Ltd.) for 6 days (29). The medium was changed every 2nd day. Differentiated cells were fixed with 3.7% formaldehyde for 10 min at room temperature and visualized with an Olympus IX70 light microscope equipped with Zeiss AxioVision 4.6.

Establishment of Stable Cell Lines—RAW264 cells were transfected with the pSUPER-mJMJD5-shRNA plasmid by Lipofectamine 2000 (Invitrogen). After 24 h, 3 µg/ml puromycin (Sigma) was added, and cultivation was continued for approximately 3 weeks to select stably transfected cells (29). Culture media were changed every 2nd day. After detecting colony formation, several colonies were individually picked up, and their knockdown efficiency was confirmed by RT-PCR.

RNA Analysis—Total RNA was extracted with TRIzol reagent (Invitrogen) according to a previous report (30). For real time RT-PCR, 500 ng of total RNA was reverse transcribed into first strand cDNA with oligo(dT) and random hexamers using a SuperScript III reverse transcriptase (Invitrogen), and cDNA was amplified with the indicated primer sets. All primer sets used are indicated in the supplemental Experimental Procedures.

Luciferase Assay—HEK293T cells in 12-well plates were transfected with the NFATc1-RE reporter plasmid (500 ng) together with each expression vector (700 ng). Transfection efficiency was normalized by co-transfection with pGL4.75 (hRluc/CMV) (5 ng). After 36 h, a luciferase assay was performed with the Dual-Luciferase Reporter assay system (Promega) according to the manufacturer’s instructions.

2-OG Decarboxylation Assay—Decarboxylation assays of ^14^C-radio labeled 2-OG were performed as reported previously (26). Briefly, 80 µM enzyme, 80 µM substrate, 24 µM ^14^C-radio labeled 2-OG, 576 µM 2-OG, 8 mM sodium ascobicte, 200 µM (NH₄)₂Fe(SO₄)₂, and 2 mM DTT were mixed in a total volume of 100 µl with 50 mM Tris-Cl (pH 7.5). A 500-µl Eppendorf tube containing 200 µl of hyamine hydroxide (Fisher Scientific) was added to each tube, and tubes were closed with a rubber septum. After incubation at 37 °C for 30 min, the reaction was stopped by setting tubes on ice for 30 min. Then Eppendorf tubes containing the hyamine hydroxide were transferred to scintillation vials and mixed with Optiphase Hisafe 2 Liquid Scintillation Mixture (Fisher Scientific). Total released ^14^C counts were quantified using an LSC-5100 scintillation counter (Aloka). Data were analyzed by two-tailed Student’s t test, and p < 0.05 was accepted as significant.

GST-fused Protein Preparation—GST-fused proteins were prepared as described previously (31–33). Briefly, established plasmids for GST-fused proteins were transformed into Escherichia coli strain Rosetta (DE3) (Novagen), which was grown at 37 °C in LB medium containing 100 µg/ml ampicillin. GST-fused protein was induced by 0.5 mM isopropyl β-D-thiogalactoside and incubation at 25 °C for 5 h. Cell pellets were resuspended with LEW-Tween 20 buffer (50 mM NaH₂PO₄, 300 mM NaCl, 0.05% Tween 20, pH 7.4) containing 1 mg/ml lysozyme (Sigma) and sonicated with a UD-200 ultrasonic disruptor (Tomy). After centrifugation, the supernatants were mixed with glutathione-Sepharose 4B (GE Healthcare), and bound proteins were eluted with 50 mM glutathione (Wako). Ion exchange chromatography (ÄKTA Mono Q column, GE Healthcare) yielded highly pure GST-fused proteins that were visualized by Coomassie Brilliant Blue staining.

Immunocytochemistry—HEK293T cells were transfected with pcDNA3-FLAG-mJMJD5-ZsGreen plasmid. After 24 h of transfection, cells were plated in Lab-TekII chamber slides (Nalge Nunc International). After 48 h of transfection, cells were fixed with 2% paraformaldehyde in PBS and permeabilized in 0.05% Triton X-100 in PBS (PBST). Cells were blocked with 5% skim milk in PBST, and the indicated primary antibodies were bound overnight at 4 °C. Primary antibody signals were generated with a rhodamine-conjugated secondary antibody (Molecular Probes). The slides were mounted with mounting medium containing DAPI (Vector Laboratories, Inc.) and visualized with a fluorescence confocal microscope (LSM510, Carl Zeiss, Inc.) (28). The following primary antibodies were used: H3K4me2 (Upstate, 07-030), H3K4me3 (Abcam, ab8580), H3K9me1 (Upstate, 07-450), H3K9me2 (Abcam, ab1220), H3K27me1 (Upstate, 07-448), H3K27me2 (Upstate, 07-452), H3K27me3 (Upstate, 07-449), H3K36me1 (Abcam, ab9048), and H3K36me2 (Upstate, 07-369).

