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Zfp521 Is a Target Gene and Key Effector of Parathyroid Hormone-Related Peptide Signaling in Growth Plate Chondrocytes

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SUMMARY

In the growth plate, the interplay between parathyroid hormone-related peptide (PTHrP) and Indian hedgehog (Ihh) signaling tightly regulates chondrocyte proliferation and differentiation during longitudinal bone growth. We found that PTHrP increases the expression of Zfp521, a zinc finger transcriptional coregulator, in prehypertrophic chondrocytes. Mice with chondrocyte-targeted deletion of *Zfp521* resembled PTHrP$^{-/-}$ and chondrocyte-specific PTHR1$^{-/-}$ mice, with decreased chondrocyte proliferation, early hypertrophic transition, and reduced growth plate thickness. Deleting *Zfp521* increased expression of Runx2 and Runx2 target genes, and decreased Cyclin D1 and Bcl-2 expression while increasing Caspase-3 activation and apoptosis. Zfp521 associated with Runx2 in chondrocytes, antagonizing its activity via an HDAC4-dependent mechanism. PTHrP failed to upregulate Cyclin D1 and to antagonize Runx2, Ihh, and collagen X expression when Zfp521 was absent. Thus, *Zfp521* is an important PTHrP target gene that regulates growth plate chondrocyte proliferation and differentiation.

INTRODUCTION

In the growth plate, endochondral longitudinal bone growth requires the tight regulation of entry into and exit from each stage of chondrocyte differentiation, which correspond to the well-defined resting, proliferative, prehypertrophic, and hypertrophic zones. Alterations in this regulation disrupt the normal differentiation program, resulting in distorted growth plate architecture that leads to skeletal dysplasias with pronounced limb shortening (Kronenberg, 2003).

Parathyroid hormone-related peptide (PTHrP) is a key regulator of growth plate development (Kronenberg, 2006). It slows the progression from proliferating cells to hypertrophy by inducing Cyclin D1 expression, which promotes proliferation (Beier et al., 1999, 2001), and by repressing expression of Runx2 (Guo et al., 2006; Iwamoto et al., 2003; Li et al., 2004), which drives chondrocyte differentiation (Kim et al., 1999; Zheng et al., 2003). In addition to slowing the transition of prehypertrophic cells to the hypertrophic state, PTHrP promotes the survival of hypertrophic chondrocytes by inducing the expression of the anti-apoptotic protein Bcl-2 (Amling et al., 1997). Consequently, genetic changes that eliminate (Amizuka et al., 1994; Lanske et al., 1999) or constitutively activate (Schipani et al., 1997; Weir et al., 1996) PTHrP-induced signaling result in major alterations in the structure of the growth plate and longitudinal bone growth.

Zfp521 (also known as Evi3 in mice, EHZF in humans) is a 180 kDa transcriptional coregulator that contains 30 Krüppel-like C2H2 zinc fingers (Bond et al., 2004; Justice et al., 1994). Zfp521 is highly expressed in hematopoietic and neural stem cells. Although the role of Zfp521 in the coordination of neuronal differentiation is still elusive, it is thought to have an inhibitory function in hematopoietic stem cell differentiation because overexpression of Zfp521 favors the expansion of hematopoietic progenitors while blocking their differentiation (Bond et al., 2004). In hematopoiesis and oncogenesis, Zfp521 exerts some of its main effects on B cells and erythrocyte maturation by binding and antagonizing Ebf (Bond et al., 2008; Matsubara et al., 2009). In murine B cell lymphomas, the *Zfp521* gene is frequently altered by retroviral insertions adjacent to the initiation codon, which leads to increased expression of the proteins (Warming et al., 2003). Significant levels of *Zfp521* transcript are also found in several human acute myeloid leukemias (Bond et al., 2008; Yamasaki et al., 2010), suggesting that altered Zfp521 activity contributes to these human hematological malignancies, possibly by increasing tumor resistance to NK cells (La Rocca et al., 2009).

