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# Control of *Fiat* (Factor Inhibiting ATF4-Mediated Transcription) Expression by Sp Family Transcription Factors in Osteoblasts

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## ABSTRACT

FIAT (factor inhibiting ATF4-mediated transcription) represses *Osteocalcin* gene transcription and inhibits osteoblast activity by heterodimerizing with ATF4 to prevent it from binding DNA. It thus appears important to identify and characterize the molecular mechanisms that control *Fiat* gene expression in osteoblasts. In silico sequence analysis identified a canonical GC-box within a 1,400 bp region of the proximal *Fiat* gene promoter. Electrophoretic mobility shift assays (EMSA) with MC3T3-E1 osteoblastic cells nuclear extracts indicated that the transcription factors Sp1 and Sp3, but not Sp7/OSTERIX, bound this proximal GC-box. Chromatin immunoprecipitation confirmed interaction of the two transcription factors with the *Fiat* promoter GC-element in living osteoblasts. Transient transfection studies showed that Sp1 dose-dependently activated the expression of a *Fiat*-luciferase reporter construct while both the long or short isoforms of Sp3 dose-dependently inhibited transcription from the *Fiat* reporter construct. Transfection of an Sp7/OSTERIX expression vector did not affect expression of the *Fiat*-luciferase reporter. Co-transfection of increasing amounts of the Sp3 expression vector in the context of maximal Sp1-dependent *Fiat*-luciferase activation led to dose-dependent repression of the expression of the reporter. Using RNA knockdown, we measured a reduction in steady-state *Fiat* expression when Sp1 was inhibited, and a reciprocal increase upon Sp3 knockdown. In parallel, treatment of osteoblasts with WP631, which prevents Sp1/DNA interactions, strongly inhibited the expression of *Fiat* and reduced the occupancy of the *Fiat* promoter proximal GC-box by Sp1. Taken together, our results suggest an interplay between Sp1and Sp3 as a mechanism involved in the control of *Fiat* gene expression in osteoblasts. J. Cell. Biochem. 114: 1863–1870, 2013. 

**KEY WORDS:** FIAT; Sp1; Sp3; TRANSCRIPTIONAL REGULATION; OSTEOBLASTS

The leucine zipper protein, FIAT (factor inhibiting ATF4-mediated transcription), forms inactive heterodimers with the transcription factor ATF4 that prevent it from binding to DNA [Yu et al., 2005, 2008]. Because ATF4 regulates so many aspects of osteoblast biology [Yang et al., 2004], the net result of the binding of FIAT to ATF4 is an inhibition of osteoblast activity. For instance, transgenic mice over-expressing FIAT under the control of the osteoblast specific α1(I) collagen promoter exhibit reduced *Osteocalcin* gene transcription and are osteopenic, characterized by reduced bone mineral density, lowered trabecular volume, and decreased bone rigidity [Yu et al., 2005]. Reciprocally, siRNA-mediated knockdown of *Fiat* expression augments ATF4 activity and results in increased *Osteocalcin* transcription, type I collagen synthesis, and mineralization [Yu et al., 2009b].

These results suggest that FIAT is a significant regulator of ATF4 function and raise the importance of identifying and characterizing the molecular mechanisms that control *Fiat* gene expression in osteoblasts. As a first step towards this goal, we subcloned a 1,400 bp fragment upstream from the *Fiat* coding region and looked for potential regulatory elements by in silico analysis. We focused on a proximal canonical GC-box with the potential to bind Sp family transcription factors, including the osteoblast-specific family member, Sp7/OSTERIX [Nakashima et al., 2002].

Conflict of interest: nothing to declare.

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Sp7/OSTERIX is a zinc finger transcription factor expressed in osteoblasts that plays a crucial role during osteoblast differentiation, intramembranous and endochondral bone formation, and in bone homeostasis. Furthermore, *Osterix*-null mice form a complete cartilaginous skeleton but fail to form bone, indicating that Sp7/OSTERIX functions specifically in osteoblasts [Nakashima et al., 2002]. The DNA-binding domain of Sp7/OSTERIX is located at its C-terminus and contains three $C_2H_2$-type zinc finger domains that share a high degree of identity with motifs in Sp1, Sp3, and Sp4. Sp7/OSTERIX subcellular localization is restricted to the nucleus where it regulates the expression of many important genes in osteoblast differentiation including *Runx2*, Osteonectin (*Sparc*), osteopontin (*Spp1*), *Osteocalcin*, and alkaline phosphatase (*Tnap*) [Niger et al., 2011].

Within the Sp family of transcription factors, Sp1 and Sp3 are ubiquitously expressed [Saffer et al., 1991; Kingsley and Winoto, 1992]. Sp1 and Sp3 act through promoter GC-boxes [Gidoni et al., 1984; Letovsky and Dynan, 1989; Kingsley and Winoto, 1992] to regulate basal transcription and housekeeping expression of numerous genes involved in many cellular functions such as differentiation, proliferation, and apoptosis [Li et al., 2004]. Depending on the promoter, Sp1 typically acts as an activator, while Sp3 serves as either a repressor or an activator [Hagen et al., 1994; Li et al., 2004]. For example, in osteoblasts, Sp1 interacts with RUNX2 to mediate PTH-induction of the matrix gla protein (*Mgp*) promoter, while Sp3 inhibits transcription from this promoter [Suttamanatwong et al., 2009].

