Reciprocal Regulation of Notch and Nuclear Factor of Activated T-cells (NFAT) c1 Transactivation in Osteoblasts*

Stefano Zanotti‡§1, Anna Smerdel-Ramoya‡, and Ernesto Canalis‡§

From the ‡Department of Research, Saint Francis Hospital and Medical Center, Hartford, Connecticut 06105 and the §University of Connecticut School of Medicine, Farmington, Connecticut 06030

Notch are transmembrane receptors involved in the determination of cell fate. Nuclear factor of activated T-cells (NFAT)c are transcription factors that control cell differentiation and function. We tested whether Notch and NFAT signaling pathways interacted in osteoblastic cells. Notch signaling was induced in ST-2 cells using vectors expressing Notch1 in osteoblastic cells by Cre recombinase-mediated excision of a loxP-flanked STOP cassette cloned between the Rosa26 promoter and NICD. NFATc1 was induced in RosaNotch osteoblastic cells by transducing an adenoviral vector expressing constitutively active NFATc1. Notch inhibited NFAT transactivation and NFATc1 transcription. In ST-2 cells, suppression of NFAT transactivation by Notch was reversed by constitutively active cGMP-dependent protein kinase type II. NFATc1 inhibited the transactivation of Notch target genes, and competed for binding to DNA with the Notch interacting protein Epstein-Barr virus latency C promoter binding factor-1, suppressor of hairless, Lag-1 (CSL). Co-immunoprecipitation and confocal microscopy demonstrated that NFATc1 and CSL interacted. Studies on the effects of NICD and NFATc1 on the differentiation and function of osteoblastic cells demonstrated that NICD and NFATc1 inhibited expression of osteoblast gene markers in RosaNotch osteoblasts, but only NICD suppressed the commitment of bone marrow stromal cells to the osteoblastic lineage. In conclusion, NICD and NFATc1 reciprocally inhibit their signaling pathways, and form a regulatory network to control their activity in osteoblasts.

Notch proteins are single pass transmembrane receptors that play a critical role in cell fate decisions. Notch regulates several developmental processes, and contributes to the regulation of cell renewal in multiple organs and cell systems (1). Notch is involved in skeletal development and homeostasis, because it inhibits chondrocyte proliferation and differentiation, and suppresses osteoblast differentiation and osteoclastogenesis (2–6). There are four Notch receptors (Notch1 to 4) and five canonical ligands, which are Serrate/Jagged1 and 2, and Delta-Like 1, 3, and 4 (7). Notch-ligand interactions result in the proteolytic cleavage and release of the Notch intracellular domain (NICD) (8). In the canonical Notch signaling pathway, NICD translocates to the nucleus and interacts with Epstein-Barr virus latency C promoter binding factor 1, suppressor of hairless and Lag-1 (CSL), which is bound to DNA and suppresses gene expression by recruiting transcriptional co-repressors. The binding of NICD to CSL induces the formation of a ternary complex with Mastermind-like proteins, which displaces the transcriptional co-repressors and recruits co-activators of transcription (9). These events induce the expression of Hairy Enhancer of Split (Hes) and Hairy/HES related with YRPF motif (Hey) transcription factors (10–13).

Nuclear factors of activated T-cells (NFAT) are five transcription factors (NFATc1 to c4 and NFAT5) involved in vertebrate development and in the growth and differentiation of multiple cell types (14–17). In unstimulated cells, NFATc1 to c4 are highly phosphorylated and reside in the cytoplasm. Activation of the phosphatase calcineurin dephosphorylates specific serine residues in the SRR and SPXX repeat motifs of the regulatory domain of NFAT. This induces NFAT translocation to the nucleus, and activation of transcription of NFAT target genes (18). NFAT phosphorylation by protein kinases, such as glycogen synthase kinase 3β (GSK3β), induces the nuclear export of NFAT preventing its transactivation (19–21). Activity of GSK3β is suppressed by phosphorylation on serine 9, which is a target of protein kinases, such as cGMP-dependent protein kinase II (cGKII), the product of the protein kinase cGMP-dependent type II (Prkg2) gene (22). cGKII activity is induced by increased cGMP levels and it is sustained by autophosphorylation on serine 126 (23).

The fate of mesenchymal cells and their differentiation toward cells of the osteoblastic lineage is controlled by a network of extracellular and intracellular signals (24, 25). We have reported that the overexpression of NICD in vitro inhibits osteoblast differentiation and that its overexpression in vivo causes osteopenia by reducing osteoblast number (5, 26, 27). Accordingly, the conditional deletion of Notch1 and Notch2 in the skeleton increases bone volume and induces the

2 The abbreviations used are: NICD, Notch intracellular domain; Ache, acetylcholinesterase; α-MEM, α-minimum essential medium; Ad, adenovirus; AP, alkaline phosphatase; Bnp, natriuretic peptide type B; ca, constitutively active; cGKII, cGMP-dependent protein kinase II; CSL, Epstein-Barr virus latency C promoter binding factor 1/suppressor of hairless/Lag-1; Ddx, Distal-less homeobox; EBNA2, Epstein-Barr virus nuclear antigen 2; Hes, Hairy Enhancer of Split; Hey, Hairy/Hes related with YRPF motif; hnRNA, heterogeneous nuclear RNA; NFAT, nuclear factor of activated T-cells; OC, osteocalcin; Prkg2, protein kinase cGMP-dependent type II; Rcn, regulators of calcineurin; Rpl38, ribosomal protein L38; Runx, Runt-related transcription factor; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole.
commitment of mesenchymal precursor cells toward cells of the osteoblastic lineage (3). NFATc1 is expressed during osteoblast growth and differentiation (28). The function of the calcineurin/NFAT pathway in osteoblasts is controversial, and both stimulatory and inhibitory effects on osteoblastic differentiation and function have been reported (28–34). Notch1 regulates NFAT signaling in keratinocytes, but interactions between the two signaling pathways in osteoblasts have not been reported (14).

We hypothesized that Notch and NFATc1 could interact in osteoblasts. In the present study, the effects of Notch1 and the products of its target genes Hey1 and Hey2 on NFAT transactivation were explored in cells of the osteoblastic lineage. In addition, the effects of NFATc1 on Notch signaling and the effects of these signaling pathways on osteoblastic differentiation were investigated.