Immunoblotting and Immunoprecipitation—Cells were washed with PBS and resuspended with TNE buffer (10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, and protease inhibitors). Total cell lysates were resolved in SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk in PBST, and primary antibody was bound overnight at 4 °C. After washing with PBST, horseradish peroxide (HRP)-conjugated secondary antibody (Dako) was bound for 1 h at RT. Immunoreactive signals were detected with ECL Western blotting detection reagent (GE Healthcare) (32). Antibodies against FLAG (clone M2) and Myc (clone 4A6) epitopes were purchased from Sigma and Millipore, respectively. NFA4tc1 (sc-7294) and β-actin (sc-1616) antibodies were purchased from Santa Cruz Biotechnology. JMJD5 (ab36104), p65 (ab790), and p105/50 (ab7971) antibodies were purchased from Abcam. Antibodies against VHL (2738), ubiquitin (Z0458), and GAPDH (MAB374) were purchased from Cell Signaling Technology, Dako, and Chemicon, respectively. The following primary antibodies were used to detect histone modifications: H3K4me1 (Upstate, 07-436), H3K4me2 (Abcam, ab7766), H3K4me3 (Abcam, ab1012), H3K9me1 (Upstate, 07-395), H3K9me2 (Upstate, 07-441), H3K9me3 (Abcam, ab6001), H3K27me1 (Upstate, 07-448), H3K27me2 (Upstate, 07-452), H3K27me3 (Upstate, 07-449), H3K36me1 (Abcam, ab9048), and H3K36me2 (Upstate, 07-369).
Then immunoprecipitation was performed by incubating precleared lysates with 2 μg of anti-NFATc1 antibody overnight at 4 °C. After incubation with Protein G-Sepharose 4 fast flow for 1 h at 4 °C, immunoprecipitated proteins were washed five times with TNE buffer and analyzed by immunoblotting.

For immunoprecipitation assays with anti-FLAG antibody, total cell lysates were incubated with anti-FLAG M2-agarose affinity gel (Sigma). After 5 h of incubation at 4 °C, immunoprecipitated proteins were washed five times with TNE buffer and eluted with 400 μg/ml FLAG peptide (Sigma). Eluted proteins were analyzed by immunoblotting.

**RESULTS**

**JMJD5 Represses Osteoclastogenesis by Reducing Expression of Several Osteoclast-specific Genes**—To clarify epigenetic regulation during osteoclastogenesis, we focused on JmjC domain-containing proteins, which are histone demethylases. Their physiological roles in bone metabolism have not
been characterized. We first observed their expression profiles during osteoclastogenesis. RAW264 cells were differentiated to mature osteoclasts by RANKL treatment, and total RNA was prepared from each differentiation stage to assess the expression levels of JmjC domain-containing proteins. Through a real time RT-PCR analysis, we detected their various expression patterns. The expression levels of JMJD1a/2a transcripts increased, whereas those of JMJD5/3 decreased (Fig. 1A and supplemental Fig. S1). On the other hand, the expression level of JMJD4 transcripts was unaltered during osteoclastogenesis.

Although various factors, such as NFATc1, promote osteoclastogenesis and are induced during osteoclastogenesis (9), little is known about intracellular negative regulators of osteoclastogenesis. Because JMJD5/3 expression levels decreased during osteoclastogenesis, we reasoned that their functions were related to negative regulation. To test this idea, stable cell lines constitutively expressing JMJD5- or JMJD3-specific shRNAs with the puromycin-resistant gene were established from RAW264 cells by puromycin selection. Each cell line was then treated with RANKL for osteoclast formation. The shRNA knockdown was confirmed by attenuated expression levels of either JMJD3 or JMJD5 transcripts in the stable lines (Fig. 1B and supplemental Fig. S2). The stable RAW264 JMJD3 knockdown line differentiated normally into osteoclasts and displayed normal expression levels of osteoclast-specific genes Acp5, DC-STAMP, and Nfatc1 (supplemental Fig. S2). However, when JMJD5 was knocked down, osteoclastic maturation was accelerated (Fig. 1C and supplemental Fig. S3). The same results were observed in bona fide osteoclasts derived from murine bone marrow macrophages (supplemental Fig. S4). Consistent with the accelerated formation of osteoclasts, the induced expression of Ctsk, DC-STAMP, and Acp5 genes was also observed (Fig.