Interestingly, our results show that Sp7/OSTERIX does not bind the *Fiat* promoter GC-box, but that the binding site mediates *Fiat* gene transcription through opposing effects of the other family members, Sp1 and Sp3. Binding of Sp1 activates *Fiat* transcription, while Sp3 acts to inhibit *Fiat* expression.

## MATERIALS AND METHODS

### IN SILICO PROMOTER ANALYSIS

Potential transcription factor binding sites were identified using the TFSEARCH algorithm (http://www.cbrc.jp/research/db/TFSEARCH.html) on 1,400 bp of *Fiat* 5′-sequence upstream from the translation start site.

### CELL CULTURE

MC3T3-E1 osteoblastic cells [Sudo et al., 1983] were maintained in alpha minimum essential medium supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% $CO_2$.

### ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

A 200 bp double-stranded oligonucleotide corresponding to the most proximal 5′-promoter fragment from the murine *Fiat* gene was synthesized with an overhang and labeled with $^{32}$P-deoxynucleotide triphosphates by Klenow fill-in using standard protocols [Ausubel et al., 1993]. Nuclear extracts from MC3T3-E1 cells were prepared and used in EMSA as previously described [Andrews and Faller, 1991; Yu et al., 2005]. For supershifting, 2 μg of anti- Sp1, 2, 3, 4, or 7 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added to the binding reaction for 30 min prior to the addition of the labeled probe. The bound mixtures were size-fractionated on a nondenaturing 4% polyacrylamide gel at 180 V in 0.5× TBE buffer. The gel was subsequently dried and autoradiographed.

### CHROMATIN IMMUNOPRECIPITATION (CHIP)

MC3T3-E1 cells were plated on 100-mm-diameter dishes to grow until confluence. Chromatin immunoprecipitation was performed as previously described [Akhouayri et al., 2005]. Immunoprecipitations were performed with anti-Sp1, anti-Sp3, or anti-ribosomal protein S6 polyclonal antibodies (Santa Cruz). Five microliters of purified DNA was used as template for PCR with the following primer sequences which amplified the promoter region of the *Fiat* gene: Forward, 5′-CCCAAAATTTTACGCTCCCCAC-3′; Reverse, 5′-GCGTTACCTCCTCAAGCCGAGT-3′. Primer sequences from the *Fiat* gene coding sequence (anchored at +5.4 kb, forward, and +5.7 kb, reverse) were used as a negative control for primer specificity. In the experiment targeting the Sp1–DNA interaction, cells were treated with 100 nM of WP631 methanesulfonate (Sigma–Aldrich, Oakville, ON, Canada) for 4 h prior to ChIP. Samples were subjected to quantitative real-time PCR using SYBR Green QuantiFast Master Mix (Qiagen, Inc., Toronto, ON, Canada) as previously described [Meury et al., 2010]. The reaction conditions were as follows: 95°C for 5 min, 40 cycles of 95°C for 20 s (denaturation), 65°C for 1 min (annealing), and 72°C for 1 min (extension). Real-time PCR was performed on an ABI Prism 7700 sequence detection system (Life Technologies, Grand Island, NY). Data are expressed relative to expression levels from the input template for each ChIP.

### VECTOR CONSTRUCTION

The *Fiat* promoter fragment was obtained by PCR cloning using standard protocols and synthetic oligonucleotides that allowed amplification of a 1.4 kb genomic fragment from the 5′-flanking region of *Fiat* (primer sequences available upon request). This promoter fragment was purified and subcloned into the pGL3-basic vector (Promega Corp., Madison, WI). Site-specific mutation of the GC response element within the 1.4 kb genomic *Fiat* promoter fragment was performed using a PCR-based strategy [Yu et al., 2008] with primers containing a GCG to AAA mutation (Fig. 1A, primer sequences available upon request). The mutated promoter fragment was ligated into the pGL3-basic vector to yield the p*Fiat*-Mut-Luc reporter vector.

The Sp1 cDNA in the pBluescript-M13-SK+ vector was obtained from Dr. James Kadonaga, University of California at San Diego. The Sp1 cDNA was removed using *Eco*RI and *Nco*RI, gel-purified, amplified by PCR (primer sequences available upon request) and ligated into the pCI plasmid (Promega) to yield the pCI-Sp1 expression vector. The Sp3-si (short isoform) expression vector was constructed by removing a 718 bp *Mef*I–*Bam*HI fragment from the Sp3 cDNA long isoform (li) sequence within the commercially available pCMV6-Entry-Sp3vector (OriGene Technologies, Rockville, MD) (details available upon request). The Sp7/OSTERIX expression vector [Nakashima et al., 2002] was a generous gift from Dr. Benoit De Crombrugghe, MD Anderson Cancer Centre.