**EXPERIMENTAL PROCEDURES**

*Expression Vectors—* A 2.4-kilobase (kb) DNA fragment containing the murine NICD coding sequence (J. S. Nye, Columbia University, New York) was cloned into either pcDNA 3.1 (Invitrogen) for use in acute transfection experiments, or into the retroviral vector pLPCX (Clontech, Palo Alto, CA) for the creation of stably transduced cell lines (35). 1- and 1.1-kb DNA fragments containing the respective coding sequences of murine Hey1 and Hey2 (T. Iso, University of Southern California, Los Angeles, CA) were cloned into pcDNA 3.1, and used in acute transfection experiments (13, 36). A 2.3-kb DNA fragment containing the coding sequence of murine Prkg2, where a serine 126 mutation to glutamic acid mimics the autophosphorylated active form of cGKII (cGKII<sup>126E</sup>), was obtained from B. Hogema (Erasmus University Medical Center, Rotterdam, The Netherlands) (23). This construct was cloned into pcDNA 3.1 for use in acute transfection experiments (pcDNA-cGKII<sup>126E</sup>). A 2.1-kb DNA fragment containing the coding sequence of murine Nfatc1, where multiple serine to alanine mutations in the SRR and SPXX repeat motifs of the regulatory domain render it constitutively active, was obtained from A. Rao (Addgene plasmid 11793; Harvard Medical School, Boston, MA) (37). This construct was used to create an adenoviral vector directing the expression of constitutively active (ca) NFATc1 under the control of the cytomegalovirus (CMV) promoter (Ad-CMV-caNFATc1; Vector Biolabs, Philadelphia, PA). Cell Cultures, Creation of Transduced Cell Lines, and Adenoviral Infection—ST-2 cells, established from Whitlock-Witte type long-term bone marrow culture of BC8 mice (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) were grown in a humidified 5% CO<sub>2</sub> incubator at 37 °C in α-minimum essential medium (α-MEM, Invitrogen), supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) (38–40). The retroviral vectors pLPCX and pLPCX-NICD were transfection into Phoenix packaging cells (American Type Culture Collection, ATCC, Manassas, VA) with TransFast transfection reagent, according to the manufacturer’s instructions (Promega Corporation, Madison, WI) and cells were selected in 2 μg/ml of puromycin (Sigma), as described (26). Retrovirus-containing conditioned medium was harvested, filtered through a 0.45-μm membrane, and used to transduce ST-2 cells by replacing the culture medium with retroviral conditioned medium in the presence of 8 μg/ml of Polybrene (Sigma-Aldrich) (26). Transduced ST-2 cells were grown, trypsinized, and selected in the presence of 2 μg/ml of puromycin. After selection, cells were cultured until confluent in α-MEM supplemented with 10% FBS.

Osteoblasts were isolated from parietal bones of 3 to 5 day old Rosa<sup>Notch</sup> mice by sequential collagenase digestion, as described (41). Bone marrow stromal cells were harvested by centrifugation of femurs aseptically removed from 1-month-old Rosa<sup>Notch</sup> mice, as described (42). Transgenic Rosa<sup>Notch</sup> mice were obtained from D. A. Melton (Harvard University, Cambridge, MA) (43). In these mice, the Rosa26 locus is targeted with a DNA construct encoding NICD, preceded by a STOP cassette flanked by loxp sites, 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. Calvarial osteoblasts were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with non-essential amino acids, 20 mM HEPES, 100 μg/ml of ascorbic acid, and 10% FBS at 37 °C in a humidified 5% CO<sub>2</sub> incubator. Bone marrow stromal cells were cultured in α-MEM supplemented with 20 mM HEPES and 15% FBS at 37 °C in a humidified 5% CO<sub>2</sub> incubator.

At 70% confluence, ST-2 cells, bone marrow stromal cells, and 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 CMV promoter (Ad-CMV-Cre, Vector Biolabs) was delivered to Rosa<sup>Notch</sup> 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. In selected experiments, Rosa<sup>Notch</sup> cells were co-transduced with Ad-CMV-caNFATc1, or Ad-CMV-GFP as control, to test the effects of NFAT on transactivation of Notch downstream genes, on the formation of nuclear protein complexes with a consensus CSL oligonucleotide, and on osteoblastic gene markers. To analyze the cellular localization of CSL and NFATc1, Rosa<sup>Notch</sup> osteoblasts transduced with Ad-CMV-caNFATc1 were co-transduced with Ad-CMV-Cre or with an adenoviral vector expressing β-galactosidase (β-gal) under control of the CMV promoter (Ad-CMV-β-gal, Vector Biolabs), as control. In selected experiments, expression of osteoblastic gene markers was studied in ST-2 cells transduced with Ad-CMV-caNFATc1, or Ad-CMV-GFP as control. After transduction, cells were washed with versene (Invitrogen) and cultured in the presence of DEMEM containing 10% FBS.

**Transient Transfections—*To verify activation of Notch signaling, ST-2 stromal cells transiently transfected with NICD or pcDNA 3.1 as control, were either co-transfected with a construct containing six multimerized dimeric CSL binding sites, linked to the β-globin basal promoter (12xCSL-Luc; L. J.
Notch and NFATc1

Strobl, Munich, Germany) or with 2.9- and 2.0-kb fragments of the Hey1 (Hey1-Luc; M. Maier, Wuerzburg, Germany) and Hey2 (Hey2-Luc; T. Iso) promoters, cloned upstream of the Luciferase gene (44–47). To test the effects of Notch signaling on NFAT transactivation, ST-2 stromal cells transiently transfected with NICD, Hey1 and Hey2 expression vectors, or pcDNA 3.1, as control, were co-transfected with a construct containing 9 copies of an NFAT response element, cloned upstream of the Rcan1.4 (Rcan1.4-Luc; B. Rothermel, University of Texas, Dallas, TX), which contains 15 NFAT consensus binding sites, cloned upstream of the Luciferase gene, was used (49, 50). To test the mechanism of Notch action on NFAT transactivation, ST-2 stromal cells transiently transfected with pcDNA-NICD, or pcDNA 3.1 as control, were co-transfected with pcDNA-cGKI{sub 126E} or control empty vector, and with 9xNFAT-Luc.

To test activation of Notch signaling and its effects on NFAT transactivation in primary osteoblasts, Rosa{sup Notch} osteoblasts transfused with Ad-CMV-Cre or with control were transfected with 12xCSL-Luc, 9xNFAT-Luc, or Rcan1.4-Luc. To test the mechanisms of NICD action on NFAT transactivation, transfused osteoblasts were transfected with pcDNA-cGKI{sub 126E} or control empty vector, and with 9xNFAT-Luc. To test the effects of NFATc1 on Notch signaling, transfused osteoblasts were co-transfused with Ad-CMV-caNFATc1, and with Ad-CMV-GFP as control, and transiently transfected with a 12xCSL-Luc reporter construct or with Hey1-Luc or Hey2-Luc promoter fragments. Transfections were conducted in cells cultured to 70% confluence using FuGENE 6 (3 µl FuGENE 6/2 µg of DNA), according to the manufacturer’s instructions (Roche Applied Science). A CMV-directed β-galactosidase expression construct (Clontech) was used to control for transfection efficiency. Cells were exposed to the FuGENE 6/DNA mixture for 16 h, transferred to fresh medium for 24 h, and harvested. Luciferase and β-galactosidase activities were measured using an Opto-comp luminometer (MGM Instruments, Hamden, CT). Luciferase activity was corrected for β-galactosidase activity.