**FIGURE 2.** Overexpressed JMJD5 did not affect methylation levels on tested histone H3 lysine residues. A, HEK293T cells were transfected with JMJD5-ZsGreen. After 36 h of transfection, cells were fixed and analyzed by immunocytochemistry. JMJD5 was visualized by the tagged ZsGreen protein (green). Histone H3 lysine methylation levels were observed by staining with the indicated primary antibodies (red). IgG antibody was used for the negative control. DAPI was used to stain nuclei (blue). B, HEK293T cells were transfected with FLAG-mJMJD5. After 48 h of transfection, chromosomal extracts were prepared and analyzed by immunoblotting with the indicated primary antibodies. Histone H3 was used as loading control for immunoblotting.

**JMJD5 Is Novel Osteoclastogenic Repressor**
These results implied that JMJD5 is a negative regulator for osteoclast differentiation.

**JMJD5 Represses Transcriptional Activity of NFATc1 by Reducing Level of Its Protein**—JMJD5 is reportedly an H3K36me2 demethylase (34). To verify its histone demethylase activity, we measured the methylation level of histone H3K36 in HEK293T cells overexpressing JMJD5 using immunofluorescence and immunoblotting analyses. However, we could not detect the expected alteration in the level of H3K36me2 (Fig. 2A). Although other methylated histone lysine residues were tested with several methylation-specific histone antibodies, we did not detect altered methylation levels on any tested lysine residues of histone H3 (Fig. 2, A and B). Thus, from these observations, we assumed that JMJD5 is not a histone demethylase.

To assess other enzymatic functions of JMJD5, we examined the cellular distribution of JMJD5 protein during osteoclastogenesis based on its solubility differences in lysis buffers. JMJD5 was present in the chromatin fraction as expected, and its distribution appeared to be unaffected by RANKL stimulation (supplemental Fig. S5). JMJD5 was also abundant in the soluble nuclear fraction, suggesting that JMJD5 serves as a soluble transcriptional co-regulator in the nucleus rather than a histone demethylase bound to chromatin.

To test this idea, we examined the association of JMJD5 with the primary transcription factors for osteoclastogenesis, i.e. NFATc1, NFκB, and AP-1. We expressed and purified recombinant affinity-tagged full-length proteins from *E. coli* and then performed *in vitro* pulldown assays. His-tagged JMJD5 was an interactant with tested GST-fused transcription factors NFATc1/β, c-Jun, and NFkB (p105/50) but not c-Fos (supplemental Fig. S6). To further address their association, we tested the *in vivo* interactions within cells. Total cellular extracts were prepared from HEK293T cells expressing FLAG-JMJD5 and Myc-NFATc1, and these proteins were immunoprecipitated by anti-FLAG antibody. The bound proteins were eluted with FLAG peptides and observed by immunoblotting with anti-Myc and anti-NFkB (p65/50) antibodies. Among the tested factors, only NFATc1 was detected (Fig. 3A). The interaction of JMJD5 with NFATc1 was still significant with a catalytically inactive mutant form of JMJD5 (JMJD5-K334A) in which the Lys-334 was substituted by alanine (Fig. 3A, last lane).

NFATc1 is a primary transcription factor for osteoclastogenesis, and it regulates the expression of osteoclast-specific genes (9, 16). From its interaction with JMJD5, we hypothesized that JMJD5 co-regulated the transcriptional activity of NFATc1. To examine this possibility, we constructed an NFATc1-RE reporter plasmid by inserting the −485 to +525 region of the Acp5 gene in front of the luciferase gene and measured the transcriptional activity of NFATc1 when JMJD5 was overexpressed. The luciferase activity was increased 15-fold when NFATc1 was expressed, whereas it was significantly decreased by co-expression of JMJD5 (Fig. 3B). To characterize the repressive function of JMJD5, we then analyzed the lysates used to pull down the recombinant full-length proteins and observed a nonspecific band.
JMJD5 Is Novel Osteoclastogenic Repressor

FIGURE 4. JMJD5 reduced level of NFATc1 protein by post-translational regulation. A, HEK293T cells were transfected with human JMJD5-specific siRNA. After 48 h of siRNA transfection, cells were also transfected with human JMJD5-specific siRNA together with FLAG-NFATc1 and FLAG-JMJD5-WT or FLAG-JMJD5-K334A. After 48 h, total cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies. β-Actin was used as a loading control for immunoblotting. The signals obtained from the immunoblotting were quantified with the ImageJ program. Ctrl, control.