We recently observed that PTHrP regulates Zfp521 expression in osteoblasts and osteocytes, where Zfp521 represses

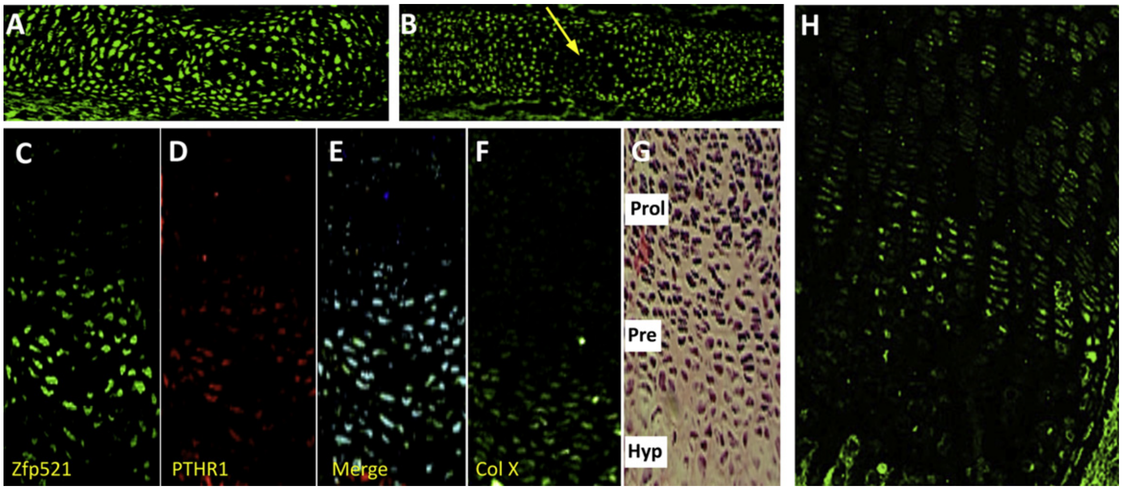

Figure 1. Zfp521 Expression during Skeletal Development
Whole tibia at E12.5 (A) (25×) and E14.5 (B) (10×) and proximal tibia growth plate at E17.5 (C–G) (10×) and 2 weeks (H) (10×) were immunostained with an antibody against Zfp521 (A–C and H), PTHR1 (D), or collagen type X (F) or with hematoxylin and eosin (G). (A) Zfp521 was highly expressed throughout the cartilage anlage. (B) Zfp521 staining decreased in the forming primary center of ossification (arrow). (C–E) In the formed growth plate, Zfp521 and PTHR1 were most highly expressed in prehypertrophic chondrocytes. Prol, proliferative; Pre, prehypertrophic; Hyp, hypertrophic.

Runx2's transcriptional activity and, indirectly, expression (Wu et al., 2009). In situ hybridization (ISH) also revealed Zfp521 expression around mesenchymal condensations as early as E12.5, in the perichondrium and in early chondrocytes, and in the developing growth plate (Wu et al., 2009). Based on its pattern of expression, its response to PTHrP, and its effects on Runx2, we hypothesized that Zfp521 could regulate growth plate development, possibly downstream of PTHrP. We now report that Zfp521 is an important downstream effector of PTHrP in the regulation of chondrocyte proliferation and differentiation in the growth plate, ensuring proper postnatal longitudinal bone growth.

RESULTS

Zfp521 Is Highly Expressed in Prehypertrophic Chondrocytes

Immunostaining of developing long bones showed expression of Zfp521 as early as E12.5 in all chondrocytes within the cartilage anlage (Figure 1A). At E14.5, Zfp521 expression decreased in the area of hypertrophic differentiation (Figure 1B). During the late stages of embryonic development (E17.5) (Figure 1C) and postnatally at 2 weeks (Figure 1H), Zfp521 was preferentially expressed within the growth plate in prehypertrophic chondrocytes, where it colocalized with the peak expression of the PTHrP receptor (PTHR1) (Figures 1D and 1E).