RNA Interference (RNAi)—To down-regulate NFATc1 in ST-2 cells, a 19-mer double-stranded small interfering (si) RNA, targeted to the murine NFATc1 mRNA sequence, was obtained commercially (siRNA ID 288360, Invitrogen) (51). A scrambled 19-mer siRNA with no homology to known mouse or rat sequences was used as control (Invitrogen) (52). NFATc1 and scrambled siRNA at 20 nM were transfected into 60% confluent ST-2 cells using siLentFect lipid reagent, in accordance with the manufacturer’s instructions (Bio-Rad). To test for the effects of NFATc1 down-regulation on osteoblastic differentiation, alkaline phosphatase activity was determined 96 h following the transfection of siRNAs, and mRNA levels for markers of osteoblast differentiation were determined by real time RT-PCR in total RNA extracted from parallel cultures. To ensure adequate down-regulation, mRNA levels of NFATc1 were determined by real time RT-PCR in total RNA extracted from parallel cultures.

RNA Decay Experiments—To assess the effects of Notch on the stability of NFATc1 mRNA, ST-2 cells transfused with pLPX-NICD or pLPXC, as control, and Rosa{sup Notch} osteoblasts transfused with Ad-CMV-Cre or with Ad-CMV-GFP, as control, were grown to confluence and exposed to 5,6-dichloro-1-β-D-ribofuransylbenzimidazole (DRB), 75 µM (BioMol, Plymouth Meeting, PA), to arrest transcription. Subsequently, total RNA was extracted with the RNeasy Kit, according to the manufacturer’s instructions (Qiagen, Valencia, CA), and subjected to real time reverse transcription (RT)-PCR analysis to determine NFATc1 mRNA levels and establish the rate of transcript decay.

Real Time RT-PCR—Total RNA was extracted and changes in mRNA levels were determined by real time RT-PCR (53, 54). For this purpose, 0.5–1 µg of total RNA was reverse transcribed using the SuperScript III Platinum Two-step qRT-PCR kit (Invitrogen), according to the manufacturer’s instructions, and amplified in the presence of specific primers (Table 1, A) and Platinum Quantitative PCR SuperMix-UDG (Invitrogen) at 60 °C for 45 cycles. mRNA copy number was estimated by comparison with a standard curve constructed using alkaline phosphatase, bone sialoprotein, type I collagen, osteopontin (all from ATCC), distal-less homeobox 5 (DLX5; Open Biosystems, Huntsville, AL), Hey1 and Hey2 (both from T. Iso), Prkg2 (B. Hogema), NFATc1 (A. Rao), osteocalcin, and runt-related transcription factor 2 (Runx2) (both from J. B. Lian, University of Massachusetts, Worcester, MA) cDNAs, and corrected for Rpl38 (ATCC) expression (23, 36, 37, 55, 56).

To assess transcription of NFATc1, heterogeneous nuclear RNA (hnRNA) levels were determined by real time RT-PCR. For this purpose, 0.5 µg of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen), in the presence of specific antisense primers targeted to intron 6 of NFATc1 (Table 1, B). Reverse transcribed cdNA was amplified in the presence of specific primer pairs spanning the exon 6-intron 6 junction of NFATc1 hnRNA (Table 1, C) and Platinum Quantitative PCR SuperMix-UDG (Invitrogen) at 60 °C for 45 cycles. NFATc1 hnRNA copy number was estimated by comparison with a standard curve created with a specific DNA template, and data were corrected for Rpl38 expression. The template for NFATc1 hnRNA was created by cloning primers flanking the region amplified by the primers used for real time RT-PCR, designed with a 5′ extension containing an EcoRI restriction site (Table 1, D) and used to amplify murine genomic DNA. The PCR product was cloned into the EcoRI site of pBluescript SK (Stratagene, Santa Clara, CA) to create a DNA template for NFATc1 hnRNA. Fold-changes in NFATc1 hnRNA, corrected for Rpl38 expression, were determined by performing amplification reactions in a CFX96 real time PCR detection system (Bio-Rad), and analyzing the results with the 2−ΔΔCT method using as reference the corrected expression levels of hnNFATc1 in control cells (57). All other reactions were conducted in a 96-well spectrofluorometric thermal iCycler.
TABLE 1
Forward (Fwd) and reverse (Rev) primer pairs used for real time RT-PCR analysis of mRNA and hnRNA levels

| Primer     | Strand | Sequence                                                                 |
|------------|--------|--------------------------------------------------------------------------|
| A<sup>a</sup> |        |                                                                          |
| Alkaline phosphatase | Fwd | 5′-CGTGGAGCTCCACGATTTAC(FAM)G-3′ |
|             | Rev   | 5′-GTTGTCTTGGCATGAGTGTC-3′                                              |
| Bsp        | Fwd   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
|             | Rev   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
| Dlx5       | Fwd   | 5′-CCGAGATCTGGCAGTCCG-3′                                                |
|             | Rev   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
| Col1a      | Fwd   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
|             | Rev   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
| Hey1       | Fwd   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
|             | Rev   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
| Hey2       | Fwd   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
|             | Rev   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
| NFATc1     | Fwd   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
|             | Rev   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
| Notch1     | Fwd   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
|             | Rev   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
| Osteocalcin | Fwd   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
|             | Rev   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
| Osteopontin| Fwd   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
|             | Rev   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
| Runx2      | Fwd   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
|             | Rev   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
| RPL38      | Fwd   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |
|             | Rev   | 5′-GCCAGATCTGGCAGTCCG-3′                                                |

<sup>a</sup> A, primers used for amplification of the indicated mRNA.
<sup>b</sup> B, primer used for reverse transcription of the NFATc1 hnRNA.
<sup>c</sup> C, Fwd and Rev primers used for the amplification of the reverse transcribed NFATc1 hnRNA.
<sup>d</sup> D, Fwd and Rev primers used to create the template for NFATc1 hnRNA.

(Bio-Rad). Fluorescence was monitored during every PCR cycle at the annealing step.

**Western Blot Analysis and Co-immunoprecipitation**—The levels of NICD, Hey1, Hey2, and NFATc1 proteins were tested in ST-2 cells or Rosa<sub>Notch</sub> osteoblasts, either under overexpression of NICD or basal conditions. In selected experiments, protein levels of caNFATc1 were assessed in Rosa<sub>Notch</sub> osteoblasts transduced with Ad-CMV-caNFATc1, either in the context, or not, of NICD induction. To facilitate protein detection, ST-2 cells were treated overnight with 10 μM of the proteasome inhibitor MG132 (Sigma), and nuclear extracts were harvested as described (58, 59). The layer of Rosa<sub>Notch</sub> osteoblasts was washed in phosphate-buffered saline (PBS, Amresco, Solon, OH) and extracted in cell lysis buffer (Cell Signaling Technology, Beverly, MA) in the presence of protease and phosphatase inhibitors and 1 mM dithiothreitol (DTT) (all from Sigma) at 4 °C for 30 min. Protein concentrations were determined using a DC protein assay kit (Bio-Rad) and 20 μg of total protein was fractionated by gel electrophoresis in 10% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Billerica, MA).

