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Zfp521 antagonizes Runx2, delays osteoblast differentiation *in vitro*, and promotes bone formation *in vivo*

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Abstract

Zfp521, a 30 C2H2 Kruppel-like zinc finger protein, is expressed at high levels at the periphery of early mesenchymal condensations prefiguring skeletal elements and in all developing bones in the perichondrium and periosteum, in osteoblast precursors and osteocytes, and in chondroblast precursors and growth plate prehypertrophic chondrocytes. Zfp521 expression in cultured mesenchymal cells is decreased by BMP-2 and increased by PTHrP, which promote and antagonize osteoblast differentiation, respectively. *In vitro*, Zfp521 overexpression reduces the expression of several downstream osteoblast marker genes and antagonizes osteoblast differentiation. Zfp521 binds Runx2 and represses its transcriptional activity, and Runx2 dose-dependently rescues Zfp521's inhibition of osteoblast differentiation. In contrast, *osteocalcin* promoter-targeted overexpression of Zfp521 in osteoblasts *in vivo* results in increased bone formation and bone mass. We propose that Zfp521 regulates the rate of osteoblast differentiation and bone formation during development and in the mature skeleton, in part by antagonizing Runx2.

Keywords

Zfp521; Runx2; osteoblast; bone formation; differentiation

Introduction

OBs arise from mesenchymal stem cells that can differentiate into a number of specialized cell types [1,2]. The progression from multipotent cells to the fully differentiated and bone matrix-producing osteoblast is regulated by numerous transcription factors, among them Runx2, a member of the Runt domain family of transcription factors considered to be a master regulator that is essential for osteoblast commitment and early stages of osteoblast differentiation [3–

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6] and thus essential for bone formation. Runx2 induces the expression of Osterix, a transcription factor specifically expressed in all developing bones [7], which together with the autocrine Wnt-induced activation of β -catenin [8] promotes the progression to fully mature osteoblasts. The activities of these key transcription factors are modulated by a variety of factors and mechanisms [9–13].

Zfp521 (also termed Evi3 in mice and EHZF in humans) is a zinc finger protein consisting almost entirely of 30 C2H2 Kruppel-like zinc fingers [14,15]. Both Zfp521 and the homologous Zfp423 (also termed OLF/EBF associated-zinc finger protein [OAZ]) inhibit cell differentiation in some cell lineages [15–17]. We have now found that Zfp521 is expressed in osteoblast and chondrocyte precursors in the periosteum, endosteum and perichondrium, in prehypertrophic chondrocytes in the growth plate, and in osteoblasts and osteocytes in bone. Zfp521 expression increased during *in vitro* osteoblast differentiation, and BMPs and PTHrP regulated its expression. Zfp521 bound Runx2 and repressed the expression of several osteoblast marker genes and *in vitro* osteoblast differentiation, while overexpressed Runx2 dose-dependently reversed this effect. In contrast to the effect of Zfp521 on *in vitro* osteoblast differentiation, however, targeted overexpression of Zfp521 in mature osteoblasts in transgenic mice resulted in marked increases in bone formation. We propose that the balance between Zfp521 and Runx2 contributes to the regulation of the rate of osteoblast differentiation and bone formation during development and in the mature skeleton.

Materials and Methods

Plasmids

Zfp521 was cloned from a mouse brain lambda phage library (Clontech, Mountain View, CA). The open reading frame was amplified by PCR (HA-Zfp521 F and HA-Zfp521 R primers; Table 1) and ligated into the pCMV-HA vector (Clontech) between the Sal1/Not1 sites. P. Ducy (Columbia University, New York) provided pCMV-FLAG-Runx2 encoding the p57 type II Runx2 isoform (N-terminal MASNSL..) and *OG2-luciferase*, G. Rawadi (Galapagos, Romainville, France) provided *Runx2-luciferase*, and S. Warming (National Cancer Institute, Frederick, MD) provided *mEBFAZ* cDNA, which was subcloned into the pCMV-HA vector. Zfp521 shRNA constructs were generated by subcloning annealed oligonucleotides (PS3; Table 1) into the pSilencer vector, resulting in shRNA with a 19-nucleotide sequence that NCBI Blast analysis showed to be specific for Zfp521.

Cell culture

Primary calvarial osteoblasts were obtained from 1–3 day-old mice as previously described [18]. All cells were cultured in α -MEM with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μ g/ml streptomycin (all Invitrogen, Carlsbad, CA). Differentiation was induced by adding BMP-2 (100 ng/ml; Galapagos, Romainville, France) to confluent cells (day 0). Calvarial osteoblasts and MC3T3-E1 cells stably transfected with Zfp521 were differentiated in 50 μ M ascorbic acid and 5 mM β -glycerolphosphate (Sigma-Aldrich).

Cell transfection

Cells at 80% confluence were transfected using a ratio of 2 μ g DNA to 3 μ l FuGENE6 transfection reagent (Roche) as described by the manufacturer.

