Nuclear Factor of Activated T-cells (NFAT)c2 Inhibits Notch Receptor Signaling in Osteoblasts*

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Background: Notch and nuclear factor of activated T-cells (NFAT)c signaling regulate cell function and interact in osteoblasts.

Results: Notch stabilizes NFATc2 transcripts, NFATc2 inhibits Notch by competing for DNA binding with the Notch transcriptional complex, and Notch and NFATc2 suppress osteoblast gene markers.

Conclusion: Notch and NFATc2 interact to inhibit osteoblast function.

Significance: Notch and NFAT signaling suppress osteoblastogenesis.

Notch receptors regulate osteoblastogenesis, and Notch activation induces cleavage and nuclear translocation of the Notch intracellular domain (NICD), which associates with Epstein-Barr virus latency C-promoter binding factor-1/suppressor of hairless/lag-1 (CSL) and induces transcription of Notch target genes, such as hairy enhancer of split-related with YRPW motif (Hey)1 and Hey2. Nuclear factors of activated T-cells (NFAT) are transcription factors that regulate osteoblastogenesis, but their function in osteoblasts is not clear. Notch inhibits NFATc1 transcription, but interactions between Notch and NFAT are understood poorly. To determine the regulation of NFAT expression by Notch, osteoblasts from RosaNotch mice, where NICD is transcribed following excision of a loxP flanked STOP cassette, were used. Alternatively, wild-type C57BL/6 osteoblasts were exposed to the Notch ligand Delta-like (Dll)1 to induce Notch signaling or to bovine serum albumin as control. In RosaNotch osteoblasts, Notch suppressed NFATc1 expression, increased Nfatc2 mRNA by post-transcriptional mechanisms, and had no effect on NFATc3 and NFATc4 transcripts. Induction of Nfatc2 transcripts by Notch was confirmed in C57BL/6 osteoblasts exposed to Dll1. To investigate NFATc2 function in osteoblasts, constitutively active NFATc2 was overexpressed in RosaNotch osteoblasts. NFATc2 suppressed Notch transactivation and expression of Hey genes. Electrophoretic mobility shift assays revealed that NFATc2 and CSL bind to similar DNA sequences, and chromatin immunoprecipitation indicated that NFATc2 displaced CSL from the Hey2 promoter. The effects of NICD and NFATc2 in RosaNotch osteoblasts were assessed, and both proteins inhibited osteoblast function. In conclusion, Notch stabilizes Nfatc2 transcripts, NFATc2 suppresses Notch signaling, and both proteins inhibit osteoblast function.

The Notch signaling pathway develops developmental processes, cell renewal, and cell fate (1). Notch1 to Notch4 are a family of transmembrane receptors that interact with transmembrane ligands expressed by neighboring cells (2). Ligand-receptor interactions result in the proteolytic cleavage and release of the Notch intracellular domain (NICD) (3). Epstein-Barr virus latency C promoter binding factor 1, suppressor of hairless and lag-1 (CSL), also known as Rbp-jc in mice, is a nuclear protein constitutively bound to DNA, able to suppress gene expression by recruiting transcriptional co-repressors. In the canonical signaling pathway, NICD translocates to the nucleus and forms a multimeric protein complex with CSL, displacing the transcriptional co-repressors and recruiting co-activators of transcription (4). These events result in the expression of Notch target genes, such as hairy enhancer of split (Hes) and Hes related with YRPW motif (Hey)1 and Hey2 (5).

Nuclear factors of activated T-cells (NFAT)c1 to NFATc4 are transcription factors that regulate growth and differentiation of multiple cell types. NFAT transactivation is induced by calcineurin, a phosphatase that dephosphorylates specific serine residues in the SRR and SPXX repeat motifs of the regulatory domain of NFAT (6). Dephosphorylated NFAT translocates to the nucleus, binds to specific DNA consensus sequences, such as those present upstream of exon 4 of regulator of calcineurin (Rcan1.4), and as a consequence induces expression of NFAT target genes (6, 7). NFAT phosphorylation by protein kinases, such as casein kinase 1 and glycogen synthase kinase 3β, induces NFAT nuclear export, preventing its binding to DNA, and as a consequence inhibiting NFAT transactivation (6).