To determine interactions between NFATc1 and CSL, Rosa<sub>Notch</sub> osteoblasts transduced with Ad-CMV-caNFATc1, or control, were examined in the context, or not, of NICD induction. The cell layer was washed in PBS and extracted in 50 mM Tris-Cl, pH 7.5, 15 mM EGTA, 100 mM NaCl, and 0.1% Triton X-100 (all from Sigma), in the presence of protease and phosphatase inhibitors and 1 mM DTT at 4 °C for 30 min. Samples were centrifuged at 8000 X g for 15 min at 4 °C to obtain the cytoplasmic fraction. Protein concentrations were determined using the DC protein assay kit and 500 μg of total protein were mixed for 1.5 h at 4 °C with either NFATc1 or CSL antibodies (Santa Cruz Biotechnology), or IgG, as control. Samples were mixed with Protein A/G-agarose beads (Santa Cruz Biotechnology) for 1 h at 4 °C, washed in PBS, and the immunoprecipitates were released by boiling for 5 min. Eluates were fractioned on 10% polyacrylamide gels and transferred to Immobilon-P membranes.

Membranes were blocked with 3% bovine serum albumin (BSA) in PBS and exposed to a 1:1000 dilution of a rabbit polyclonal antibody to NICD (Cell Signaling Technology), Hey1 (Santa Cruz), or Hey2 (Abcam, Cambridge, UK), to a 1:1000 dilution of murine monoclonal antibody to NFATc1 (Santa Cruz Biotechnology), or to a 1:1000 dilution of goat polyclonal antibody to CSL (Santa Cruz Biotechnology). All blots were exposed to anti-rabbit, anti-mouse, or anti-goat IgG conjugated to horseradish peroxidase (Sigma), incubated with a chemiluminescence detection reagent (Bio-Rad) and acquired with a cooled charged coupled device camera mounted on the Chemidoc XSR molecular imager (Bio-Rad). To assess even loading of the samples, antibodies were removed by washing in 70 mM glycine, 0.1% SDS, and 1% Tween.
Notch and NFATc1

TABLE 2

Double-stranded oligonucleotides containing Csl and Nfatc1 consensus sites used in the EMSA

The oligonucleotides containing the wild type (WT) Csl and Nfatc1 consensus sequences from the EBNA2 and Bnp promoters were used as radiolabeled probes or as homologous unlabeled competitors in binding reactions. The oligonucleotides containing the Csl and Nfatc1 mutant sequences from the EBNA2 and Bnp promoters and the oligonucleotides containing the WT Csl and Nfatc1 sequences from the Ren, and Ache promoters were used as unlabeled competitors in binding reactions. Mutated bases are underlined.

| Consensus | Sequence | Promoter |
|-----------|----------|----------|
| Csl       | WT       | 5′-GGAAACACGGCCGTGGGAAAAATTGAGG-3′ | EBNA2 |
|        | Mutant   | 5′-GGAAACACGGCCGTGGGAAAAATTGAGG-3′ | Ren |
|        | WT       | 5′-CCTGGGACCCCCCCGCAAC-3′ | Bnp |
|        | Mutant   | 5′-CCTGGGACCCCCCCGCAAC-3′ | Ache |

20 (Sigma), pH 2.2, followed by rinsing with PBS and 0.1% Tween 20 in PBS. Membranes were blocked and re-probed with a 1:1000 dilution of a murine antibody against heat shock protein 90 (Hsp90) or a 1:1000 dilution of a goat polyclonal antibody against actin (Santa Cruz Biotechnology), exposed to either anti-mouse or anti-goat IgG conjugated to horseradish peroxidase, and images were acquired as described above.

Electrophoretic Mobility Shift Assay (EMSA)—For gel shift assays, nuclear extracts were obtained from Rosa<sup>Notch</sup> osteoblasts transduced with Ad-CMV-caNFATc1 or control, either in the context or not, of NICD overexpression (58, 59). Synthetic double-stranded oligonucleotides containing consensus binding sequences for Csl or Nfatc1, 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<sup>32</sup>]<sub>P</sub>ATP using T<sub>4</sub> polynucleotide kinase (Promega) (Table 2) (60, 61). Nuclear extracts and labeled 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). To determine whether nuclear extracts specifically bind to the radiolabeled oligonucleotides, unlabeled oligonucleotides containing homologous or mutated consensus sequences were added in 200-fold excess (Table 2). To assess whether CSL and NFATc1 recognize reciprocal consensus sequences, binding to the radiolabeled Csl oligonucleotide was performed in the presence of a 200-fold excess of unlabeled oligonucleotides containing Nfatc1 consensus sequences from the Bnp or acetylcholinesterase (Ache) promoters (61, 62). In the converse experiment, binding to the radiolabeled Nfatc1 oligonucleotide was performed in the presence of a 200-fold excess of unlabeled oligonucleotides containing Csl consensus sequences from the EBNA2 or Renin (Ren) promoters (Table 2) (60, 63). In selected experiments, the binding reaction was followed by a 15-min incubation at room temperature with a 1:1000 dilution of a murine monoclonal antibody raised against NFATc1 or Csl (Santa Cruz Biotechnology), or with normal murine or goat IgG (Sigma), as control. DNA-protein complexes were resolved on non-denaturing, non-reducing 6% polyacrylamide gels, and the complexes were visualized by autoradiography.

Immunofluorescence and Laser Confocal Scanning Microscopy—To confirm an interaction between CSL and NFATc1, dual immunofluorescence labeling was performed in Rosa<sup>Notch</sup> osteoblasts transduced with Ad-CMV-β-gal or Ad-CMV-Cre and co-transduced with Ad-CMV-cnNFATc1. Cells were grown on glass coverslips until confluent, washed with PBS, and fixed in 4% formaldehyde in PBS for 5 min on ice, and permeabilized with 0.1% Triton X-100 in PBS for 5 min on ice (Sigma). After 3 washes with PBS, cells were incubated overnight with blocking buffer containing 3% rabbit IgG (Invitrogen) in PBS, 3% BSA, and 20 mM HEPES (Sigma). Cells were incubated for 1 h at 4 °C with a 1:25 dilution of CSL and a 1:50 dilution of NFATc1 antibodies in blocking buffer. After 3 washes in PBS, cells were exposed to a 1:1000 dilution of rabbit anti-goat or anti-mouse secondary antibodies conjugated with the fluorophores Alexa 633 and Alexa 488 (Invitrogen), respectively. To test for specificity of the fluorescent signal, parallel control reactions without either the CSL or the NFATc1 antibody were performed. Cells were washed with PBS and mounted in 50% glycerol. Confocal scanning images were obtained using an LSM 510 confocal microscope mounted on an Axiovert 100M and equipped with a Plan-Apochromat ×100/1.40 oil objective (Carl Zeiss, Thornwood, NY). The signal from the Alexa 633 fluorophore, obtained by excitation with a HeNe<sub>2</sub> laser emitting at 633 nm, was detected with a 650-nm low pass emission filter. The Alexa 488 fluorophore was excited with an argon laser emitting at 488 nm and the signal was detected with a 505–530-nm band pass emission filter. Images were acquired with a photomultiplier tube using Zeiss LSM software, by single track scanning with a ×2 optical zoom. For each track, uniform pinhole opening, laser output, and detector and amplifier gain were used for all the conditions studied. For detection of co-localization, images were analyzed using the co-localization module in the Imaris version 7.0.0 software (Bitplane Inc., Saint Paul, MN). The intensity thresholds for co-localization of the red and green channels were set above the background immunofluorescence observed in the control reactions. Identical threshold settings were used for each image analyzed, and co-localized pixels were identified when the intensity surpassed the preset thresholds in both channels.