B

C

in the luciferase assay by immunoblotting, testing whether JMJD5 regulated an event after transcription. Unexpectedly, the NFATc1 protein level was significantly reduced by expression of JMJD5, indicating that decreased NFATc1 transcriptional activity is due to the reduced protein level of NFATc1 (Fig. 3C). Such JMJD5 action was not seen for NFkB (p50/65) (supplemental Fig. S7, A and B). Importantly, the suppressive actions of JMJD5 on the transcriptional activity and protein level of NFATc1 were not observed when the JMJD5-K334A mutant was expressed (Fig. 3C, last lane), suggesting that the repressive function of JMJD5 against NFATc1 depended on its 2-OG-dependent activity.

Post-translational Destabilization of NFATc1 Protein by JMJD5—To determine whether JMJD5 was capable of modulating the stability of NFATc1 protein, the level of exogenous NFATc1 protein was monitored during the knockdown of JMJD5. Human JMJD5-specific siRNA was introduced into HEK293T cells, and the level of exogenously expressed NFATc1 was measured by immunoblotting. As anticipated, reduced expression of JMJD5 was seen, and the NFATc1 protein level was significantly elevated (Fig. 4A). Furthermore, when JMJD5 was overexpressed in these knockdown cells, the NFATc1 protein level was indistinguishable from that in the control cells. The JMJD5-K334A mutant was not effective in regulating the protein level of NFATc1 (Fig. 4A).

Next, we asked whether the level of endogenous NFATc1 protein was indeed regulated by JMJD5. Using the stable cell lines expressing JMJD5-specific shRNA, total RNA and cellular extracts were prepared for RT-PCR and immunoblotting. Although the level of NFATc1 transcripts was unaltered (Fig. 4B), the level of NFATc1 protein was greater than that in the control knockdown cells (Fig. 4C). Taken together, it appears that the mechanism of down-regulating the level of NFATc1 protein by JMJD5 is post-translational.

JMJD5 Hydroxylates NFATc1 Protein through Its Enzymatic Activity—Recently, some members of JmjC domain-containing proteins have been shown to serve as protein hydroxylases, for example JMJD6 and FIH1 (24, 27). Because JMJD5 exhibits sequence homology with JMJD6 and FIH1, it is conceivable that JMJD5 is a protein hydroxylase. This idea is further supported by the present findings that a catalytically inactive JMJD5 mutant was impaired in the regulation of the NFATc1 protein level.

To address this idea, we performed an in vitro “2-OG decarboxylation assay” with GST-fused recombinant proteins (25, 26). Recombinant NFATc1 deletion mutant proteins were expressed and purified from E. coli (Fig. 5A), and each mutant was incubated with the purified GST-tagged JMJD5 to induce 14CO2 formation. After confirming FIH1 activity for the known hydroxylase substrate (p105) (Fig. 5B, lanes 7 and 8) (26), we tested whether NFATc1 is a hydroxylase substrate for JMJD5. The C-terminal domain of NFATc1 was found to serve as a substrate, although its 2-OG decarboxylation was less than that for p105 (Fig. 5B, lanes 5 and 6). To confirm that the observed decarboxylation activity of JMJD5 mediated by the JmjC domain, the JMJD5-K334A mutant was also used for this assay. As anticipated, a reduction in 14CO2 formation was seen (Fig. 5C). These findings suggest that JMJD5 has hydroxylase activity and that NFATc1 is its substrate.