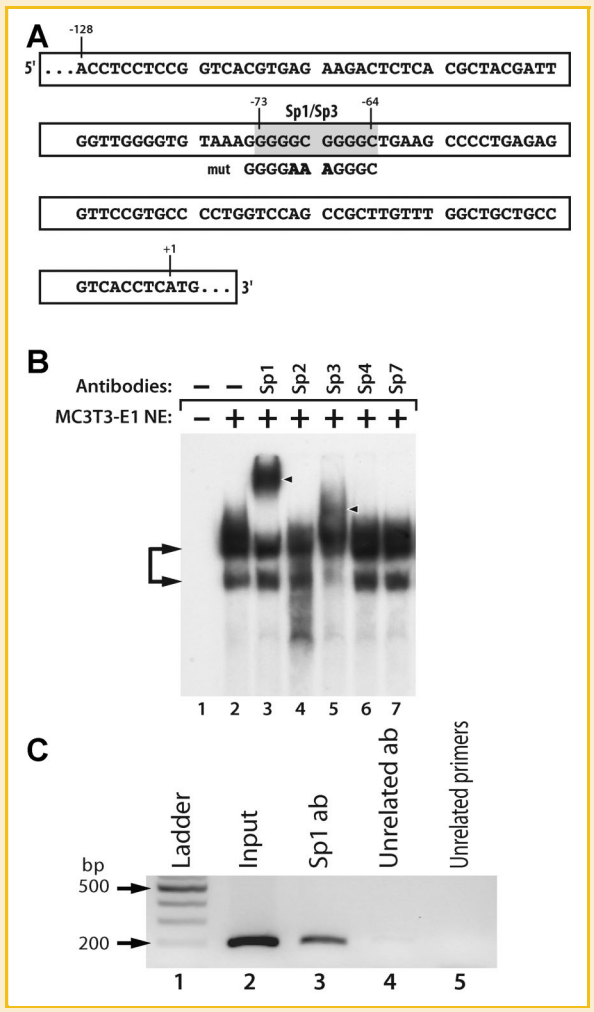


Fig. 1. Binding of Sp family members to the proximal *Fiat* promoter GC-box. A: Fiat proximal promoter sequence (−128 to +3 relative to the translation start site). The canonical GC-box (−73 to −64) is shaded in gray, with the mutated sequence listed underneath. B: Electrophoretic Mobility Shift Assay using a synthetic 200 bp oligonucleotide probe from the murine proximal *Fiat* promoter (−200 to +2) and nuclear extracts from osteoblastic MC3T3-E1 cells. A specific complex can be detected (arrow) that was "supershifted" (arrowheads) in the presence of anti-Sp1 (lane 3) or anti-Sp3 (lane 5) protein antibodies. C: Chromatin Immunoprecipitation assay. Immunoprecipitation was performed with formaldehyde-crosslinked chromatin from confluent MC3T3-E1 cells and antibodies (ab) against Sp1 or a control, unrelated antibody (anti-ribosomal protein S6). Ethidium bromide-stained agarose gels of PCR products obtained with primers flanking the *Fiat* GC-box (lanes 2–4) or primers from the FIAT coding sequence (unrelated primer, lane 5) are shown. Input, amplification of DNA prior to immunoprecipitation; Ladder, molecular size markers.

## TRANSIENT TRANSFECTION ASSAYS

MC3T3-E1 cells were plated at 1.0–1.5 × $10^5$ cells/well in a six-well plate. Cells were transfected with 50–200 ng of the reporters (p*Fiat*-Luc or p*Fiat*-Mut-Luc) and various concentrations of the expression vectors (pCI-Sp1, 25–50 ng; pCMV6-Sp3 si or li, 25–1,000 ng; pTriEx-1.1-Sp7, 25–50 ng) by using the GenePORTER reagent (Genlantis, San Diego, CA) according to the manufacturer's instructions. The total amount of transfected DNA was adjusted to 2 μg in each well by using pBlueScript (Stratagene, La Jolla, CA). At 24 or 48 h post-transfections, cells were lysed and 20 μl of cell lysate was used to measure luciferase activity following the manufacturer's procedure (Promega) and analyzed with a Sirius single tube luminometer (Berthold Technologies, Oakville, TN). Each transfection was repeated three times with triplicate samples, and the data shown represent the mean and the standard error of the mean (SEM) of a representative experiment.

## RNA INTERFERENCE

Five different commercially available small hairpin RNAs (shRNAs) against mouse Sp1 were tested; shRNA 1606 (Sigma–Aldrich) was judged the most efficacious and used for subsequent experiments. Lentiviruses were produced in HEK293 cells as previously described [Wazen et al., 2006]. Conditioned media containing the viral particles was used directly to infect MC3T3-E1 cells. As a control, a nontarget "nonsense" shRNA (pLK0.1-NT; Sigma–Aldrich) was used. MC3T3-E1 cells were plated at 8.0 × $10^4$/well in a six-well plate overnight and infected with either 1 or 3 ml of HEK293 lentivirus-containing media in the presence of 8 μg/ml of polybrene (Sigma–Aldrich) for 8 h. Medium was changed, cells were grown until confluence, passaged into 10 cm dishes, and 5 μg/ml of puromycin was added for selection. After a few days, cells were passaged again and seeded in six-well plates. At confluence, cells were collected for RT-qPCR and Western blot analysis as detailed below. For Sp3 knockdown, MC3T3-E1 cells were plated at 1.0 × $10^5$ cells/well in a six-well plate and transiently transfected with 120 nM of DsiRNA targeting mouse Sp3 or a scrambled universal negative control RNA duplex (NC1; Integrated DNA Technologies, Coralville, IA) using X-tremeGene transfection reagent (Roche Diagnostics Canada, Laval, QC) based on the instruction provided by the manufacturer. Cells were harvested for RNA and protein analysis at 48-h post-transfection.