Generation of stable cell lines

C3H10T1/2 and MC3T3-E1 cells were infected with concentrated Zfp521 or lacZ lentivirus and cultured overnight in 6-well dishes (1 ml/well containing 6 mg/ml polybrene). Fresh

medium was added one day post-infection. Cells were cultured for 3 weeks in 2 mg/ml (C3H10T1/2) or 5 mg/ml (MC3T3-E1) blasticidin to eliminate uninfected cells.

Retrovirus and adenovirus production and gene transduction

shRNA and control retroviruses were generated by transfecting PhoenixE cells using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). 72 hours post-transfection, retrovirus was harvested, combined with medium and polybrene (4 mg/ml) in a total volume of 4 ml, and immediately overlaid on CD1 calvarial osteoblasts plated the previous night at 275,000 cells/6 cm culture dish. After 24 hours, medium was changed to α -MEM with 10% FBS, 100 units/ml penicillin and 100 μ g/ml streptomycin. One day later, cells were split into two 10 cm dishes and placed in selection medium containing 2 μ g/ml puromycin. Cells were cultured until uninfected cells died, then harvested, counted and replated for experiments.

To produce Runx2 adenovirus, the Runx2 open reading frame of the pCMV-FLAG-Runx2 plasmid (type II isoform) was amplified and cloned into the pShuttle-CMV vector (Qbiogene). Linearized plasmid (PmeI - New England Biolabs, Ipswich, MA) was purified and electroporated into E. coli strain BJ5183-AD-1 (Stratagene) for recombination with the pAdEasy-1 plasmid. Positive colonies were identified by restriction digestion with PacI (New England Biolabs). Adenovirus was generated as previously described [19]. Calvarial osteoblasts were seeded at a density of 50,000 cells/well in a 6-well tissue culture dish and infected overnight once confluent.

Antibodies

Mouse monoclonal anti-HA, mouse monoclonal anti-Myc, mouse polyclonal anti-PEBP2aA, goat anti-OAZ and mouse monoclonal anti-C23 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), mouse monoclonal anti-actin from Chemicon International (Temecula, CA), and mouse anti-V5 from Invitrogen. Affinity-purified rabbit polyclonal antibodies against Zfp521, produced by Bethyl Laboratories (Montgomery, TX) using peptide antigens CT³⁶⁹MVEAAPPKSRGR [anti-Zfp521(369)] and CQ¹¹⁷⁷VSPMPRISPSQSDEKK [anti-Zfp521(1177)], were used at 1:500 for Western blotting, 5 μ g/ml for immunoprecipitation and 20 μ g/ml for immunofluorescence. Antibody specificity, including lack of cross-reactivity with the closely related Zfp423, was confirmed by Western blot (data not shown).

In-situ hybridization

To generate Zfp521 probes, the 907–1219 and 3306–3611 fragments of the cDNA were amplified by PCR with primers containing HindIII and BamHI restriction sites and inserted into the HindIII/BamHI sites of pBSKII. ³⁵S-labeled sense and antisense riboprobes were synthesized from linearized plasmids using the Promega transcription kit and ³⁵S-UTP (Amersham). *In situ* hybridization of Zfp521 mRNA with cRNA probes was performed on sections from E12.5, E15.5 and E18.5 mouse embryo and neonatal mouse skeleton as previously described [20]. Sections were digitally photographed and analyzed using Adobe Photoshop 6.0.

Immunofluorescence microscopy

Humeri of 6 month old mice were fixed and decalcified in 4.1% EDTA, 5% PVP (pH 7.4) for 3–4 days. Tissue samples and bones were washed in PBS after fixation and kept in 40% sucrose until sectioned. Cells were plated on glass coverslips and cultured overnight, then fixed in 3.7% formaldehyde/PBS for 10 min and washed twice with PBS. Nuclei were stained with ToPro-3 iodide (1:1000, Molecular Probes, Eugene, OR). Zfp521 was labeled with the anti-Zfp521 (369) antibody. Cells and cryostat sections were mounted in FluorSave (Calbiochem, San Diego, CA) and imaged using an LSM 510 Meta confocal microscope (Carl Zeiss Inc.,

Thornwood, NY). Images were recorded, composite images were compiled and enhancements performed using Adobe Photoshop 8.0.

Analysis of alkaline phosphatase activity and mineralized nodules

Cells were washed twice with PBS and fixed for 10 minutes in 3.7% formaldehyde, then stained using Fast BlueAlkaline phosphatase (Sigma-Aldrich) according to the manufacturer's instructions. Mineralized nodules were stained by the Von Kossa method [18], or with alizarin red by incubating cells with 2% (w/v) of alizarin red S (Sigma-Aldrich) in water at pH 4.2 for 10 minutes.

Northern blot analysis

RNA was isolated and Northern blots were performed using a gel electrophoresis-purified Zfp521 probe excised from pASV4 by EcoR1 digestion as previously described [18].