Osteoblast cell fate and function are regulated by signaling networks that include the Notch and calcineurin/NFAT signal-

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††The abbreviations used are: NICD, Notch intracellular domain; Ad, adenovirus; ca, constitutive active; CSL, Epstein-Barr virus latency C-promoter binding factor-1/suppressor of hairless/lag-1; Dll1, Delta-like 1; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; Hey, hairy enhancer of split; Hey, Hes-related with YRPW motif; hnRNA, heterogeneous nuclear RNA; Nfatc, nuclear factor(s) of activated T-cells c; qPCR, quantitative PCR; qRT-PCR, quantitative reverse transcription-PCR; Rpl38, ribosomal protein I38; Runx, runt-related transcription factor; luc, luciferase.
Notch promotes osteoblast proliferation, possibly by inducing expression of cyclin D1 and D3, and suppresses osteoblast differentiation by inhibiting Wnt/β-catenin signaling and by opposing runt-related transcription factor (Runx)2 transactivation (10). The role played by NFAT proteins in the regulation of osteoblast differentiation and function is not clear, and suppression of NFAT signaling by inactivation of calcineurin in mice has generated controversial results (11–13). We demonstrated that overexpression of a constitutively active form of NFATc1 suppresses the expression of osteoblast gene markers in vitro (14). Accordingly, NFATc1 inhibits osteoblastic differentiation and recruits histone deacetylases to the osteocalcin promoter to suppress osteocalcin expression (15).

The role played by NFAT proteins in the regulation of osteoblast differentiation and function is (Runx)2 transactivation (10). The role played by NFAT proteins in the regulation of osteoblast differentiation and function is not clear, and suppression of NFAT signaling by inactivation of calcineurin in mice has generated controversial results (11–13). We demonstrated that overexpression of a constitutively active form of NFATc1 suppresses the expression of osteoblast gene markers in vitro (14). Accordingly, NFATc1 inhibits osteoblastic differentiation and recruits histone deacetylases to the osteocalcin promoter to suppress osteocalcin expression (15).

Unexpectedly, global Nfatc1 null mice are osteopenic and display defective bone formation, although indirect nonspecific effects are possible (16, 17). Similarly, global Nfatc2 null mice display osteopenia and suppressed osteoblastic function, but hyperproliferation of B and T-cells may be responsible for this skeletal phenotype (18). In osteoblasts, Notch suppresses NFATc1 transcription and NFAT transactivation (14), but the effects of Notch on the expression of alternate NFAT paralogs have not been reported, and interactions between these two signaling pathways are poorly understood.

In the present study, we investigated the effects of Notch on the expression of the four NFATc paralogs in primary calvarial osteoblasts. In addition, we explored the regulation of Notch canonical signaling by NFATc2 and assessed the effects of Notch and NFATc2 on osteoblast function.

**EXPERIMENTAL PROCEDURES**

Cell Cultures—Osteoblast-enriched cells were isolated by sequential collagenase digestion from parietal bones of 3–5-day-old RosaNotch mice, generated in a 129SvJ/C57BL/6 mixed genetic background (D. A. Melton, Harvard University, Cambridge, MA, obtained from The Jackson Laboratory, Bar Harbor, ME), or wild-type C57BL/6 mice, as described (19, 20). In RosaNotch mice, the Rosa26 locus is targeted by homologous recombination with a DNA construct encoding NICD, preceded by a STOP cassette flanked by loxp sequences, cloned downstream of the Rosa26 promoter. Expression of NICD from the targeted Rosa26 locus occurs following the excision of the STOP cassette by CRE recombination of loxp sequences. Notch receptors can be activated by Notch ligands adherent to the cell culture substrate (21). Tissue culture plates were exposed to 250 ng/ml of the Notch ligand Delta-like (Dll)1 (R&D Systems, Minneapolis, MN) in phosphate-buffered saline (PBS, Amresco, Solon, OH) for 1 h at room temperature to obtain immobilized Dll1 at a density of 62.5 ng/cm². As control, an equimolar amount of bovine serum albumin (BSA, Sigma-Aldrich) in PBS was immobilized per cm² of cell culture substrate. Wild-type C57BL/6 osteoblasts were seeded on immobilized Dll1 to induce Notch signaling or seeded on BSA as control. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies) supplemented with nonessential amino acids (Life Technologies), 20 mM HEPES, 100 μg/ml ascorbic acid (both from Sigma-Aldrich), and 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), at 37°C in a humidified 5% CO₂ incubator.