Destabilization of NFATc1 by JMJD5 Mediates Ubiquitin-mediated Proteasomal Degradation—Here, we showed that JMJD5 destabilizes NFATc1 protein, presumably through its hydroxylase activity. To determine whether this process was related to protein degradation, we first examined the effect of a proteasome inhibitor (MG132). In HEK293T cells expressing exogenous NFATc1, NFATc1 protein did indeed accumulate in a time-dependent manner during MG132 treatment (Fig. 6A, lanes 4 – 6). Moreover, the reduced protein level of NFATc1 caused by co-expression of JMJD5 was completely reversed by MG132 treatment (Fig. 6A, lanes 7 and 8). We also confirmed in osteoclasts derived from bone marrow macrophages that endogenous NFATc1 protein accumulated during MG132 treatment to the same level as that seen when JMJD5 was knocked down (supplemental Fig. S8).

Next, we examined whether the proteasomal degradation of NFATc1 was ubiquitin-dependent. Immunoprecipitation by anti-NFATc1 antibody was performed in HEK293T cells exogenously expressing NFATc1 and JMJD5 after which the ubiquitinated NFATc1 was detected with an anti-ubiquitin antibody. Ubiquitinated NFATc1 was barely detectable when MG132 was used (Fig. 6B, upper panel, lane 4), whereas it was...
significantly increased by co-expression of JMJD5 (Fig. 6B, upper panel, lane 6). In addition, when the immunoprecipitants were probed with anti-FLAG antibody, the band shifts representing ubiquitinated NFATc1 were made more evident by JMJD5 co-expression (Fig. 6B, middle panel, lanes 5 and 6). These findings suggest that JMJD5 facilitates the ubiquitin-dependent proteasomal proteolysis of NFATc1.

Because JMJD5 does not contain a specific domain to ubiquitinate, it appeared likely that ubiquitin ligases were required for NFATc1 degradation by JMJD5. Among them, we focused on VHL (a component of the multimeric E3 ubiquitin ligase) because this factor is known to directly bind to HIF1α protein through a hydroxylated proline residue on HIF1α for subsequent ubiquitination and proteasomal degradation (35–37).

Therefore, we asked whether degradation of NFATc1 by JMJD5 was mediated by VHL. Total cellular extracts of HEK293T cells overexpressing NFATc1 and JMJD5 were immunoprecipitated with anti-FLAG antibody and probed with an anti-VHL antibody. VHL was visible when the cells were treated with MG132, indicating that it interacted with NFATc1 (Fig. 6C, lane 4). Furthermore, the interaction of NFATc1 with VHL was potentiated when JMJD5 was overexpressed (Fig. 6C, lane 6). Taken together, these results imply that JMJD5 facilitates the association of NFATc1 with VHL E3 ligase, and as a result, NFATc1 is prone to undergo ubiquitin-mediated proteasomal degradation (Fig. 6D).

**DISCUSSION**

**JMJD5 Attenuates Osteoclastogenesis**—In the present study, we showed that JMJD5 functions as a novel repressor for osteoclastogenesis. JMJD5 was found to serve as a protein hydroxylase for NFATc1, thereby facilitating NFATc1 protein degradation through association with VHL E3 ubiquitin ligase. This is a novel mechanism of regulating NFATc1 activity during osteoclastogenesis at a post-translational level. Interestingly, the expression level of JMJD5 was decreased the 1st day after
RANKL treatment in contrast to NFATc1 expression during osteoclastic differentiation (Fig. 1A). It appears that JMJD5 functions to maintain the undifferentiated state of osteoclastic progenitor cells. Because JMJD5 appears to be a co-regulator of NFATc1 at the post-translational level, this factor represents a previously uncharacterized class of regulators in osteoclastogenesis. The present study also implies the possible presence of other co-regulators of NFATc1 at other levels. Additionally, it is quite conceivable from recent reports of epigenetic co-regulators of DNA-binding transcription factors that co-regulators of other important transcription factors directing osteoclastogenesis remain to be identified.

It is currently believed that epigenetic regulation through histone methylation/demethylation plays a central role in gene regulation during differentiation of many types of cells, presumably also bone cells. In particular, such enzymes (histone methyltransferases and demethylases) would transcriptionally co-regulate the function of transcription factors governing osteoclastic differentiation. For example, the JmJ domain-containing protein NO66 reportedly acts as a negative regulator of Osterix, an essential transcription factor in osteoblastogenesis (38). Consistent with biochemical characterization of NO66 as a direct interactant with Osterix, NO66 co-repressed the Osterix-mediated gene cascade through demethylation of active histone methylation marks (H3K4 and H3K36). Gene regulation by epigenetic marks is also evident during osteoclastic differentiation. Likewise, promoter activity of the \( Nfatc1 \) gene appears to be under negative control by methylation of histone H3K4me3 and H3K27me3 at an undifferentiated stage of osteoclasts (39). In contrast, the inactive methylation markers of H3K27me3 are removed for \( Nfatc1 \) gene induction along with RANKL-induced osteoclastogenesis (39). Therefore, it is quite conceivable that JmJ domain-containing proteins are critical for proper regulation of nuclear events during osteoclastic differentiation through histone demethylation.