Real-time PCR

RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized using 1 µg of total RNA and SuperScript 2 (Invitrogen) according to the manufacturer's instructions. 50 ng of cDNA was mixed with 12.5 µl iQ SYBR green supermix (BioRad, Hercules, CA), 10.5 µl of water, and 0.5 µl of sense and antisense oligonucleotide (10 µM; Table 1), denatured at 95 °C for 3 minutes and amplified by 40 cycles of 95 °C/57 °C/72 °C for 30 seconds each using an iCycler (BioRad). All samples were normalized to GAPDH and relative expression of Zfp521 was determined using the $2^{-\Delta\Delta CT}$ method. Data are presented as fold change relative to control samples.

Transcriptional activation assay

MC3T3-E1 cells were plated in 6-well tissue culture plates (1×10^5 cells/well) and transfected with pCMV-HA-Zfp521 (2.0 µg) and pCMV-FLAG-Runx2 (1.5 µg) per well for 24h using FuGENE 6 at a 6:1 FuGENE 6 to DNA ratio. The Runx2-luc and OG2-luc constructs were used at 1.0 µg/well. *Renilla luciferase* cDNA (0.017 µg/well, Promega) was included as control for transfection efficiency. Total DNA was maintained at 4.5 µg/well with empty vector (pcDNA3.1). Dual-Luciferase assay (Promega) was performed according to the manufacturer's instructions. Results corrected for transfection efficiency were normalized to empty vector.

Transgenic mice

OG2-tTA mice were described elsewhere [21]. TetOp-Zfp521 mice were generated by subcloning the SalI-NotI fragment of Zfp521 from pCMV-HA-Zfp521 into the TetOP-tTA vector (E. Nestler, Southwestern Medical Center, Dallas) from which the tTa gene was excised. Linearized DNA was microinjected into pronuclei of oocytes from SJL $\times$ C57BL6 mice F2 mouse ova as previously described [21]. When crossed with the OG2-tTA mice, two TetOp-Zfp521 lines (03 and 05) generated double-transgenic OG2-tTA $\times$ TetOp-Zfp521 (OG2 $\times$ Zfp521) mice that expressed Zfp521. Animal protocols were approved by the Yale Animal Care and Use Committee.

Bone histomorphometry

Histomorphometric analysis of 4 and 12 week-old OG2 $\times$ Zfp521 mice and control littermates was performed as previously described [18].

In vivo bone resorption analysis

Collagen type 1 C-telopeptide fragments in serum samples collected at 4 and 12 weeks of age were analyzed using the RatLaps ELISA kit (Nordic Biosciences Diagnostics) according to the manufacturer's instructions.

Statistical analysis

Statistical significance was calculated using the Student's t test. Standard error was calculated using one-tailed analysis.

Results

Zfp521 is expressed in osteoblasts and mesenchymal cells

Zfp521 is present in many cell types [14], and Northern blots revealed that it is expressed at high levels in calvarial osteoblasts as well as in C3H10T1/2 and MC3T3-E1 mesenchymal cell lines (Fig. 1A). In contrast, Zfp521 was not detected in mature osteoclasts generated *in vitro*, osteoclast precursors or the monocytic RAW 264.7 cell line (not shown), indicating that the protein is either not expressed or expressed at much lower levels in osteoclasts than in osteoblasts.

In situ hybridization confirmed these findings in developing skeletal elements (Fig 1B–F), with high expression of Zfp521 at the periphery of mesenchymal condensations (Fig. 1B), in the developing skull (Fig. 1C) and in perichondral and periosteal cells (Fig. 1D–F), suggesting a possible role in regulating early skeletal development. Immunocytochemistry using affinity-purified antibodies directed against Zfp521 (Fig. 1G) confirmed the pattern of expression in developing long bones seen by *in situ* hybridization. In the growth plate, Zfp521 expression was low in resting and proliferating chondrocytes, markedly higher in the prehypertrophic chondrocytes, then low again in hypertrophic cells (upper panels). In postnatal bone, Zfp521 was strongly expressed in the periosteum and the endosteum where it was restricted to osteoblasts and one or two layers of pre-osteoblastic cells (middle panels), and in osteocytes (lower panels). No staining was detected in osteoclasts.

Zfp521 is a nuclear protein in the chondrocyte and osteoblast lineages

Consistent with Zfp521 being a nuclear transcription factor, Zfp521 was most highly enriched in the nuclei of C3H10T1/2 cells, especially the nucleoli (Fig. 1H). Some Zfp521 was also detected in the cytoplasm. Together, the immunocytochemical and *in situ* hybridization data suggested a regulated pattern of expression with high levels in partially differentiated chondrocytes (but not in hypertrophic cells), as well as in pre-osteoblasts, mature osteoblasts and osteocytes. The nuclear localization of Zfp521 suggests that it plays a role in the regulation of gene transcription during osteoblast differentiation.

Zfp521 antagonizes osteoblast differentiation

The expression of Zfp521 at the periphery of mesenchymal condensations, in areas of transition from precursors to more mature chondrocytes or osteoblasts (growth plate, perichondrium and periosteum), and in mesenchymal cell lines suggested that it might regulate the differentiation of early mesenchymal cells during bone development and formation. To determine whether Zfp521 affected the process of osteoblastogenesis positively or negatively, we used small hairpin RNA (shRNA) to deplete Zfp521 mRNA by approximately 40% in calvarial OBs (Fig. 2A). The shRNA increased nodule formation and mineralization (Fig. 2B), indicating that Zfp521 has an inhibitory effect on osteoblast differentiation and maturation in cultures of early osteoblast precursors.