Deleting *Zfp521* in Chondrocytes Affects Longitudinal Bone Growth and the Structure of the Growth Plate

To examine its role in the growth plate, we deleted *Zfp521* in chondrocytes using Cre recombinase driven by the *collagen II* promoter (Col II-Cre) (Long et al., 2001). The deletion efficiency was confirmed in primary rib chondrocytes by qPCR (see Figure S1A available online). Although Zfp521$^{fl/fl}$ Cre^{+} (cKO) mice were similar to floxed-control (Zfp521$^{fl/fl}$ Cre^{-}) mice at birth (Figure S1B), growth retardation was evident by 1–2 weeks of age (Figure 2A) and still present at 8 weeks (Figure S1E) and 12 weeks (data not shown). Consistent with the pattern of Col II expression, bones that formed by endochondral ossification were smaller, whereas craniofacial structures that develop by intramembranous ossification and the cranium base synchondroses appeared normal (Figures S1C and S1D). No other gross anatomical alterations were seen in the Zfp521 cKO mice.

Histomorphometric analysis of proximal tibia from 2-week-old mice revealed a 30% reduction in the mutant growth plate thickness, affecting all zones (Figures 2B and 2C). The resting zone contained only one or two layers of cells, rather than five or more layers in the Zfp521$^{fl/fl}$Cre^{-} growth plates. Proliferating cells were properly oriented along the long axis of the bone and had a normal flat morphology, but the number of cell columns and cells per column were reduced (Figure 2D). The transition to the hypertrophic zone was closer to the epiphysis. The hypertrophic zone thickness was also reduced (Figures 2B and 2C) with fewer chondrocytes, each of increased cellular height (Figures 2D and 2E), and about 50% less extracellular matrix (ECM) (Figures 2F and 2H), resulting in a reduced matrix-to-cell ratio (Figure 2G), suggesting impaired matrix production. The onset of ECM mineralization also occurred prematurely (Figure 2I), due to decreased proliferation, accelerated differentiation, or both. Consequently, whereas an average of six layers of hypertrophic chondrocytes were present between the prehypertrophic chondrocytes and the leading edge of the mineralization front in the Zfp521$^{fl/fl}$ Cre^{-}, only three layers were present in the cKO mice.

In situ hybridization confirmed the altered growth plate architecture in the absence of Zfp521 (Figure 3). Reduction in the thickness of the entire growth plate and the proliferative and hypertrophic zones were revealed by Col II expression (Figure 3A), the hedgehog receptor subunit patched (Ptc) (Figure 3B), and Col X, respectively (Figure 3C). Indian hedgehog