Adenoviral Infection—At 70% confluence, osteoblasts were transferred to medium containing 2% FBS for 1 h and exposed overnight to 100 multiplicity of infection of replication defective recombinant adenoviruses. An adenoviral vector expressing CRE recombinase under the control of the cytomegalovirus (CMV) promoter (Ad-CMV-CRE, Vector Biolabs, Philadelphia, PA) was delivered to RosaNotch cells to induce recombination of the loxp sequences and NICD expression. An adenoviral vector expressing green fluorescent protein (GFP) under the control of the CMV promoter (Ad-CMV-GFP, Vector Biolabs) was used as control.

A 2.8-kb DNA fragment containing the sequence coding for amino acids 98–106 of the human influenza hemagglutinin (HA) followed by the coding sequence of murine Nfatc2, where serum to alamine mutations in the SRR and SPXX repeat motifs of the regulatory domain render NFATc2 constitutively active, was created by A. Rao (Harvard Medical School, Boston, MA) (22). This construct was obtained from Addgene (Cambridge, MA, Addgene plasmid 11792) and used to create an adenoviral vector where the expression of constitutively active NFATc2 (caNFATc2) is directed by the CMV promoter (Ad-CMV-caNFATc2; Vector Biolabs). Wild-type C57BL/6 osteoblasts were transduced with Ad-CMV-caNFATc2 or with control Ad-CMV-GFP, and in selected experiments, RosaNotch osteoblasts transduced with Ad-CMV-CRE or with control Ad-CMV-GFP were co-transduced with Ad-CMV-caNFATc2 or with control Ad-CMV-GFP. After transduction, cells were cultured in the presence of DMEM containing 10% FBS.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)—Total RNA was extracted with the RNeasy mini kit, according to the manufacturer’s instructions (Qiagen, Valencia, CA), and changes in mRNA levels were determined by qRT-PCR (23, 24). 0.5–1 μg of total RNA was reverse-transcribed using either the SuperScript III Platinum two-step qRT-PCR kit (Life Technologies) or the iQ SYBR Green supermix kit (Bio-Rad) at 60°C for 45 cycles. cDNA copy number was estimated by comparison with a standard curve constructed using alkaline phosphatase liver/bone/kidney (Alpl), bone sialoprotein (Bsp), collagen type I α1 (Col1a1), osteopontin, and Rcan1.4 (all from American Type Culture Collection (ATCC), Manassas, VA), distal-less homeobox (Dlx)5 (Source BioScience UK Ltd., Nottingham, UK), Nfatc1 (Addgene plasmid 11793) and Nfatc2 (both created by A. Rao), Nfatc3 and Nfatc4 (both from Open Biosystems, Huntsville, AL), Hey1 and Hey2 (both from T. Iso, University of Southern California, Los Angeles, CA), and osteocalcin and Runx2 (both from J. B. Lian, University of Vermont, Burlington, VT) cDNAs and corrected for ribosomal protein L18 (Rpl18; ATCC) expression (22, 25–27). Amplification reactions were conducted either in a 96-well spectrophotometric thermal cycler (Bio-Rad) or in a CFX96 real time system (Bio-Rad).