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

Statistical Analysis—Data are expressed as mean ± S.E. Statistical differences were determined by Student’s t test,
Notch signaling (Fig. 1, panel A) was transiently transfected with control pcDNA (pcDNA, white bars) or pcDNA NICD (NICD, black bars) expression vectors, and co-transfected with a Notch-dependent 12xCSL-Luc reporter (12xCSL), or Hey1-Luc (Hey1) and Hey2-Luc (Hey2) promoter fragments, and a CMV/β-galactosidase (β-gal) expression vector, and harvested after 48 h. Data shown represent luciferase/β-gal activity. Bars represent mean ± S.E. for 6 observations. In panel B, ST-2 cells transduced with control pLPCX (pLPCX, white bars) or pLPCX-NICD (NICD, black bars) retroviral vectors were cultured for 72 h after confluence; total RNA was extracted, reverse transcribed, and amplified by real time RT-PCR. Data are expressed as Notch1, Hey1, and Hey2 copy number, corrected for Rpl38 expression. Values are mean ± S.E., n = 4. In panel C, transduced ST-2 cells were incubated for 24 h with MG132, or vehicle control at confluence. Nuclear extracts were fractioned with gel electrophoresis and transferred to Immobilon-P membranes, which were incubated with antibodies against NICD, Hey1, and Hey2. Membranes were stripped and reprobed with an antibody against Hsp90. In panel D, ST-2 cells were transiently transfected with control pcDNA, or with NICD, Hey1, or Hey2 expression vectors, and co-transfected with an NICD-dependent 9xNFAT-Luc reporter or Rcan1.4-Luc promoter fragment, and a CMV/β-gal expression vector, and harvested after 48 h. Data shown represent luciferase/β-gal activity. Bars represent mean ± S.E. for 6 observations. *, significantly different from control cells, p < 0.05.

FIGURE 1. Effects of Notch signaling on NFAT transactivation in ST-2 stromal cells. In panel A, ST-2 cells were transiently transfected with control pcDNA (pcDNA, white bars) or pcDNA NICD (NICD, black bars) expression vectors, and co-transfected with a Notch-dependent 12xCSL-Luc reporter (12xCSL), or Hey1-Luc (Hey1) and Hey2-Luc (Hey2) promoter fragments, and a CMV/β-galactosidase (β-gal) expression vector, and harvested after 48 h. Data shown represent luciferase/β-gal activity. Bars represent mean ± S.E. for 6 observations. In panel B, ST-2 cells transduced with control pLPCX (pLPCX, white bars) or pLPCX-NICD (NICD, black bars) retroviral vectors were cultured for 72 h after confluence; total RNA was extracted, reverse transcribed, and amplified by real time RT-PCR. Data are expressed as Notch1, Hey1, and Hey2 copy number, corrected for Rpl38 expression. Values are mean ± S.E., n = 4. In panel B, transduced ST-2 cells were transcriptionally arrested with DRB (time 0) and harvested at the indicated times. Total RNA was extracted, reverse transcribed, and amplified by real time RT-PCR. Data are expressed as percent of Nfatc1 mRNA copy number corrected for Rpl38 expression, relative to the time of DRB treatment, plotted versus time indicated in hours (h). Data are mean ± S.E., n = 4. In panel D, nuclear extracts from ST-2 cells were fractioned with gel electrophoresis and transferred to Immobilon-P membranes, which were incubated with antibodies against NICD, Hey1, and Hey2. Membranes were stripped and reprobed with an antibody against Hsp90. In panel D, ST-2 cells were transiently transfected with pcDNA (control, white bars) or pcDNA-NICD (NICD, black bars) expression vectors in conjunction with a 9xNFAT-Luc reporter and a CMV/β-galactosidase (β-gal) expression vector, and co-transfected with a constitutively active cGKIIS126E (cGKII) expression vector or pcDNA control, and harvested after 48 h. Data shown represent luciferase/β-gal activity. Bars represent mean ± S.E. for 6 observations. *, significantly different between experimental and control cells, p < 0.05.

FIGURE 2. Mechanisms of Notch action on the NFAT pathway in ST-2 stromal cells. In panels A, B, and E, ST-2 cells transfected with pLPCX (control, white bars) or pLPCX-NICD (NICD, black bars) retroviral vectors were cultured for 24 (C) or 72 h (A and E) after confluence; total RNA was extracted, reverse transcribed, and amplified by real time RT-PCR. Data are expressed as Nfatc1 (A) and Prkg2 (E) copy number corrected for Rpl38 expression, or as ratio of Nfatc1/hnRNA copy number corrected for Rpl38 expression, relative to the control (C). Values are mean ± S.E., n = 4. In panel B, transduced ST-2 cells were transcriptionally arrested with DRB (time 0) and harvested at the indicated times. Total RNA was extracted, reverse transcribed, and amplified by real time RT-PCR. Data are expressed as percent of Nfatc1 mRNA copy number corrected for Rpl38 expression, relative to the time of DRB treatment, plotted versus time indicated in hours (h). Data are mean ± S.E., n = 4. In panel D, nuclear extracts from ST-2 cells were fractioned with gel electrophoresis and transferred to Immobilon-P membranes, which were incubated with antibodies against NICD, Hey1, and Hey2. Membranes were stripped and reprobed with an antibody against Hsp90. In panel D, ST-2 cells were transiently transfected with pcDNA (control, white bars) or pcDNA-NICD (NICD, black bars) expression vectors in conjunction with a 9xNFAT-Luc reporter and a CMV/β-galactosidase (β-gal) expression vector, and co-transfected with a constitutively active cGKIIS126E (cGKII) expression vector or pcDNA control, and harvested after 48 h. Data shown represent luciferase/β-gal activity. Bars represent mean ± S.E. for 6 observations. *, significantly different between experimental and control cells, p < 0.05.

The effects of Notch1 on NFAT transactivation were investigated in ST-2 stromal cells transiently transfected with NICD, Hey1, and Hey2 expression vectors or with pcDNA 3.1, in conjunction with a 9xNFAT-Luc reporter construct. NICD, Hey1, and Hey2 suppressed the transactivation of the 9xNFAT-Luc reporter and the activity of a transfected Rcan1.4-Luc promoter fragment, which contains 15 copies of an NFAT binding site (Fig. 1D) (49).