The effect of Zfp521 on alkaline phosphatase (ALP) production, a marker of early osteoblast differentiation, was tested by transiently transfecting wild-type calvarial cells with Zfp521 and staining for ALP activity 6 days after transfection. The Zfp521-transfected cells had less ALP activity than mock-transfected cells (Fig. 2C). Moreover, stable overexpression of Zfp521 (Fig. 2D) reduced ALP expression in MC3T3-E1 cells and in BMP-2-treated C3H10T1/2 cells (which do not express ALP unless treated with BMP-2) (Fig. 2E). Thus, Zfp521 delays differentiation in calvarial osteoblasts and mesenchymal cell lines.

Zfp521 expression is regulated during osteoblast differentiation

If Zfp521 is a physiologically important brake on osteoblast differentiation, its expression might be regulated during differentiation of osteoblast precursors. We therefore determined if agents that modulate osteoblast differentiation affected endogenous Zfp521 expression (Fig. 3). Endogenous Zfp521 mRNA levels increased steadily with time in culture in mesenchymal cell lines and primary calvarial osteoblasts. Treatment with BMP-2, which induces osteoblast differentiation and maturation, repressed Zfp521 expression (Fig. 3A, B). In contrast, continuous treatment of calvarial osteoblasts with PTHrP 1–34, which favors proliferation [22] but antagonizes osteoblast differentiation [23], increased Zfp521 expression (Fig. 3C).

Zfp521 interacts with Runx2 and inhibits Runx2-induced activation of the Runx2 and osteocalcin promoters

Runx2 is a key regulatory factor downstream of BMPs and PTHrP [24]. We therefore examined the effect of Zfp521 on Runx2-induced activation of *Runx2-luciferase* (Runx2-luc) and *osteocalcin-luciferase* (OG2-luc) reporter genes in MC3T3-E1 cells (Fig. 4A). As previously reported [3,6,25], Runx2 activated both reporter genes. Zfp521 alone had little effect on either reporter, but it strongly repressed the activation of the two reporters by Runx2, indicating that Zfp521 indeed antagonizes Runx2 transcriptional activity. The antagonism of Runx2 activity by Zfp521 suggested that the two proteins might interact, either directly or indirectly, and we confirmed this by co-immunoprecipitating HA-tagged Zfp521 and FLAG-tagged Runx2 from transiently transfected 293VNR cell lysates (Fig. 4B). Thus, Zfp521 both associates with Runx2 and represses Runx2 transcriptional activity, indicating that Zfp521's ability to antagonize osteoblast differentiation could be a consequence, at least in part, of its interaction with Runx2.

Osteoblast-targeted overexpression of Zfp521 inhibits ex vivo osteoblast differentiation but promotes in vivo bone formation

Zfp521's antagonism of *in vitro* osteoblast differentiation and Runx2 transcriptional activity suggested that overexpressing the protein would inhibit bone formation *in vivo*. To test this possibility and since we had observed Zfp52 expressed in mature osteoblasts, we generated TetOP-Zfp521 mice and crossed them with the osteoblast-restricted OG2-tTA mice, in order to express the protein specifically in osteoblasts [26]. Two lines of OG2-tTA × TetOp-Zfp521 (OG2 × Zfp521) mice expressed two-fold more Zfp521 in bone but not in fat or heart (Fig. 5A), consistent with the previous characterization of the OG2-tTA mice [21].

Before examining the effect of the overexpressed Zfp521 on the *in vivo* bone formation, we examined the *ex vivo* differentiation of calvarial cells from the mice to confirm that the overexpressed Zfp521 antagonized the differentiation of calvarial cells in a manner consistent with our earlier observations. Prior to induction of osteogenic differentiation, the cells derived from transgenic mice already expressed 1.5- to 2-fold more Zfp521 than cells from control littermates (data not shown), notwithstanding the characterization of osteocalcin as a marker of relatively mature osteoblasts [27]. The OG2 × Zfp521 cultures had less nodule formation and mineralization than the control cultures (Fig. 5B), consistent with our *in vitro* results (Fig. 2). Moreover, expression of several early osteoblast marker genes and Runx2 target genes

(*Runx2*, *Osterix*, *alkaline phosphatase*, *osteopontin*, *osteocalcin*, *bone sialoprotein*) was decreased in the Zfp521 calvarial cells (Fig. 5C), further confirming that Zfp521 antagonizes Runx2 and *in vitro* osteoblast differentiation.

We also examined the effect of expressing increasing amounts of Runx2, using a Runx2 adenovirus, on the differentiation of the OG2 $\times$ Zfp521 calvarial cells, in order to determine if Runx2 could reverse the inhibitory effect of Zfp521. Runx2 dose-dependently reversed the Zfp521-induced inhibition of ALP expression by Runx2 (Fig. 5D), providing further evidence that Zfp521 acts at least in part by antagonizing Runx2 activity.