## REVERSE TRANSCRIPTION QUANTITATIVE PCR (RT-qPCR)

MC3T3-E1 cells were plated at 1.0 × $10^5$ cells/well in a six-well plate. The cells were serum-starved in 0.5% FBS overnight and the following day treated with or without 100 nM WP631 methanesulfonate (Sigma–Aldrich). The cells were collected after 4 h of treatment with WP631. RNA was extracted using TRIzol (Life Technologies-Invitrogen) by following the manufacturer's instructions. RNA was reversed transcribed into cDNA using the High Capacity cDNA Archive kit in accordance with the manufacturer's recommendations (Life Technologies-Applied Biosystems). Real-time PCR amplification was performed on an Applied Biosystems 7700 instrument using the TaqMan Universal Master Mix and specific TaqMan assays for *Fiat*, *Sp1*, *Sp3*, and *Gapdh*. Relative expression level of each mRNA was quantified by the standard curve method (User bulletin #2; ABI Prism 7700 sequence detection system) and normalized to *Gapdh* levels.

## WESTERN BLOTTING

This assay was performed as described previously [Yu et al., 2008]. Protein concentrations were measured by Bradford assay and equal amounts were loaded for SDS gel electrophoresis and Western blotting. Anti-Sp1 and anti-Sp3 antibodies at 1:500 dilutions (Santa

Cruz) and anti-rabbit antibody conjugated with horseradish peroxidase at 1:5,000 dilutions (GE Healthcare Bio-Sciences, Baie d'Urfé, QC) were used.

## RESULTS

### Sp FAMILY MEMBERS BIND TO THE CANONICAL GC-BOX WITHIN THE *FIAT* PROXIMAL PROMOTER

Based on computer sequence analysis, we identified a canonical GC element (5′-GGGGCGGGGC-3′) located at −73 to −64 relative to the FIAT translation start site (Fig. 1A). Considering the role of the GC-box binding Sp7/OSTERIX transcription factor in osteoblast biology [Nakashima et al., 2002], we tested the possibility that members of the Sp family interact with this element in osteoblasts. Nuclear extracts from MC3T3-E1 osteoblastic cells were used in electrophoretic mobility shift assays (EMSA) with a 200 bp $^{32}$P-labeled double stranded oligonucleotide spanning the proximal mouse *Fiat* 5′-flanking sequence. As shown in Figure 1B, we observed two retarded complexes when MC3T3-E1 nuclear extracts were incubated with the labeled probe (compare lanes 1 and 2). To determine which Sp family member(s) bind(s) to the promoter fragment, supershift assays were performed using anti-Sp1, -Sp2, -Sp3, -Sp4, and -Sp7 antibodies (lanes 3–7). Antibodies specifically recognizing Sp1 partially supershifted the slowest-migrating complex (lane 3), while antibodies directed against Sp3 affected migration of the faster-migrating complex (lane 5). Anti-Sp2, anti-Sp4, and anti-Sp7 antibodies did not affect migration.

To confirm the binding of Sp1 to the GC-box within the *Fiat* promoter in vivo, we performed Chromatin Immunoprecipitation (ChIP). DNA was cross-linked to proteins using formaldehyde and sonicated chromatin from whole MC3T3-E1 cell lysates was co-immunoprecipitated using anti-Sp1 or negative control (anti-ribosomal S6) antibodies. DNA fragments that co-precipitated with Sp1 proteins were purified upon reversal of protein/DNA cross-links and used as template for PCR reactions with proximal *Fiat*-specific primers (−180 to +25) or negative control primers anchored within the FIAT coding sequence (+5.4 to +5.7 kb). The predicted 205-bp amplimer diagnostic of Sp1 binding to the *Fiat* promoter was specifically observed (Fig. 1C).

These results confirm the binding of Sp1 to the *Fiat* proximal 5′-flanking region both in vitro and in vivo and suggest the possible regulation of *Fiat* transcription by Sp1 in osteoblasts.

### TRANSCRIPTIONAL REGULATION OF *FIAT* EXPRESSION BY Sp FAMILY MEMBERS

We next examined whether over-expression of Sp1 could modulate *Fiat* expression. We co-transfected a reporter in which 1.4 kb of Fiat 5′-sequence upstream from the translation start site was inserted upstream from the luciferase gene (p*Fiat*-Luc) together with increasing amounts of an Sp1 expression vector in MC3T3-E1 cells. Sp1 dose-dependently increased the expression of the luciferase reporter under the control of the *Fiat* proximal region (Fig. 2A). To confirm the contribution of the GC element to *Fiat* promoter activity we mutated the core GC-box nucleotides (5′-GGGaaaGGGC-3′) to engineer the p*Fiat*-Mut-Luc reporter construct (Fig. 1A). Increasing amounts of transfected Sp1 expression vector