Mechanisms of Notch suppression of NFAT transactivation in ST-2 Stromal Cells—To verify the results obtained in transient transfection experiments, ST-2 cells were stably transfected with pLPCX-NICD or pLPCX, as control. In accordance with the inhibition of NFAT transactivation, Nfatc1 mRNA and nuclear protein levels were reduced in NICD overexpressing cells (Fig. 2, A and D). To investigate the mechanism of action of NICD on Nfatc1 activity, the stability of Nfatc1 transcripts was assessed in cells transcriptionally arrested with DRB. NICD did not alter the half-life of the Nfatc1 mRNA, indicating that Notch may regulate the transcription of Nfatc1 (Fig. 2B). To verify this possibility, we measured levels of Nfatc1 hnRNA by real time RT-PCR,
Notch and NFATc1

FIGURE 3. Effects of Notch signaling on NFAT transactivation in Rosa<sup>Notch</sup> calvarial osteoblasts. Osteoblasts were infected with Ad-CMV-Cre (CRE, black bars) or with Ad-CMV-GFP (GFP, white bars) as control. In panels A and D, cells were cultured to subconfluence and transiently transfected with a Notch-dependent 12xCSL-Luc reporter (A), a 9xNFAT-Luc (9xNFAT) reporter or an Rcan1.4-Luc (Rcan1.4) promoter fragment (D) and a CMV/β-galactosidase (β-gal) expression vector and harvested after 48 h. Data shown represent luciferase/β-gal activity. Bars represent mean ± S.E. for 6 observations. In panels B, E, and H, osteoblasts were cultured for 24 h (B) or 72 h (B and E) after confluence; total RNA was extracted, reverse transcribed, and amplified by real time RT-PCR. Data are expressed as Notch1, Hey1, and Hey2 (B), and NFATc1 (E) copy number corrected for Rpi38 expression, or as ratio of NFATc1 hnRNA copy number corrected for Rpi38 expression, relative to the control group (H). Values are mean ± S.E., n = 4. In panels C and F, total cellular extracts were fractioned by gel electrophoresis and transferred to Immobilon-P membranes, which were incubated with antibodies against NICD, Hey1, Hey2, and NFATc1 (F). Membranes were stripped and reprobed with an antibody against actin. In panel G, osteoblasts were transcriptionally arrested with DRB (time 0) and harvested at the indicated times. Total RNA was extracted, reverse transcribed, and amplified by real time RT-PCR. Data are expressed as percent of Nfatc1 mRNA copy number, corrected for Rpi38 expression, relative to the time of DRB treatment, and plotted versus time (h). Data are mean ± S.E., n = 4, *, significantly different between experimental and control cells, p < 0.05.

Effects of Notch Signaling on NFAT Transactivation in Rosa<sup>Notch</sup> Osteoblasts—To confirm the results obtained in ST-2 cells, NICD suppressed transactivation of the 9xNFAT-Luc reporter, and the Rcan 1.4-Luc promoter fragment (Fig. 3D). Accordingly, NICD suppressed NFATc1 transcripts and protein levels (Fig. 3, E and F). NICD did not alter the half-life of NFATc1 mRNA in transcriptionally arrested osteoblasts, whereas it suppressed NFATc1 hnRNA levels, confirming the transcriptional down-regulation of NFATc1 observed in ST-2 cells (Fig. 3, G and H). Prkg2 mRNA levels were suppressed by 60% in Rosa<sup>Notch</sup> osteoblasts transduced with Ad-CMV-Cre (not shown). However, expression of cGKIIS126E did not reverse the inhibitory effects of Notch on NFAT transactivation, indicating that in committed osteoblasts this mechanism requires additional co-regulators to operate (not shown).

Effects of NFATc1 on Notch Signaling—To test whether NFATc1 modulates the transactivation of Notch downstream target genes, Rosa<sup>Notch</sup> osteoblasts were transduced with an adenoviral vector where the CMV promoter controls the expression of caNFATc1 (Ad-CMV-caNFATc1). Overexpression of the NFATc1 protein was documented by Western blot analysis, and NICD decreased NFATc1 protein levels in cells transduced with either Ad-CMV-GFP or Ad-CMV-caNFATc1 (Fig. 4A). Suppression of caNFATc1 expression by NICD indicates a post-translational effect, because regulation of the CMV promoter by NICD is not probable (66, 67). Cells were transfected with either the 12xCSL-Luc reporter, or Hey1-Luc and Hey2-Luc promoter fragments, and the effects of Ad-CMV-caNFATc1 on their transactivation were ana-
lyzed. Notch induced transactivation of the 12xCSL-Luc, Hey1-Luc, and Hey2-Luc constructs, and NFATc1 opposed these effects (Fig. 4B). Accordingly, mRNA levels for Hey1 and Hey2 were suppressed 50–60% by Ad-CMV-caNFATc1 in control cells and in the context of NICD induction, demonstrating that NFATc1 suppresses canonical Notch signaling (Fig. 4C).

**Competition between CSL and NFATc1 for Binding to DNA**—
The nature of the inhibitory action of NFATc1 on Notch signaling was analyzed further by EMSA in Rosa<sup>Noch</sup> osteoblasts. Nuclear extracts bound to a CsI consensus oligonucleotide both in control and NICD expressing cells, although the binding was less pronounced in the context of Notch induction (68). An excess of unlabeled oligonucleotides containing an Nfatc1 consensus sequence from the Ache promoter markedly decreased binding of the nuclear extracts to the CsI consensus oligonucleotide, indicating that CSL can bind to Nfatc1 consensus sequences (Fig. 5A). To verify whether NFATc1 participated in the formation of nuclear protein-DNA complexes, cell extracts were incubated with an antibody against NFATc1, or with normal murine IgG, as
FIGURE 6. Analysis of CSL and NFATc1 interaction in Rosa\textsuperscript{Notch} calvarial osteoblasts. In panel A, the interaction between CSL and NFATc1 was investigated by co-immunoprecipitation in osteoblasts transduced with Ad-CMV-caNFATc1, and co-transduced with Ad-CMV-Cre (CRE) to induce Notch, or with Ad-CMV-GFP (GFP) as control. Cells were grown to confluence and cytoplasmic extracts were incubated with protein A/G-agarose in the presence of control immunoglobulin G (IgG), or immunoprecipitated (I.P.) in the presence of anti-CSL (α-CSL; left), or anti-NFATc1 (α-NFATc1; right) antibodies. Immunoprecipitates were analyzed by Western blot (W.B.) with antibodies against NFATc1 (left) or CSL (right). In panel B, cellular localization of CSL and NFATc1 was analyzed by confocal microscopy in osteoblasts transduced with Ad-CMV-caNFATc1, and co-transduced with Ad-CMV-Cre (CRE), or with Ad-CMV-β-gal (β-gal), as control. Cells were fixed, and CSL and NFATc1 were detected by indirect immunolabeling with anti-goat Alexa 633 (red) and anti-mouse Alexa 488 (green) fluorophore-conjugated antibodies, respectively. Single track images obtained with 650 low pass and 505–530 band pass filters are shown in the first and second rows, whereas merged images are shown in the third row. Merged images, where yellow pixels represent co-localization of the red and green channels, are shown in the lowest row. Scale bar is 10 μm.