Unexpectedly, however, Von Kossa-stained sections of proximal tibiae from the OG2 $\times$ Zfp521 animals showed denser trabecular bone and thicker cortices (Fig. 6A). Histomorphometric analysis of tibiae from 4 and 12 week-old OG2 $\times$ Zfp521 mice and non-Zfp521 littermates (Fig. 6B, Table 2) revealed increased bone volume, osteoblast number and bone formation rate in the OG2 $\times$ Zfp521 mice. Osteoclast number and serum collagen cross-links, a marker of bone resorption, were essentially unchanged (Table 2, Fig. 6C), indicating that Zfp521 overexpression did not alter bone resorption and that *osteocalcin* promoter-driven overexpression of Zfp521 leads to an increase in bone mass due to a marked increase in osteoblast numbers and bone-forming activity. Thus, *osteocalcin* promoter-driven stable expression of Zfp521 in osteoblast precursors hindered the progression of both calvarial cells and mesenchymal cell lines towards a mature osteoblast phenotype *in vitro*, but promoted bone formation in the whole animal.

Discussion

Our results show that Zfp521 inhibits *in vitro* osteoblast differentiation and that overexpressing Runx2, whose transcriptional activity is decreased by Zfp521, reverses this inhibition. In addition, Zfp521 regulates *in vivo* bone formation when its expression is targeted to osteoblasts via the *osteocalcin* promoter, although in this case, Zfp521 increases bone formation, the opposite of what would be predicted from its antagonism of *in vitro* osteoblast differentiation. Thus, this report establishes Zfp521 as a novel and important player in the regulation of osteoblast differentiation and bone formation, which on the one hand antagonizes Runx2 but on the other hand favors bone formation *in vivo*, at least when expression is targeted to osteoblasts by the *osteocalcin* promoter.

Both Zfp521 and the homologous Zfp423 exert inhibitory effects on the differentiation and maturation of several cell types [14,16,17,28], suggesting that this family of proteins might function generally as “switch proteins”, controlling the transition from immature to mature cells. Several of our observations suggest that Zfp521 restricts the developmental progression of mesenchymal cells in the developing skeleton. We found that it is expressed in early mesenchymal cell precursors during skeletal development, around early mesenchymal condensations, in the perichondrium, periosteum, and specific regions of the growth plate of developing bones, in osteocytes, and also in primary osteoblasts and mesenchymal cell lines *in vitro*. Its expression was strongly repressed by treatment of the cells with BMP-2, which induces osteoblast differentiation, and increased by PTHrP, which prevents differentiation in favor of proliferation. Overexpressing Zfp521 in primary osteoblasts and a multipotent mesenchymal cell line delayed or reduced the expression of early osteoblast differentiation markers (Runx2, alkaline phosphatase, collagen 1) and the formation of mineralized nodules. Conversely, depleting Zfp521 with shRNA favored osteoblast differentiation and mineralization. Together, these results strongly suggest that Zfp521 acts to maintain osteoblast precursors in a less differentiated state.

Appropriately balancing the advance of some cells towards a more mature state while retaining others in a less differentiated state requires tight regulation of the relative activities of factors that favor the maturation of the cells and those that arrest differentiation. Multiple transcription factors (i.e., *Msx*, *Sox9*, *Runx2*, β -catenin, *Osterix*, members of the AP-1 family) act at highly specific steps during the differentiation of early mesenchymal cells to mature chondrocytes or osteoblasts to either retain or advance the cell through specific checkpoints [2]. Appropriate timing of the activity of these key transcription factors is critical to the proper coordination of genes that maintain the balance of proliferation and commitment of osteoblast precursors and the progression to mature bone-forming cells. *Runx2* regulates both chondrogenesis, where it is required for final maturation of hypertrophic chondrocytes, and osteogenesis, where it plays a dual role, first as a “master regulator” that is required for osteoblast formation [3–6] and later as an antagonist of late stages of osteoblast maturation [29–31]. Similar early and late opposite effects have recently been suggested for Wnt signaling [32,33]. Since *Runx2* activates many of the osteoblast marker genes that we found to be down-regulated by *Zfp521*, we hypothesized that *Zfp521*’s inhibitory effect on osteoblast differentiation could result from antagonism of *Runx2* activity, and indeed, we observed that *Zfp521* bound *Runx2* and inhibited the *Runx2*-induced activation of *OG2-luc* and *Runx2-luc* reporter genes. In addition, overexpressed *Runx2* dose-dependently reversed the repression of ALP expression in calvarial cells from *Zfp521* transgenic mice. Thus, *Zfp521* inhibition of *in vitro* osteoblast differentiation appears to be at least in part a consequence of antagonizing *Runx2* activity.