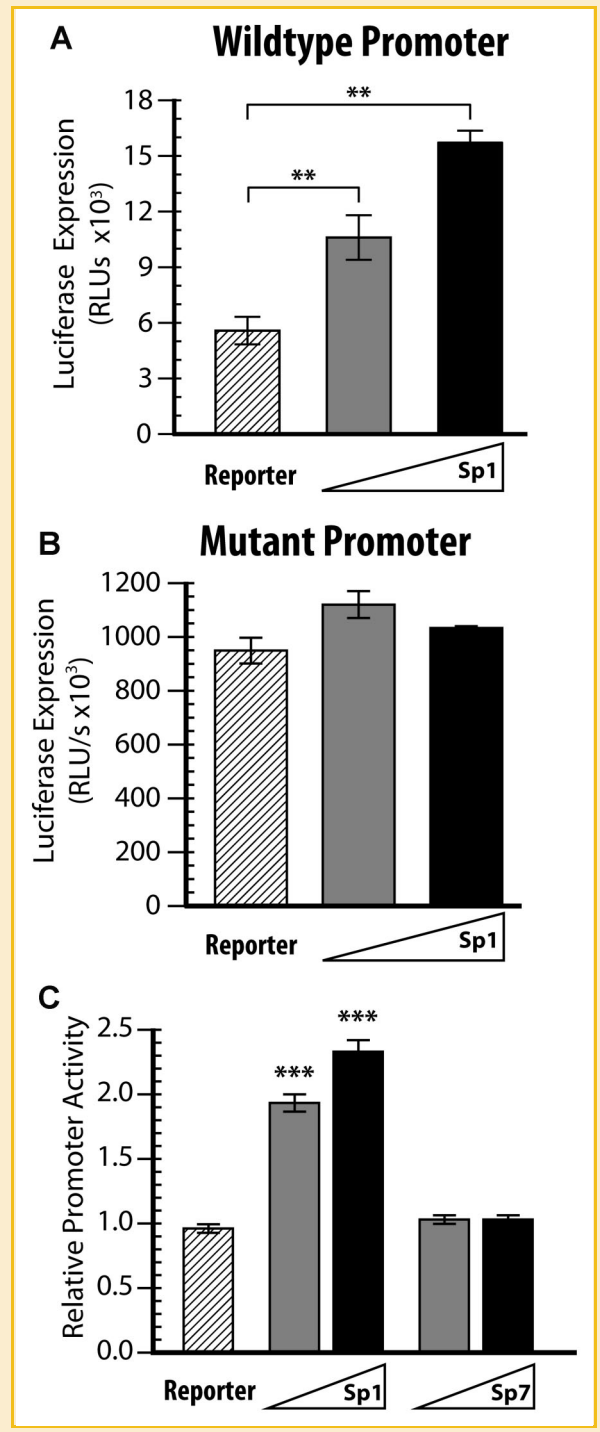


Fig. 2. Sp1 but not Sp7/OSTERIX activates transcription from the proximal *Fiat* promoter. MC3T3-E1 cells were transiently transfected with the p*Fiat*-Luc (Wildtype Promoter, panels A, C) or p*Fiat*-Mut-Luc (Mutant Promoter, panel B) reporters, and increasing amounts of expression vectors for Sp1 (A–C) or Sp7/OSTERIX (panel C). Results are mean ± SEM of representative transfections performed in triplicates. $^{**}P < 0.01$; $^{***}P < 0.001$.

had no impact on expression of the p*Fiat*-Mut-Luc reporter in transient transfection assay (Fig. 2B). In accordance with the EMSA data showing that Sp7/OSTERIX does not bind the proximal *Fiat* promoter fragment (Fig. 1B), over-expression of Sp7/OSTERIX had no effect on transcription from the p*Fiat*-Luc reporter (Fig. 2C).

Chromatin Immunoprecipitation also confirmed that the Sp3 transcription factor is bound to the endogenous *Fiat* promoter region spanning the GC-box (Fig. 3A). To understand the functional consequence of Sp3 binding on *Fiat* promoter activity, we examined the effect of individual isoforms of Sp3 on p*Fiat*-Luc expression in MC3T3-E1 cells. As shown in Figure 3B,C, transfection with either the long isoforms or the short isoforms of Sp3 led to dose-dependent inhibition of reporter gene expression. Co-transfection of increasing amounts of the Sp3 long isoform expression vector in the context of maximal Sp1-dependent *Fiat*-luciferase activation led to dose-dependent repression of the expression of the reporter (Fig. 3D). Taken together, these results suggest that an interplay between Sp1 and Sp3 may contribute to the steady-state control of *Fiat* gene expression in osteoblasts.

### MODULATION OF SP1 OR SP3 EXPRESSION AFFECTS *FIAT* TRANSCRIPTION

To further demonstrate that endogenous Sp1 is required for *Fiat* gene expression, MC3T3-E1 cells were infected with control (scrambled) or Sp1-specific shRNA lentiviruses. We achieved 70% reduction in Sp1 mRNA (Fig. 4A) and protein (Fig. 4B) levels with the Sp1-specific shRNA construct. This led to an inhibition of Fiat transcript levels that achieved near statistical significance ($P = 0.053$; Fig. 4C).