To assess Nfatc2 transcription, heterogeneous nuclear RNA (hnRNA) levels were determined. For this purpose, 0.5 μg of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies), in the
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presence of a specific antisense primer targeted to intron 6 of Nfatc2 (supplemental Table 1). Reverse-transcribed cDNA was amplified in the presence of primers flanking the exon 6 intron 6 junction of Nfatc2 hnRNA (supplemental Table 1) and Platinum quantitative PCR SuperMix-UDG (Life Technologies) at 60° C for 45 cycles. -Fold changes in Nfatc2 hnRNA, normalized to Rpl38 expression, were determined by performing amplification reactions in a CFX96 real time system and by analyzing the results with the 2−ΔΔCT method using as a reference the corrected expression levels of Nfatc2 hnRNA in control cells. Amplification efficiency was estimated by comparison with a standard curve generated by parallel amplification of a dilution series of genomic murine 129Sv/C57BL/6 DNA (28). Fluorescence was monitored during every PCR cycle at the annealing step, and specificity of the reaction was confirmed by the presence of a single peak in the melt curve analysis of PCR products.

Western Blot Analysis—To detect changes in endogenous NFATc2 protein levels or to assess overexpression of caNFATc2, RosaNotch osteoblasts were washed with PBS and extracted in 50 mM Tris, 15 mM EGTA, 100 mM NaCl, and 0.5% Triton X-100 (all from Sigma-Aldrich) or cell lysis buffer (Cell Signaling Technology, Beverly, MA), respectively. Extraction was performed in the presence of protease and phosphatase inhibitors and 1 mM dithiothreitol (DTT) (all from Sigma-Aldrich) at 4° C for 30 min, and cell debris was removed by centrifugation at 4° C. Protein concentrations were determined using a DC protein assay kit (Bio-Rad), and 20–50 μg of total protein were fractionated by gel electrophoresis in 10% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Billerica, MA). Membranes were blocked with 3% BSA in PBS and exposed to a 1:1000 dilution of primary antibody. A rabbit polyclonal antibody raised against the N terminus of NFATc2 (antibody 4389, Cell Signaling Technologies) was used for the detection of endogenous NFATc2, whereas a mouse monoclonal antibody (clone 25A10.D6.D2, Thermo Scientific) was used for the detection of caNFATc2. Blots were exposed to either anti-rabbit IgG or anti-mouse IgG conjugated to horseradish peroxidase (Sigma-Aldrich) and incubated with a chemiluminescence detection reagent (Bio-Rad), and digital images were acquired using an Optocomp luminometer (MGM Instruments, Hamden, CT).

Electrophoretic Mobility Shift Assay (EMSA)—To analyze DNA binding by CSL and NFATc2, nuclear extracts were obtained from RosaNotch osteoblasts transduced with Ad-CMV-caNFATc2 or control Ad-CMV-GFP, in the context or not of NICD overexpression (32, 33). Synthetic double-stranded oligonucleotides containing consensus sequences for Csl or Nfatc, found in the Epstein-Barr virus nuclear antigen 2 (EBNA2) or in the natriuretic peptide type B (Bnp) promoters (Integrated DNA Technology, Coralville, IA), were labeled with [γ-32P]ATP, using T4 polynucleotide kinase (Promega Corp., Madison, WI) (supplemental Table 2A) (34, 35). Nuclear extracts and radiolabeled oligonucleotides were incubated for 20 min at room temperature in 10 mM Tris buffer (pH 7.5) containing 3 μg of poly(dI-dC) (Sigma-Aldrich). To assess whether CSL and NFATc2 recognize reciprocal consensus sequences, binding to the radiolabeled Csl oligonucleotide was performed in the presence of a 200-fold excess of unlabeled oligonucleotide containing the Nfatc consensus sequence from the Bnp promoter (supplemental Table 2A) (35). In the converse experiment, binding to the radiolabeled Nfatc oligonucleotide was performed in the presence of a 200-fold excess of unlabeled oligonucleotide containing the Csl sequence from the EBNA2 promoter (supplemental Table 2A) (34). To determine specificity of the binding of the nuclear extracts to the consensus sequences, unlabeled homologous or mutated oligonucleotides were added in 200-fold excess (supplemental Table 2A). DNA-protein complexes were resolved on nondenaturing, nonreducing 6% polyacrylamide gels, and the complexes were visualized by autoradiography.