Unexpectedly, however, overexpressing *Zfp521* in osteoblasts in transgenic mice under the control of the *osteocalcin* promoter strongly increased bone formation. This difference in the *in vitro* and *in vivo* effects of overexpressed *Zfp521* was not due to the use of different promoters, since differentiation of calvarial osteoblast precursors from *OG2* $\times$ *Zfp521* mice recapitulated the results with the cell lines that expressed *Zfp521* under the control of unrelated promoters, notwithstanding the characterization of the *osteocalcin* gene as a relatively late marker of osteoblast differentiation [26]. Moreover, driving *Zfp521* expression with the more widely expressed *ENO2* promoter [18] resulted in an even stronger increase in bone formation *in vivo* (data not shown). We cannot rule out, however, the possibility that expressing the *Zfp521* transgene *in vivo* under the control of a promoter that is specifically expressed earlier in osteoblast differentiation (e.g., *Osterix*, *Collagen type 1*) would result in reduced bone, and we are currently preparing to evaluate this possibility.

It is not clear how *Zfp521*’s antagonism of *Runx2* could contribute to the high bone phenotype. Other proteins that decrease *Runx2* levels (i.e., *Schnurri3*) or antagonize its activity (i.e., *Twist*) appear to down-regulate bone formation *in vivo*, consistent with their actions *in vitro*, since deleting either gene increases bone formation [10,11]. It is however important to note here that these genetic experiments simply removed the gene of interest in an untimed manner, thereby enhancing *Runx2* levels from early time points, and only at the early time points when *Schnurri3* or *Twist* are normally expressed. It is therefore not surprising that these loss-of-function experiments lead to increased bone formation. It is however well established that *Runx2* antagonizes the final steps of osteoblast maturation and/or the bone-forming activity of fully differentiated osteoblasts [29–31,34] in addition to promoting the progression of early osteoblast precursors [3–5]. Thus, a factor that antagonizes *Runx2* might promote bone formation if expressed in more mature cells, as we did here for *Zfp521* driven by the *osteocalcin* promoter. This proposed mechanism does not, however, exclude the involvement of other proteins in the increased bone formation, since *Zfp521* also interacts with *Smads* and *EBFs* [15], both of which affect bone formation [35–37]. Future studies will determine the respective contribution of these pathways to the observed *in vivo* phenotype.

In conclusion, our data show that *Zfp521* is expressed at high levels in mesenchymal precursor cells, and that BMP and PTHrP, which modulate osteoblast proliferation and differentiation,

regulate its expression. We also found that Zfp521 slows the progression of mesenchymal precursors towards the osteoblast phenotype *in vitro*, but promotes bone formation *in vivo*. Finally, Zfp521 interacts with and inhibits the transcriptional activity of Runx2. Thus, this report establishes Zfp521 as an important player in bone formation that modulates the actions of several key regulators of osteoblast differentiation and function, including Runx2.

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Figure 1. Zfp521 is a nuclear protein expressed in developing bones

A. Left panel - Northern blot of RNA isolated from tissues of adult CD1 mice with a probe specific for Zfp521. Zfp521 is expressed in all tissues assayed, with highest expression in the brain (Br), ovaries (Ov), uterus (Ut) and heart (He) and low levels in the spleen (Sp), muscle (Mu), kidney (Ki), calvaria (Ca) and lung (Lu). Center panel - Zfp521 is strongly expressed in primary neonatal calvarial cells. Right panel - Zfp521 is expressed in C3H10T1/2 mesenchymal cells as well as in pre-osteoblastic MC3T3-E1 cells. **B–F.** *In situ* hybridization of wild-type CD1 mouse embryos with Zfp521 antisense probe. Left panels – bright field, right panels – dark field. **B.** E12.5 vertebral section. Zfp521 is expressed at high levels in the areas surrounding the mesenchymal condensations that will give rise to individual vertebrae. **C.**

Oblique section through E15.5 skull. Zfp521 is highly expressed in all the craniofacial elements, most strongly in the developing skull. **D.** E15.5 metatarsals. Zfp521 is highly expressed in the perichondrium of each developing bone in the developing limb. **E.** E15.5 humerus. Zfp521 is expressed in both the perichondrium and periosteum of the bone collar. **F.** D.0 vertebrae. Zfp521 is expressed in the perichondrium as well as the endosteal surface of the developing marrow space. **G.** Immunohistochemistry with anti-Zfp521(369) antibody. Zfp521 is highly expressed in prehypertrophic chondrocytes of the growth plate (neonatal metatarsals, top panels), in osteoblasts and one or two layers of pre-osteoblastic cells in the periosteum and the endosteum (center panels), and in osteocytes (humeri of 6 month-old mice, lower panels). **H.** Confocal immunofluorescence of Zfp521 in C3H10T1/2 cells. The anti-Zfp521(369) antibody stained the nuclei, particularly in nucleoli, with weaker cytoplasmic staining. The peptide antigen blocked the staining. Pre-immune serum gave a low diffuse signal, demonstrating specificity. Similar staining was seen with the anti-Zfp521(1177) antibody (data not shown). Bottom panels -C3H10T1/2 cells co-labeled with anti-nucleolin (α C23) and anti-Zfp521(369) antibodies, demonstrating the nucleolar localization of Zfp521 (scale bar, 5 μ m).