In parallel, we disrupted the Sp1-cognate DNA interaction in MC3T3-E1 cells by treatment with the anthracycline derivative, WP631 methane sulfonate. WP631 bisintercalates within GC-rich domains in DNA and interferes with Sp1 binding [Martin et al., 1999; Mansilla et al., 2004]. A 4 h treatment with WP631 significantly decreased the binding of Sp1 to its cognate site within the *Fiat* proximal promoter as observed by ChIP (Fig. 4D), and this was associated with a significant reduction in endogenous *Fiat* transcript levels (Fig. 4E).

We also selectively inhibited Sp3 expression using DsiRNA (Diced silencing RNA) to examine its relative contribution to *Fiat* transcription in MC3T3-E1 cells. Selective knockdown inhibition of Sp3 decreased its mRNA level by 70% and decreased the level of each of its isoforms (Fig. 5A). Knockdown of Sp3 expression led to a significant increase in endogenous *Fiat* transcript levels (Fig. 5B). These results confirm that Sp3 negatively influences steady-state *Fiat* gene transcription in osteoblastic cells.

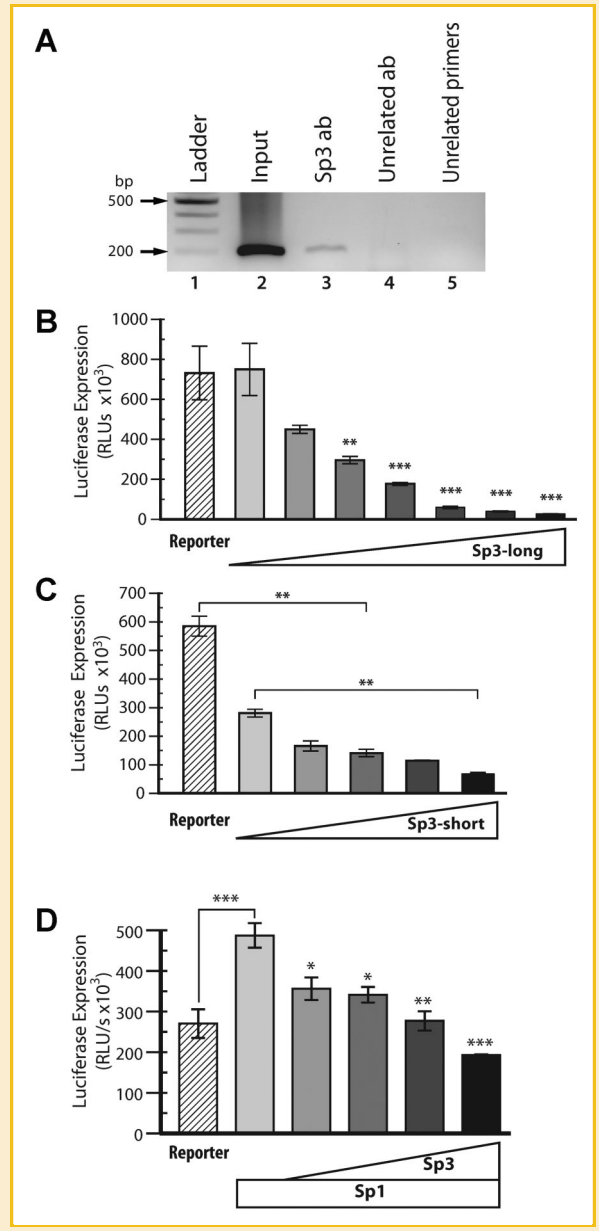


Fig. 3. Sp3 binds the proximal *Fiat* promoter GC-box to repress *Fiat* transcription. A: Chromatin Immunoprecipitation assay. Immunoprecipitation was performed with formaldehyde-crosslinked chromatin from confluent MC3T3-E1 cells and antibodies (ab) against Sp3 or a control, unrelated antibody (anti-ribosomal protein S6). Ethidium bromide-stained agarose gels of PCR products obtained with primers flanking the *Fiat* GC-box (lanes 2–4) or primers from the FIAT coding sequence (unrelated primers, lane 5) are shown. Input, amplification of DNA prior to immunoprecipitation; Ladder, molecular size markers. B–D: MC3T3-E1 cells were transiently transfected with the p*Fiat*-luc reporter, and increasing amounts of expression vectors for the long Sp3 isoform (Sp3-long, panels B, D), the short Sp3 isoform (Sp3-short, panel C), or for Sp1 (panel D), alone or in combination. Results are mean ± SEM of representative transfections performed in triplicates. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

## DISCUSSION

During osteogenesis FIAT regulates osteoblast biology by interacting with ATF4 and inhibiting its function [Yu et al., 2005, 2009b]. ATF4 is a key transcription factor that controls the expression of critical effectors of osteoblast activity including *Esp*, *Rankl*, and *Osteocalcin* [Karsenty, 2008; St-Arnaud and Mandic, 2010]. This regulatory role of FIAT makes it important to understand how *Fiat* transcription and expression is controlled. Our study is the first to identify a cognate element within the *Fiat* proximal promoter through which Sp1 and Sp3 act. We showed that Sp1 and Sp3directly bound to this GC element both in vitro and in vivo.