Chromatin Immunoprecipitation (ChIP) Assay—The association of NFATc2 to the Rcan1.4 promoter was assessed in wild-type C57BL/6 osteoblasts transduced with Ad-CMV-caNFATc2, or with Ad-CMV-GFP as control. The interactions of NFATc2 and CSL with the Hey2 promoter were investigated.
in Rosa<sup>Notch</sup> osteoblasts transduced with Ad-CMV-caNFATc2, or control Ad-CMV-GFP, in the context or not, of NICD overexpression. ChIP analysis was carried out with the Chip-IT express magnetic chromatin immunoprecipitation and sonication shearing kit following the manufacturer's instructions with slight modifications (Active Motifs, Carlsbad, CA). To cross-link DNA-interacting proteins and DNA, confluent osteoblasts were exposed to DMEM and 1% formalin (Sigma-Aldrich) at room temperature for 5 min. The cross-linking reaction was arrested by exposure to 125 mM glycine in PBS, and the cell layer was collected in PBS in the presence of protease and phosphatase inhibitors. Nuclei were released by Dounce homogenization, and DNA fragments about 400–800 bp long were obtained by sonication. DNA was incubated with constant agitation overnight at 4°C with a Chip grade rabbit polyclonal HA antibody (Abcam, Cambridge, MA), a rabbit CSL (H-50, Santa Cruz Biotechnology) antibody, or control rabbit immunoglobulin G (IgG; Santa Cruz Biotechnology), conjugated to protein magnetic beads (Cell Signaling Technologies). Beads were washed, and DNA–nuclear protein complexes were eluted. The cross-linking reaction was reversed at 65°C for 2.5 h, and following incubation with proteinase K, DNA was purified with DNeasy columns according to the manufacturer’s instructions (Qiagen). Purified DNA was amplified by quantitative PCR (qPCR) performed in the presence of specific primers flanking Nfatc consensus sequences required for the activity of the Rcan1.4 promoter, or Csl consensus sequences that regulate the activity of the Hey2 promoter (supplemental Table 2B), and IQ SYBR Green Supermix at 60°C for 40 cycles (31, 36). Amplification reactions were conducted in a CFX96 real time system, fluorescence was monitored during every PCR cycle at the annealing step, and specificity of the reaction was confirmed by the presence of a single peak in the melt curve analysis of PCR products.

Cytochemical Assays and Alkaline Phosphatase Activity—To determine mineralization of the culture, cells were rinsed in PBS, fixed with 3.7% formaldehyde, and stained with a 2% Alizarin Red solution in H<sub>2</sub>O (37). Color images were acquired with a Coolpix 995 digital camera (Nikon Inc., Melville, NY) imported to ImageJ software (38) and converted to grayscale, and threshold was defined as the intensity of gray above the background. Identical threshold settings were used for each image analyzed, and the mineralized area was defined as the area occupied by pixels surpassing the preset threshold, quantified by using the measure function of ImageJ.

Alkaline phosphatase activity was determined in 0.5% Triton X-100 cell extracts by the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol and measured by spectrophotometry at 405 nm after 10 min of incubation at room temperature, according to the manufacturer’s instructions (Sigma-Aldrich). Data are expressed as nanomoles of p-nitrophenol released per minute per μg of protein. Total protein content was determined in cell extracts by the DC protein assay.

Statistical Analysis—Data are expressed as means ± S.E. Statistical differences were determined by Student’s t test or analysis of variance with Scheffé post hoc analysis for pairwise or multiple comparisons. Statistical differences for the slopes of mRNA decay were analyzed by analysis of covariance (39).