**FIGURE 6.** JMJD5 induced ubiquitin-mediated proteasomal degradation of NFATc1. A, HEK293T cells were co-transfected with FLAG-NFATc1 and FLA
g-\( n \)MJD5 and then treated with MG132 (10 \( \mu \)M) for the indicated times. After 48 h of transfection, total cell lysates were prepared and analyzed by immunoblotting with anti-FLAG antibody. \( \beta \)-Actin was used as a loading control for immunoblotting. The signals obtained from the immunoblotting were quantified with the ImageJ program. B and C, HEK293T cells were co-transfected with FLAG-NFATc1 and Myc-mJMJD5 and then treated with MG132 (10 \( \mu \)M) for 16 h. After 48 h of transfection, total cell lysates were prepared and subjected to immunoprecipitation (IP) with an anti-NFATc1 antibody (B) or anti-FLAG M2-agarose (C). Bound proteins were detected by immunoblotting with the indicated antibodies. GAPDH was used as loading control for immunoblotting. D, suggested model.
JMJD5 Lacks Histone Demethylase Activity—It was recently reported that JMJD5 is a histone demethylase (34). However, in our experimental settings, no histone demethylase activity was detected for methylated H3K36 and the known methylated histone lysine residues (Fig. 2). Based on the abundant distribution of JMJD5 in the nucleus, we next raised the possibility that its associating protein, NFATc1, is a substrate of JMJD5 demethylase. However, methylated lysine residues were not evident on NFATc1 protein when tested by immunoblotting with anti-Lys methylation antibody (data not shown). From the fact that JMJD5 is a member of the same JmJC domain-containing protein group as FIH1 and JMJD6 protein hydroxylases (24–27, 40), we next asked whether JMJD5 functioned as a hydroxylase of NFATc1. Indeed, hydroxylation of NFATc1 by JMJD5 was detected with an in vitro 2-OG decarboxylation assay (Fig. 5), suggesting that JMJD5 bears uncharactertized enzymatic function as an NFATc1 hydroxylase. As JMJD5 is found in the soluble nuclear fraction (supplemental Fig. 5), other non-histone proteins may be hydroxylated substrates for JMJD5.

These previous and present observations suggest that JMJD5 has dual functions, and the functions are very dependent on its cellular locations, functioning as a histone demethylase in chromatin and functioning as a protein hydroxylase in soluble nucleus. In our experimental system, although distribution of JMJD5 was detected in chromatin, it was more abundantly located in soluble nucleus (supplemental Fig. 5). Thus, we think that we could easily detect its function as a hydroxylase but not its histone demethylase activity, which may occur at a level below our detection.

Hydroxylation Is One Post-translational Modification Directing NFATc1 Functions—Post-translational modifications are important to regulate the function or expression of many kinds of proteins. It is well known that NFATc1 is modified by phosphorylation (41–43). In its phosphorylated form, NFATc1 is present in the cytoplasmic compartment and is imported into the nucleus when dephosphorylated by calcineurin. Recently, it was reported that NFATc1 is ubiquitinated in the cytoplasm for its degradation during late stages of osteoclastogenesis (19). However, it remained to be uncovered whether post-translational modifications regulated NFATc1 during osteoclastogenesis. In the present study, we provide evidence of a novel regulatory system for NFATc1 protein stability by post-translational modification. Specifically, NFATc1 is hydroxylated by JMJD5 and degraded via a ubiquitin-mediated proteasomal pathway. Hypoxia-specific transcription regulator HIF1α is constitutively synthesized at a high level even under normoxia. However, its level is repressed through hydroxylation on specific proline residues by prolyl hydroxylases and subsequent proteasomal degradation until hypoxia is induced. In this respect, it will be interesting to understand at the molecular level how JMJD5 enzymatic activity is controlled during osteoclastogenesis.

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