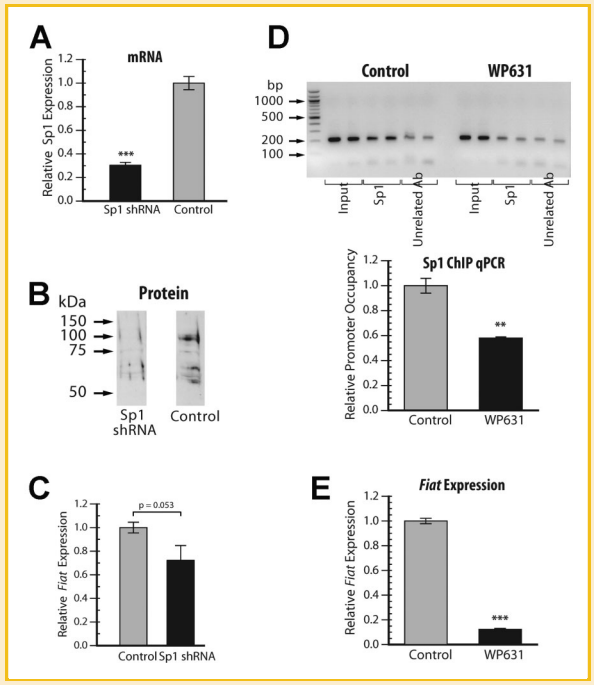


Fig. 4. Silencing of Sp1 or blocking of the Sp1/DNA interaction reduces *Fiat* expression. Sp1 mRNA (A) and protein (B) expression was significantly reduced in MC3T3-E1 cells stably transfected with an Sp1 shRNA, as compared to negative control scrambled shRNA. C: Sp1 knockdown reduced endogenous *Fiat* mRNA levels measured by RT-qPCR. D: Treatment of MC3T3-E1 cells with the anthracycline derivative, WP631 methanesulfonate, decreased Sp1 occupancy at the Fiat proximal promoter GC-box. The ethidium bromide-stained agarose gels of PCR products as well as quantitative ChIP using SYBR-green qPCR are shown. E: WP631 treatment of MC3T3-E1 cells concomitantly reduced *Fiat* mRNA levels as measured by RT-qPCR. $^{**}P < 0.01$; $^{***}P < 0.001$.

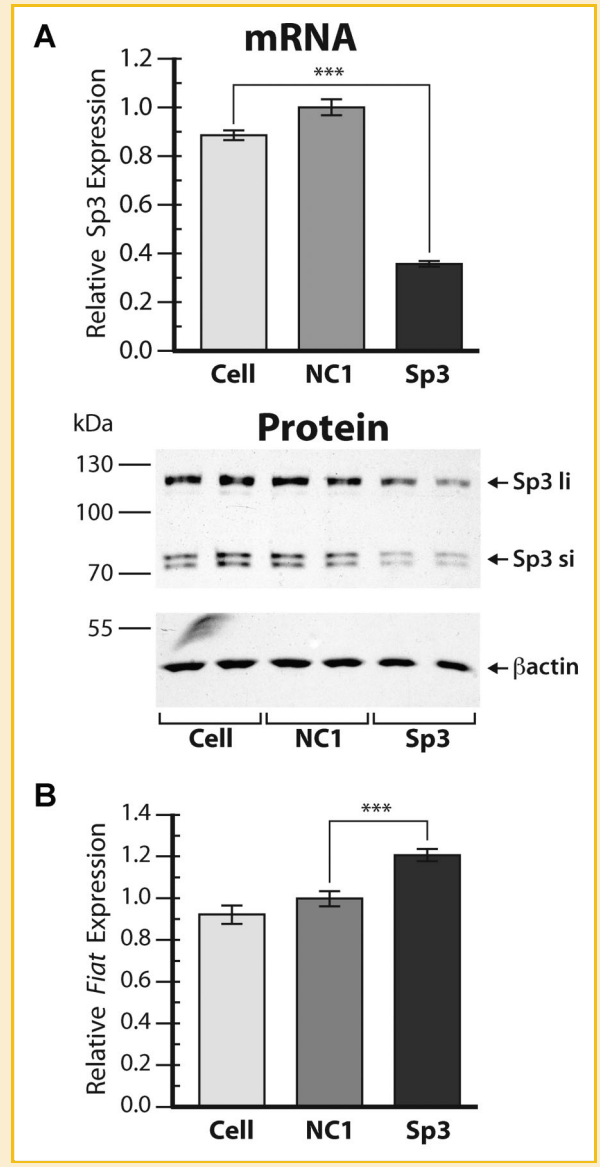


Fig. 5. Silencing of Sp3 increases *Fiat* expression. A: Sp3 mRNA and protein expression was significantly reduced in MC3T3-E1 cells transfected with an Sp3DsiRNA (Sp3), as compared to negative control (NC1) scrambled DsiRNA. Cell, control, untransfected cells; li, long isofom; si, short isoform. B: RNA was harvested 48-h post-transfection and analyzed for *Fiat* expression by RT-qPCR. $^{***}P < 0.001$.