**RESULTS**

Notch Induces the Expression of NFATc2 by Post-transcriptional Mechanisms—The effects of NICD on the expression of the four Nfatc paralogs were tested in primary calvarial osteoblast cultures. Osteoblasts from Rosa<sup>Notch</sup> mice were transduced with an adenoaviral vector expressing CRE under the control of the CMV promoter (Ad-CMV-CRE) to excise a STOP cassette flanked by lox<sup>p</sup> sequences and allow NICD expression under the control of the Rosa26 promoter. Control cultures were infected with an adenoaviral vector where the CMV promoter directs the expression of GFP (Ad-CMV-GFP). In accordance with our previous observations demonstrating that NICD inhibits the transcription of NFATc1, NICD decreased Nfatc1 mRNA levels (14). NICD induced Nfatc2 transcripts and protein levels and did not modify the expression of Nfatc3 and Nfatc4 in osteoblasts (Fig. 1, A and B). To confirm whether Notch activation induces Nfatc2 mRNA levels, wild-type C57BL/6 osteoblasts were exposed to immobilized DI11 to induce Notch signaling or to BSA as control. Following 3 days of...
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culture, osteoblasts exposed to Dll1 exhibited increased Hey2 expression in comparison with cells exposed to BSA, indicating activation of Notch signaling by Dll1. In agreement with the stimulatory effects of NICD overexpression on Nfatc2 transcripts in Rosa^Notch^ osteoblasts, Dll1 increased Nfatc2 mRNA levels, confirming that Notch induces Nfatc2 in osteoblasts (Fig. 1D). The mechanisms mediating the increase of Nfatc2 mRNA levels by Notch were investigated in Rosa^Notch^ osteoblasts. Notch did not change Nfatc2 hRNA levels, indicating that Notch does not regulate Nfatc2 transcription (Fig. 1C). To determine whether Notch increased Nfatc2 expression by post-transcriptional mechanisms, the decay of Nfatc2 transcripts was assessed in Rosa^Notch^ osteoblasts transcriptionally arrested with DRB. NICD prolonged the half-life of Nfatc2 mRNA from 4 to 13 h, demonstrating that Notch stabilizes Nfatc2 transcripts (Fig. 1E).

NFATc2 Suppresses Notch Canonical Signaling — To investigate the effects of NFATc2 on Notch signaling, Rosa^Notch^ osteoblasts transduced with Ad-CMV-CRE or Ad-CMV-GFP, and wild-type C57BL/6 osteoblasts, were transduced with an adenoviral vector where the CMV promoter controls the expression of constitutively active NFATc2 (Ad-CMV-caNFATc2) or control Ad-CMV-GFP. Expression of caNFATc2 in Rosa^Notch^ osteoblasts was documented by Western blot and qRT-PCR analysis (Fig. 2, A and B). We confirmed that Notch induced Nfatc2 mRNA in control Rosa^Notch^ osteoblasts, and Notch caused an additional increase in Nfatc2 transcripts in cells transduced with Ad-CMV-caNFATc2 (Fig. 2B). Because transactivation of the CMV promoter by Notch is not likely, this result suggests that the NFATc2 coding sequence contains regulatory motifs in part responsible for the post-transcriptional effects of Notch on Nfatc2 mRNA. In C57BL/6 osteoblasts transduced with Ad-CMV-caNFATc2 tagged with HA, ChIP analysis revealed that immunoprecipitation with an HA antibody enriched DNA fragments containing Nfatc consensus sequences required for the activity of the Rcan1.4 promoter, demonstrating direct interaction of NFATc2 with this promoter (Fig. 2C) (36). Accordingly, caNFATc2 increased Rcan1.4 mRNA levels in Rosa^Notch^ osteoblasts, although the induction was similar in control cells and in the context of Notch induction, suggesting that the increase in Nfatc2 mRNA levels caused by Notch does not translate in enhanced NFATc2 activity (Fig. 2D).

To test the effects of NFATc2 on Notch transactivation, Rosa^Notch^ osteoblasts were transfected either with the 12 × CSL-Luc reporter, which is induced by activation of Notch canonical signaling, or with fragments of the Hey1 (Hey1-Luc) and Hey2 (Hey2-Luc) promoters, which are targets of Notch signaling. NICD transactivated the 12 × CSL-Luc reporter and the Hey1-Luc and Hey2-Luc promoter constructs, whereas caNFATc2 opposed this effect (Fig. 3A). In accordance with these results, NICD induced Hey1 and Hey2 transcripts, and this effect was opposed by caNFATc2, demonstrating that NFATc2 suppresses Notch canonical signaling in osteoblasts (Fig. 3B).