control. An NFATc1 antibody induced a slower migrating nuclear protein-DNA complex in extracts from control and NICD overexpressing Rosa\textsuperscript{Notch} cells transduced with Ad-CMV-caNFATc1, suggesting binding of NFATc1 to the Csl oligonucleotide (Fig. 5B). Nuclear extracts from control and NICD overexpressing cells bound to the Nfatc1 consensus sequence, and an excess of unlabeled oligonucleotides containing Csl consensus sequences prevented the formation of the nuclear protein-DNA complexes, suggesting that NFATc1 can bind to Csl consensus sequences (Fig. 5C). These findings indicate that NFATc1 could suppress Notch canonical signaling by binding to Csl consensus sequences. caNFATc1 increased the intensity of the nuclear protein complexes with the Nfatc1 consensus sequence, and the effect was opposed by NICD overexpression (Fig. 5C). These results are in agreement with the inhibitory effects of NICD on NFAT transactivation and suggest that NICD also acts by opposing NFATc1 binding to DNA consensus sequences.

CSL and NFATc1 Interactions—To test whether CSL and NFATc1 interact in osteoblasts, co-immunoprecipitation was performed in cytoplasmic extracts from Rosa\textsuperscript{Notch} osteoblasts transduced with Ad-CMV-caNFATc1 and co-transduced with Ad-CMV-Cre or Ad-CMV-GFP, as control. Immunoprecipitates obtained with an anti-CSL antibody revealed the presence of NFATc1 either in controls or in the context of NICD overexpression (Fig. 6A). Accordingly, an anti-NFATc1 antibody immunoprecipitated a protein complex containing CSL, either in the presence or absence of NICD induction (Fig. 6A). To confirm these findings, we investigated cellular localization of CSL and NFATc1 by dual immunofluorescence labeling and confocal microscopy in Rosa\textsuperscript{Notch} osteoblasts transduced with Ad-CMV-caNFATc1 and co-transduced with Ad-CMV-Cre or Ad-CMV-LacZ, as control. In accordance with the results from co-immunoprecipitation analysis, the immunofluorescence labels for CSL and caNFATc1 co-localized both in the presence of NICD and in cells transduced with Ad-CMV-LacZ. Co-localization analysis was performed on the whole images, and the label for CSL and NFATc1 overlapped only in the nuclei (Fig. 6B). These findings suggest that the reciprocal inhibitory effects of Notch and NFAT on their signaling pathways are also mediated by an interaction between CSL and NFATc1.

Effects of Notch and NFATc1 on Commitment to the Osteoblastic Lineage—In previous work we have shown that Notch suppresses the osteoblastic differentiation of ST-2 cells (26, 27). To assess the effects of NFAT in this culture model, NFATc1 was misexpressed in ST-2 cells. caNFATc1 was transduced by adenoviral infection and upon its overexpression alkaline phosphatase or osteocalcin mRNA levels did not change (Fig. 7A). In contrast, NFATc1 down-regulation by RNAi caused a significant increase in alkaline phosphatase and osteocalcin mRNA levels and in alkaline phosphatase activity, suggesting that endogenous levels of NFATc1 are sufficient to suppress osteoblastic differentiation in ST-2 cells (Fig. 7, B and C). To verify the impact of NFATc1 in primary cultures, Rosa\textsuperscript{Notch} bone marrow stromal cells were transduced with Ad-CMV-caNFATc1, or Ad-CMV-GFP as control, either in the context of NICD induction or under basal conditions. NICD overexpression resulted in the induction of Hey1 and Hey2 mRNA levels (Fig. 8A). In agreement with previous observations, NICD suppressed alkaline phosphatase and osteocalcin mRNA levels (Fig. 8B) (5). Confirming the results obtained in ST-2 cells, overexpression of caNFATc1 did not affect alkaline phosphatase transcript levels, although in confluent cells there was a modest suppression of osteocalcin mRNA (Fig. 8B). Down-regulation of NFATc1 by RNAi re-
RESULTS
NFAT transactivation by Notch, and demonstrated that Notch inhibited NFAT transactivation in the culture models studied. A similar effect has been described in C2C12 cells, an immortalized myoblastic cell line; however, the mechanisms of Notch action were not investigated, and the regulation of NFAT in osteoblastic cells was not explored (14).

We explored the mechanisms involved in the inhibition of NFAT transactivation by Notch, and demonstrated that Notch suppressed the transcription of NFATc1 in ST-2 stromal cells and primary calvarial osteoblasts. Regulation of NFATc1 transactivation by Notch2 has been reported during osteoclastogenesis, where the Notch2 intracellular domain resulted in high cellular mortality, so that its effects could not be examined in bone marrow stromal cells.

Effects of Notch and NFATc1 on Osteoblast Function—To test the effects of Notch and NFATc1 signaling in cells committed to the osteoblastic lineage, Rosa
\textsuperscript{Notch}\textsuperscript{GFP} osteoblasts were transduced with Ad-CMV-caNFATc1 or Ad-CMV-GFP as control, either in the context of NICD overexpression or under basal conditions. Confirming the inhibitory effects of Notch on osteoblast differentiation and function, NICD repressed alkaline phosphatase, bone sialoprotein, Dlx5, Runx2, and osteocalcin mRNA levels and alkaline phosphatase activity (Fig. 9) (5). Conversely, NICD induced a modest and unexplained increase in the expression of collagen type I. NFATc1 suppressed the expression of all the osteoblastic markers analyzed, and inhibited alkaline phosphatase activity (Fig. 9). NFATc1 suppressed the expression of osteopontin, whereas Notch opposed this effect, confirming previous observations demonstrating induction of osteopontin by Notch (69). These results reveal that Notch and NFATc1, alone or in combination, modulate the expression of markers of osteoblastic differentiation and act as suppressors of mature osteoblast function in vitro.

DISCUSSION
In the present study we examined the role of Notch1 in the regulation of NFAT transactivation in ST-2 stromal cells, primary bone marrow stromal cells and calvarial osteoblasts.

The results reported demonstrate that Notch1 inhibits NFAT transactivation in the culture models studied. A similar effect has been described in C2C12 cells, an immortalized myoblastic cell line; however, the mechanisms of Notch action were not investigated, and the regulation of NFAT in osteoblastic cells was not explored (14).