Functional analysis demonstrated that over-expression of Sp1 and Sp3 in MC3T3-E1 cells significantly increased and decreased *Fiat* promoter activity, respectively. Surprisingly, we observed that the member of the Sp family that is required for osteoblast differentiation and bone formation, Sp7/OSTERIX [Nakashima et al., 2002], does not bind the *Fiat* proximal GC-box and does not regulate *Fiat* promoter activity through this element. The relevance of Sp1 and Sp3 in the steady-state expression of endogenous *Fiat* transcription was confirmed using RNAi technology in osteoblastic cells. Taken together, our results show that both ubiquitous Sp family proteins, Sp1 and Sp3, are involved in the transcriptional regulation of the *Fiat* gene in MC3T3-E1 osteoblast cells. Reciprocal Sp1-dependent activation and Sp3-mediated inhibition of transcription through the same cognate response element has been described previously as a transcriptional regulatory mechanism in mesenchymal cells, for example in the control of pro-α1(II) (*Col2a1*) gene expression [Chadjichristos et al., 2002].

The initial characterization of Sp7/OSTERIX showed that it can bind to a canonical Sp1 response element [Nakashima et al., 2002]. Functional analysis of natural promoters responding to Sp7/OSTERIX have revealed, however, that binding sites for this factor can be quite divergent from the 5′-GGGGCGGGGC-3′ canonical Sp1-binding sequence present within the *Fiat* proximal promoter. For instance, Sp7/OSTERIX sites within the pro-α1(V) (*Col5a1*) or pro-α3(V) (*Col5a3*) collagen genes show the following sequences, respectively: 5′-CCACCC-3′ [Wu et al., 2010] and 5′-TGGGCGTGG-3′ [Yun-Feng et al., 2010]. This divergence most likely explains why, even when overexpressed, Sp7/OSTERIX does not activate the *Fiat* promoter through the proximal GC-box.

It has been shown that Sp7/OSTERIX requires Sp1 (but not Sp3) to regulate *Osteocalcin* gene transcription [Niger et al., 2011]. We have performed a series of co-transfection experiments with varying ratios of Sp7/OSTERIX and Sp1 expression vectors without detecting any synergistic effect (data not shown). These experiments

rule out a role for Sp7/OSTERIX in the regulation of transcription from the *Fiat* proximal promoter through co-recruitment by or interaction with Sp1.

Sp1 and Sp3 are ubiquitously expressed in mammalian cells and are prototypic $C_2H_2$-type zinc finger-containing DNA-binding proteins [Waby et al., 2008]. They both have similar structures and bind to the same DNA domain with comparable affinities [Sapetschnig et al., 2004]. They regulate expression of large number of genes but their roles in bone physiology have not been fully understood. It has been reported that overexpression of Sp1 increases the transcription of the master osteoblast regulatory gene, *Runx2* [Zhang et al., 2009]. However, *Sp1*-deficient mice display early embryonic lethality before the formation of skeletal components [Marin et al., 1997]. The short-lived *Sp3*-ablated mice show impaired endochondral and intramembranous ossification in embryos at E18.5 [Gollner et al., 2001].

Sp3 is expressed in four different isoforms (two long and two short) that are driven from four distinct AUG translational start sites. In most of the systems described to date, the two long isoforms can act as transcriptional activators on certain promoters, whereas the two small isoforms have inhibitory potential [Sapetschnig et al., 2004]. Western blot analysis of endogenous Sp3 reveals the expression of all four isoforms in MC3T3-E1 cells (Fig. 5A). Unlike most published studies, our results showed that the long and the short isoforms have the capacity to repress *Fiat* transcription in osteoblast cells. The upregulation of *Fiat* expression measured following Sp3 knockdown in MC3T3-E1 cells support a biologically relevant inhibitory role of endogenous Sp3 on *Fiat* gene transcription in osteoblasts.

These data raise an interesting point concerning the mechanism through which Sp3 inhibits *Fiat* transcription. One possible explanation involves post-translational modification. It is well established that SUMOylation of Sp3 is associated with transcriptional repression [Waby et al., 2008]. SUMOylation takes place at the lysine 551 residue that is present in all four Sp3 isoforms. Mutation at this residue has been associated with induced transcriptional activity of both the long and short Sp3 isoforms target genes [Sapetschnig et al., 2004; Spengler et al., 2005]. It would prove interesting to test Sp3 expression vectors mutated at residue K551 [Sapetschnig et al., 2004] on the p-*Fiat*-Luc reporter.

Our analysis has focused on the proximal promoter of the *Fiat* gene since we rapidly identified a functional response element in this region. It is evident that additional regulatory sequences are involved in the physiological control of *Fiat* expression. For instance, previous studies by our group have shown that FIAT protein levels decline as osteoblastogenesis progresses [Yu et al., 2009a]. While it is tempting to suggest that the interplay between Sp1 and Sp3 transcription factors on the *Fiat* proximal promoter acts as a mechanism involved in this decrease in *Fiat* expression, results from our laboratory show that Sp1 and Sp3 exhibit the same stable profile of expression at the mRNA and protein levels in differentiating MC3T3-E1 osteoblastic cells (data not shown). Moreover, when we compared GC-box occupancy at the *Fiat* proximal promoter between undifferentiated and differentiated MC3T3-E1 cells using quantitative ChIP, we measured higher amounts of endogenous Sp1 bound to the *Fiat* proximal promoter in comparison to Sp3 in both undifferentiated and differentiated cells (data not shown). Therefore further analysis of the control of *Fiat* transcription and/or FIAT protein stability is required to fully understand how FIAT levels are regulated in bone tissue.

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