NFATc2 Competes with CSL for Binding to DNA — The mechanism of the inhibitory effect of NFATc2 on Notch canonical signaling was analyzed by EMSA in Rosa^Notch^ osteoblasts transduced with Ad-CMV-CRE or Ad-CMV-GFP and co-transduced with Ad-CMV-caNFATc2 or control Ad-CMV-GFP. A radiolabeled Csl consensus oligonucleotide was bound by nuclear protein extracts from cells overexpressing NICD and controls, and an excess of unlabeled Csl oligonucleotides prevented this effect, demonstrating specificity of the binding. An excess of unlabeled oligonucleotides containing an Nfatc consensus sequence decreased the binding of the nuclear extracts to the Csl consensus oligonucleotide, indicating that CSL can bind to Nfatc consensus sequences. In the converse experiment, nuclear extracts from osteoblasts expressing caNFATc2 bound to a radiolabeled Nfatc consensus oligonucleotide to a greater extent than control extracts, confirming increased DNA binding by NFATc2. Formation of nuclear protein complexes with the radiolabeled Nfatc oligonucleotide was opposed by unlabeled Nfatc and Csl oligonucleotides, demonstrating specificity of the binding reaction and suggesting that NFATc2 can bind to Csl consensus sequences (Fig. 4B). Unlabeled oligonucleotides containing mutated Csl or Nfatc consensus sequences did not preclude formation of nuclear protein complexes with the radiolabeled oligonucleotides, demonstrating that nuclear protein binding to DNA requires intact Csl or Nfatc consensus sequences (Fig. 4, A and B). These findings indicate that CSL and NFATc2 recognize similar consensus
sequences and that NFATc2 competes with CSL for binding to Csl consensus sequences.

The association of NFATc2 and CSL to the Hey2 promoter was investigated by ChIP analysis in Rosa\textsuperscript{Notch} calvarial osteoblasts transduced with Ad-CMV-caNFATc2 or control Ad-CMV-GFP, in the context of Notch induction, or under basal conditions. In agreement with the results obtained by EMSA, qPCR analysis revealed that in control cells co-transduced with Ad-CMV-caNFATc2, immunoprecipitation with an HA antibody, in comparison with a control IgG, enriched DNA fragments from the Hey2 promoter containing Csl consensus sequences (Fig. 5A). However, under conditions of Notch induction, association of NFATc2 to the Hey2 promoter was not detected (Fig. 5A). Under basal conditions, DNA fragments from the Hey2 promoter were immunoprecipitated with a CSL antibody, and a modest increase in this effect was observed in the presence of NICD (Fig. 5B), confirming that the Csl consensus sequences analyzed regulate the activity of the Hey2 promoter (31). This effect was opposed by caNFATc2 (Fig. 5B), confirming the competition between CSL and NFATc2 for DNA binding observed by EMSA and indicating that NFATc2 displaces CSL from promoter sequences that regulate Hey2 expression.

**Notch and NFATc2 Regulate Osteoblast Expression of Gene Markers**—The effects of NFATc2 expression on osteoblast function were determined in Rosa\textsuperscript{Notch} osteoblasts transduced with Ad-CMV-caNFATc2 or control Ad-CMV-GFP and co-transduced with Ad-CMV-CRE to induce Notch or Ad-CMV-
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DISCUSSION

In this study, we investigated the effects of Notch on the expression of the four NFAT paralogs in primary calvarial osteoblasts. In previous work, we demonstrated that Notch suppresses NFAT transactivation by inhibiting NFATc1 expression (14). We confirmed that Notch suppresses Nfatc1 mRNA levels and reported that NICD overexpression in osteoblasts induces Nfatc2 by post-transcriptional mechanisms (14). Accordingly, exposure of osteoblasts to the Notch ligand Dll1 induces Nfatc2 mRNA levels, confirming that activation of Notch signaling induces Nfatc2 expression. Transcript levels of Nfatc3 and Nfatc4 were not affected by NICD overexpression, indicating that in osteoblasts, Notch regulates NFAT by suppressing Nfatc1 and inducing Nfatc2 expression. Although NFAT activity is controlled by cycles of phosphorylation and dephosphorylation, availability of NFATc constitutes an additional level of regulation in osteoclasts, and a similar mechanism may be operational in osteoblasts in the context of Notch induction (6, 41).