We explored the mechanisms involved in the inhibition of NFAT transactivation by Notch, and demonstrated that Notch1 suppressed the transcription of NFATc1 in ST-2 stromal cells and primary calvarial osteoblasts. Regulation of NFATc1 transactivation by Notch2 has been reported during osteoclastogenesis, where the Notch2 intracellular domain
Notch and NFATc1

transactivates the NFATc1 promoter by interacting with nuclear factor-κB (70). Thus, the effects of Notch1 and Notch2 on the NFATc1 promoter are divergent. This differential regulation could be explained by the diverse cellular environments where the activities were tested, or by the recruitment of different co-regulators of transcription by the Notch1 and Notch2 intracellular domains to the NFATc1 promoter. It is also possible that Notch1 and Notch2 induce the expression of different downstream effectors, resulting in opposite outcomes on the regulation of NFATc1 transcription. The inhibitory effects of Notch1 on NFAT transactivation can be explained by the suppression of NFATc1 transcription, which occurred in both ST-2 cells and primary osteoblasts. Hey1 and Hey2 suppressed NFAT transactivation in ST-2 cells, and it is conceivable that they mediated the inhibitory effect of NICD on NFATc1 transcription. NICD suppressed the levels of a caNFATc1 protein expressed under control of the CMV promoter in RosaNotch osteoblasts. This promoter is used for constitutive protein overexpression in mammalian cells, and inhibition of its activity by NICD has not been reported (66, 67). Therefore, destabilization of the NFATc1 protein by NICD may explain the results observed.

Notch also suppressed the expression of Prkg2, which encodes cGKI, a kinase that phosphorylates and inhibits GSK3β, which in turn phosphorylates and inhibits the activity of NFAT. Forced expression of the constitutively active cGKII[S126E] mutant relieved the inhibitory effects of NICD on NFAT transactivation in ST-2 cells. Thus, the suppression of cGKI expression by Notch could contribute to its inhibitory effects on NFAT transactivation in ST-2 cells. Although Notch suppressed Prkg2 in RosaNotch osteoblasts, cGKII[S126E] did not reverse the inhibitory effects of Notch on NFAT transactivation, indicating that in these cells Notch acts primarily by suppressing NFATc1 transcription. Therefore, the inhibition of NFAT transactivation by NICD is mediated by cGKI only at defined stages of osteoblast differentiation.

NFATc1 suppressed transactivation of the 12xCSL-Luc reporter construct, and binding of caNFATc1 to a Csf1 consensus sequence was demonstrated by EMSA, suggesting that NFATc1 inhibits canonical Notch signaling by competing with CSL for binding to DNA. Conversely, induction of Notch signaling suppressed the interaction of caNFATc1 with an Nfatc1 consensus sequence, indicating that NICD suppresses NFAT transactivation by decreasing the binding of NFATc1 to DNA. These findings support the notion that NFATc1 and CSL compete for similar DNA consensus sequences, explaining the reciprocal inhibitory effects of Notch and NFAT signaling on the transactivation of their target genes. Co-immunoprecipitation and confocal microscopy demonstrated that CSL and NFATc1 interact in the nucleus of primary calvarial osteoblasts, and formation of a complex between CSL and NFATc1 can provide an additional explanation for the reciprocal inhibition of the Notch and NFAT signaling pathways. Interactions of CSL with other transcription factors have been reported in mesenchymal cells, and our results strengthen the notion that Notch canonical signaling is regulated by alternate signaling pathways (71).

In the present study, we confirmed the inhibitory effect of Notch on osteoblastic differentiation and function, although we reported a modest and unexplained increase in collagen type I mRNA levels (3, 5, 26, 27). Down-regulation of endogenous NFATc1 favored the commitment of ST-2 cells to the osteoblastic lineage, whereas constitutive activation of NFAT signaling did not affect cell fate in either ST-2 cells or in primary bone marrow stromal cells. This indicates that basal levels of NFATc1 are sufficient to hamper the commitment of stromal cells to the osteoblastic lineage and an increase in the levels of active NFATc1 do not result in further suppression of osteoblastogenesis. In primary cultures, constitutive activation of NFATc1 decreased the expression of osteoblastic markers, indicating suppression of mature osteoblastic function. Although the effects of NFATc1 on osteoblast differentiation and function have been controversial, these results are in agreement with recent work demonstrating that NFATc1 inhibits osteoblastic differentiation and suppresses the activity of the osteocalcin promoter by recruiting histone deacetylases (28–34). These results are also in agreement with findings obtained from mice where the osteoblast-specific deletion of Calcineurin, an activator of NFAT signaling, enhances osteoblastic function and trabecular bone volume (32–34). However, these results differ from those obtained following the global deletion of Calcineurin or Nfatc2 resulting in suppressed osteoblastic function, or Nfatc1 resulting in defective bone formation in the skull (28, 30, 31). It is conceivable that the impaired osteoblastic function observed in calcineurin, Nfatc1, and Nfatc2 global null mice is due to systemic effects, and this would explain the discrepancy with the results obtained in the conditional null models.

In conclusion, Notch and NFATc1 form a regulatory signaling network that controls osteoblast differentiation and function. Notch signaling suppresses NFATc1 transactivation by inhibiting its transcription and its binding to Nfatc1 consensus sequences, and NFATc1 suppresses Notch canonical signaling by competing for binding to Csf1 consensus sequences. The reciprocal inhibition of Notch and NFAT transactivation represents a novel mechanism to temper their suppressive effects on osteoblastic function. This system is redundant because both pathways oppose osteoblast activity, and in the event of failure in either the Notch or NFAT signaling pathways, suppression of osteoblastogenesis would still occur.

Acknowledgments—We thank Drs. D. A. Melton for RosaNotch mice, J. S. Nye for NICD cDNA, B. Hogema for cGKII[S126E] and Prkg2 cDNA, A. Rao for caNFATc1 cDNA, J. B. Lian for Osteocalcin and Runx2 cDNA, L. J. Strobl for the 12xCSL-Luc reporter construct, M. M. Maier for the Hey1-Luc promoter construct, and T. Iso for the Hey2-Luc promoter construct and Hey1 and Hey2 cDNAs, J. D. Molkentin for the 9xNFAT-Luc reporter construct, and B. Rothermel for the Rcan1.4-Luc promoter construct. M. Burton, T. X. Le, and M. Monarca for technical assistance, and M. Yurczak for secretarial assistance.
Notch and NFATc1

Schaffner, W. (1985) Cell 41, 521–530
67. Nelson, J. A., Reynolds-Kohler, C., and Smith, B. A. (1987) Mol. Cell. Biol. 7, 4125–4129
68. Oswald, F., Tauber, B., Dobner, T., Bourteele, S., Kostezka, U., Adler, G., Liptay, S., and Schmid, R. M. (2001) Mol. Cell. Biol. 21, 7761–7774
69. Shen, Q., and Christakos, S. (2005) J. Biol. Chem. 280, 40589–40598
70. Fukushima, H., Nakao, A., Okamoto, F., Shin, M., Kajiya, H., Sakano, S., Bigas, A., Jimi, E., and Okabe, K. (2008) Mol. Cell. Biol. 28, 6402–6412
71. Kitamura, T., Kitamura, Y. I., Funahashi, Y., Shawber, C. J., Castrillon, D. H., Kollipara, R., DePinho, R. A., Kitajewski, J., and Accili, D. (2007) J. Clin. Invest. 117, 2477–2485