Figure 2. Zfp521 regulates alkaline phosphatase expression

A. Calvarial cells were isolated from neonatal CD1 mice and infected with shRNA ps3 retrovirus or empty vector control virus (c). PCR analysis of Zfp521 mRNA levels revealed that the ps3 shRNA induced an approximately 40% reduction in Zfp521 expression. **B.** After 19 days in culture with osteogenic medium (ascorbic acid, β -glycerolphosphate), cells were stained for mineralized nodules using the Von Kossa method. Cells infected with shRNA virus (ps3) had more mineralization and nodule formation than cells infected with the control virus (c). **C.** After 3 and 6 days of culture, alkaline phosphatase activity was lower in the osteoblasts transfected with Zfp521 than in mock-transfected cells. **D.** V5-tagged Zfp521 (Z) or V5-tagged lacZ (L) were stably overexpressed in the C3H10T1/2 and MC3T3-E1 cell lines. RT-PCR analysis demonstrated that Zfp521 was overexpressed by 30-fold in the C3H10T1/2 cells and by 7-fold in the MC3T3-E1 cells (graphs, left). The expression of the stably expressed recombinant proteins was analyzed by Western blot using anti-V5 antibody (right). **E.** Overexpressing Zfp521 decreased ALP activity in both BMP-2-stimulated C3H10T1/2 (left panel) and in unstimulated MC3T3-E1 cells (right panel).

Figure 3. Zfp521 expression is inhibited by BMP-2 and stimulated by PTHrP

Confluent C3H10T1/2 (A) and MC3T3-E1 (B) cells were cultured with (black bars) or without (white bars) BMP-2 (50 ng/ml). RT-PCR analysis of Zfp521 expression was performed on RNA isolated at days 0, 3, 6, 9, and 12 post-confluence. BMP-2 decreased Zfp521 expression. (C) Calvarial osteoblasts were cultured with (black bars) or without (white bars) 100 nM PTHrP for up to 4 days, and Zfp521 expression was measured by RT-PCR. PTHrP increased Zfp521 expression.

Figure 4. Zfp521 interacts with Runx2 and inhibits Runx-2-mediated activation of the *Runx2* and *osteocalcin* promoters

A. MC3T3-E1 cells were transiently transfected with empty vector (pc), Zfp521, or Runx2 constructs as indicated, together with a *Runx2-luciferase* reporter construct (left) or a murine *osteocalcin (OG2)-luciferase* reporter (right). Zfp521 alone had little effect on the activity of either promoter. Runx2 activated both promoters. Co-expression of Zfp521 with Runx2 suppressed the Runx2-induced promoter activation (*, $p < 0.05$ relative to reporter alone; #, $p < 0.05$ relative to Runx2). **B.** Zfp521 co-immunoprecipitates with Runx2. Left panel – anti-HA immune complexes from lysates of transiently transfected 293VnR cells blotted with anti-FLAG antibody. HA-tagged Zfp521 co-immunoprecipitated FLAG-tagged Runx2 (arrow).

Right top panel – total cell lysates (TCL) blotted with anti-FLAG antibody to demonstrate FLAG-Runx2 expression. Right bottom panel – Anti-HA immune complexes blotted with anti-HA antibody to demonstrate HA-Zfp521.

Figure 5. *Ex vivo* differentiation of OG2 × Zfp521 calvarial cells is decreased

A. RT-PCR analysis of tissues from two OG2 × Zfp521 mice showed an approximately twofold overexpression of Zfp521 in bone, but not in fat or heart. **B.** Calvarial cells from OG2 × Zfp521 mice and wild-type littermates were cultured in osteogenic medium for 18, 25 or 31 days and stained with alizarin red. Cells from Zfp521 mice formed fewer mineralized nodules. **C.** QPCR analysis of RNA isolated from calvarial cells from OG2 × Zfp521 mice and control littermates cultured in osteogenic medium for 22 days. *Runx2*, *Osterix*, *alkaline phosphatase (ALP)*, *osteopontin*, *osteocalcin* and *bone sialoprotein (BSP)* mRNAs were decreased in OG2 × Zfp521 OBs (black bars) (*, $p < 0.05$). **D.** Calvarial cells from OG2 × Zfp521 mice and control littermates were infected with increasing amounts of Runx2 adenovirus and cultured in osteogenic medium to induce osteoblast differentiation. Cells were stained for ALP activity 5 days after infection. Runx2 dose-dependently reversed the reduction in ALP activity of the OG2 × Zfp521 cells.

Figure 6. Zfp521-overexpressing mice form more bone

A. Von Kossa staining shows increased bone in proximal tibia from 4 and 12 week OG2 × Zfp521 mice. **B.** Bone volume/total volume (BV/TV) and bone formation rate (BFR) were increased at 4 and 12 weeks of age in transgenic mice. **C.** Serum C-telopeptide crosslinks (sCTX) were unchanged at both ages.