To our knowledge, this is the first report demonstrating post-transcriptional control of Nfatc2 expression. Previously, we had shown that cortisol, an inducer of Notch1 and Notch2 expression in osteoblasts, stabilizes collagenase 3 transcripts by inducing the formation of cytosolic protein complexes with the 3’-untranslated region (UTR) of the collagenase 3 mRNA (42, 43). Activation of Notch signaling might stabilize Nfatc2 mRNA levels by a similar mechanism. Post-transcriptional control of Nfatc2 by Notch could involve the regulation of microRNAs, which are short RNA molecules that bind to target mRNAs and block protein translation and induce transcript degradation (44). It is important to note that NICD stabilized native Nfatc2 transcripts as well as transcripts derived from the caNFATc2 adenovector transduced into osteoblasts, an effect that appears to be due to post-transcriptional mechanisms.3 Because the cDNA transcribed from the adeno viral

3 S. Zanotti and E. Canalis, unpublished observations.
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construct lacks the native Nfatc2 3′-UTR, stabilization of the Nfatc2 mRNA is dependent, at least in part, on Nfatc2 sequences present in the coding region. However, this does not exclude the presence of additional regulatory elements in the 3′-UTR.

NFATc2 suppressed Hey1 and Hey2 expression, and inhibition of the 12×CSL-Luc reporter suggested that this effect is mediated by suppression of Notch canonical signaling. Results obtained by EMSA suggested that NFATc2 bound to CSL consensus sequences and that NFATc2 and CSL interact with similar DNA sequences. ChIP analysis of fragments of the Hey2 promoter containing CSL consensus sequences confirmed these results and suggested that NFATc2 reduced binding of CSL to the Hey2 promoter. Therefore, NFATc2 appears to suppress Notch canonical signaling by competing with CSL for the interaction with CSL consensus sequences that regulate the expression of Notch target genes. Although NFATc2 displaced CSL from the Hey2 promoter, either in the context of NICD overexpression or under basal conditions, association of NFATc2 with the Hey2 promoter was not observed under conditions of Notch stimulation. This apparent discrepancy may be due to the formation of Notch transcriptional complexes that modify chromatin organization of Notch target genes and prevent detection of NFATc2 associated to the Hey2 promoter. Suppression of Notch canonical signaling by NFATc2 is reminiscent of the inhibitory effects of NFATc1 on Notch transactivation, and due to the extensive conservation of protein sequences among the NFAT family members, it is conceivable that inhibition of Notch signaling is a common function of NFAT proteins (13).

In contrast to the results from EMSA, ChIP analysis indicated that in the context of Notch induction, the CSL antibody immunoprecipitated Hey2 promoter sequences to a greater extent than under basal conditions. Although these results are not consistent with the most widely accepted model of Notch canonical signaling, the data are in agreement with previous reports indicating that NICD increases CSL binding to DNA (45–47). As an alternative, association of NICD with CSL may have exposed epitopes not previously available for binding by the CSL antibody, resulting in enhanced immunoprecipitation of Hey2 promoter sequences in the presence of NICD.

We confirmed the inhibition of osteoblastic function by Notch, and we reported that constitutive activation of NFATc2 had a similar but less pronounced effect (48, 49). However, the role played by NFAT transcription factors in osteoblasts is not clear (13). In mice, the osteoblast-specific conditional deletion of calcineurin, an activator of NFAT signaling, enhances osteoblastic function and trabecular bone volume, suggesting that NFAT signaling inhibits osteoblast differentiation and function (12, 15, 50). Similarly to the in vitro effects of NFATc1 in osteoblasts, we report that constitutive activation of NFATc2 suppresses osteoblast function, strengthening the notion that calcineurin/NFAT signaling suppresses osteoblast function (14, 15). In contrast, global Nfatc2 null mice are osteopoenic due to suppressed osteoblastic function (11, 17). However, Nfatc2 null mice display hyperproliferation of B- and T-lymphocytes and increased systemic levels of interleukin 4 and other inflammatory molecules, which probably contribute to the osteopenic phenotype (18).

In conclusion, Notch induces Nfatc2 by post-transcriptional mechanisms, overexpression of NFATc2 suppresses Notch canonical signaling, and Notch and NFATc2 are inhibitors of osteoblast function.

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