Table 1
Sequences of Oligonucleotides Used in This Study

<u>Plasmid construction primers</u>	
HA-Zfp521 F	5'-ATATATGCGGCCGCAGGATGTCTCGCCGCAAG-3'
HA-Zfp521 R	5'-CTCTCTGCGGCCGCCTTGCACTAACTGC-3'
<u>shRNA</u>	
ps3 sense	5'-GATCCCGTTCCCAACCAAGAGTCCTTTCAAGAGAAGGACTCTTGGTTGGGGAATTTTGGAAA -3'
ps3 antisense	5'-AGCTTTTCCAAAAAATCCCAACCAAGAGTCCTTCTCTTGAAAGGACTCTTGGTTGGGGAACGG -3'
<u>TetOP-Zfp521 genotyping primers</u>	
Forward	5'-GTGTCAGATGGTTTCTACAACGAGTGGG -3'
Reverse	5'-GTCATAGATCTTGTCTTCTTGCCATGGGC -3'
<u>RT-PCR primer sequences</u>	
ALP F	5'-ACTGATGTGGAATACGAACTGGATGAGAAGG -3'
ALP R	5'-CAGTCAGGTTGTTCGATTCAATTCATACTGC -3'
BSP F	5'-GGGAATGGCCTGTGCTTTCTCGATG -3'
BSP R	5'-TCACTGCCTCCCTGGACTGG -3'
Collagen 1 F	5'-AACCTGGTGCGAAAGGTGAA -3'
Collagen 1 R	5'-AGGAGCACCAACGTTACCAA -3'
GAPDH F	5'-GGTCGGTGTGAACGGATTTGGC -3'
GAPDH R	5'-GCAGTGATGGCATGGACTGTGG -3'
Osteocalcin F	5'-TCTCTCTGACCTCACAGATGCCAAGC -3'
Osteocalcin R	5'-GGACTGAGGCTCCAAGGTAGCG -3'
Osteopontin F	5'-ACCCATCTCAGAAGCAGAATCTCCTTGC -3'
Osteopontin R	5'-TGTGTTTCCAGACTTGGTTCATCCAGC -3'
Osterix F	5'-TCTGCTTGAGGAAGAAGCTCACTATGGC -3'
Osterix R	5'-AGGCAGTCAGACGAGCTGTGC -3'
Runx2 F	5'-TCCACAAGGACAGAGTCAGATTACAG -3'
Runx2 R	5'-CAGAAGTCAGAGGTGGCAGTGTATC -3'
Zfp521 F	5'-CAACGAGTGGGACATCCAGGTTTC -3'
Zfp521 R	5'-CATCGTATGATTCTGTAGCTCTGTTTGG -3'

Table 2

Static and Dynamic Histomorphometric Analysis of OG2-tTA × TetOp-Zfp521 Transgenic Mice and Non-transgenic Littermate Controls

	4 weeks		12 weeks	
	Control	ZFP521	Control	ZFP521
BV/TV	4.26 ± 0.54	8.54 ± 1.48 [*]	6.67 ± 0.99	8.94 ± 0.29 [*]
Tb.Th	27.87 ± 1.45	38.18 ± 5.85 [*]	37.03 ± 3.54	42.09 ± 2.56 [*]
Tb.Sp	663.35 ± 84.42	396.13 ± 30.56 [*]	571.68 ± 62	407.87 ± 34.11
Tb.N	1.52 ± 0.16	2.32 ± 0.15 [*]	1.76 ± 0.17	2.13 ± 0.08 [*]
OS/BS	16.37 ± 5.38	30.39 ± 10.25	8.06 ± 1.8	15.18 ± 2.55 [*]
Ob.S/BS	13.42 ± 3.39	13 ± 1.29	28.47 ± 2.85	30.98 ± 3.57
Oc.S/BS	0.99 ± 0.73	1.7 ± 0.73	2.36 ± 0.35	2.02 ± 0.54
N.Ob/T.Ar	14.58 ± 0.45	34.58 ± 4.69 [*]	168.34 ± 24.92	211.66 ± 50.9 [*]
N.Oc/T.Ar	1.53 ± 0.5	0.69 ± 0.44	4.48 ± 0.74	5.26 ± 0.84
OV/TV	0.65 ± 0.24	1.06 ± 0.55	0.21 ± 0.09	0.45 ± 0.09 [*]
OV/BV	8.95 ± 2.57	12.32 ± 2.81	2.15 ± 0.51	4.75 ± 1.08P
O.Th	9.57 ± 1.39	8.51 ± 0.58	4.54 ± 0.64	6.07 ± 0.59
MS/BS	7.72 ± 3.41	18.52 ± 3.27 [*]	19.71 ± 2.28	28.97 ± 2.64 [*]
MAR	0.73 ± 0.41	0.86 ± 0.57	0.44 ± 0.17	1.42 ± 0.19 ^{**}
BFR/BS	42.33 ± 10.55	68.81 ± 6.8	52.35 ± 27.45	165.83 ± 27.56 ^{***}
BFR/BV	296.1 ± 96.8	404.96 ± 90.4	197.07 ± 88.43	988.67 ± 155.4 ^{***}
BFR/TV	33.61 ± 20.54	20.33 ± 15.53	15.55 ± 6.36	89.19 ± 17.75 ^{***}

* P< 0.01

** p<0.001

*** p<0.0001