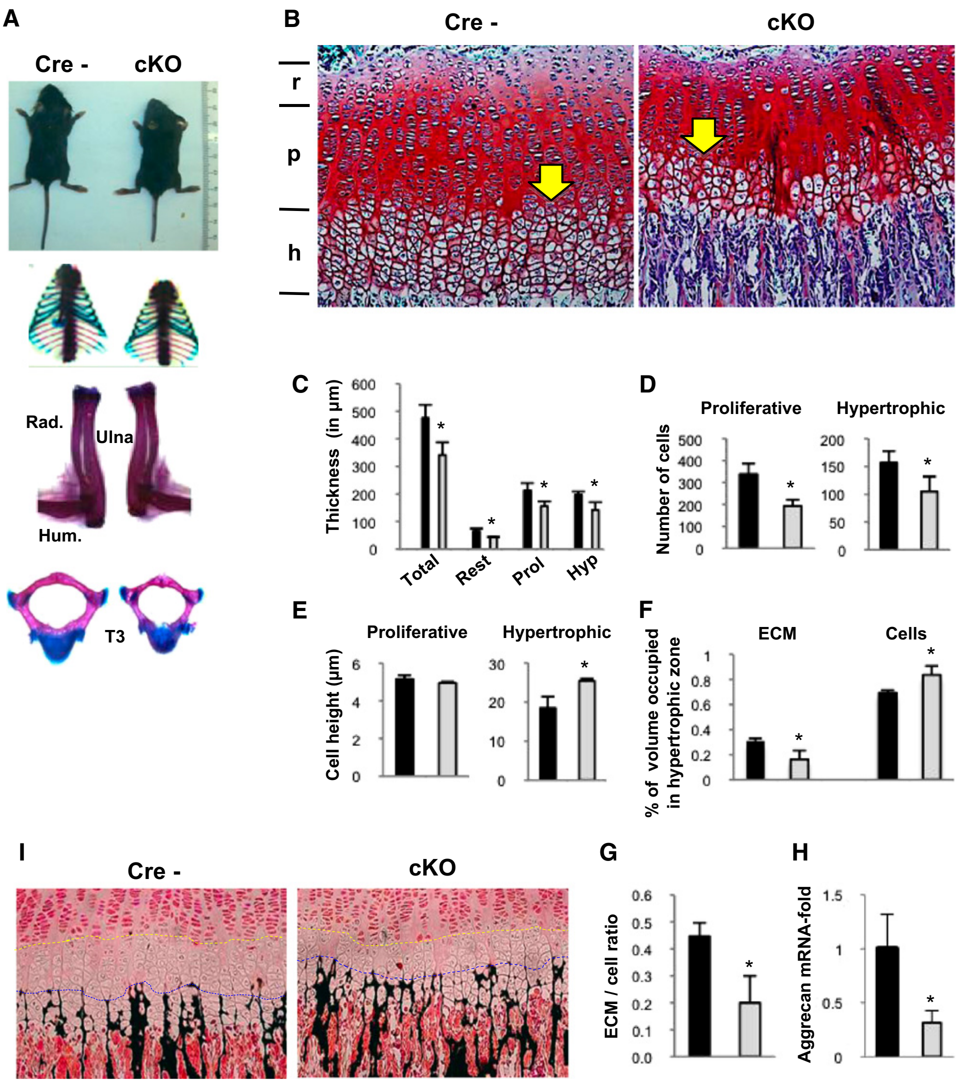

Figure 2. Deleting Zfp521 in Chondrocytes Alters Growth Plate Structure and Skeletal Development

(A) Two-week-old Zfp521 cKO mice (right) exhibited postnatal dwarfism. Rib cage, long bones, and vertebrae of the Zfp521 cKO mice were smaller than in the floxed control.

(B) Proximal tibia growth plates from 2-week-old mice were stained with safranin O (10×). A reduction in the thickness of all layers was observed in the Zfp521 cKO growth plates (r, resting; p, proliferative; h, hypertrophic). In addition the hypertrophic zone was closer to the epiphysis (yellow arrows), suggesting early hypertrophic differentiation.

(C–H) Data derived from Zfp521 cKO mice are shown in white, and data obtained in floxed control mice are presented in black (C) Histomorphometric analysis of proximal tibia growth plates from the Zfp521 cKO and floxed control mice confirmed an approximate 30% reduction in the thickness of all layers. Also, there were fewer cell columns with fewer cells per column in the proliferative zone (339 ± 48 versus 193 ± 28) and a reduced cell population in the hypertrophic zone (157 ± 21 versus 105 ± 27) (D). (E) The cell height of hypertrophic chondrocytes in Zfp521 cKO growth plates was increased (17.7 ± 2.7 µm versus 23.7 ± 2.9µm), whereas proliferative chondrocytes were similar in floxed control and mutant growth plates. (F) The percentage of the hypertrophic zone volume occupied by ECM was reduced in mutant growth plates (31% ± 0.02% versus 16% ± 0.07%), a comparable increase in the percentage occupied by cells (69% ± 0.02% versus 84% ± 0.07%), which generated a decrease in the ECM/cell ratio (0.44 ± 0.05 versus 0.20 ± 0.07) (G). (H) The expression of aggrecan mRNA is reduced by about 50% in the growth plate of Zfp521 cKO mice.

(I) von Kossa staining showed earlier chondroid mineralization (black) in the Zfp521 cKO growth plates. Note the reduction in the number of layers of hypertrophic chondrocytes between the prehypertrophic chondrocytes and the leading edge of the mineralization front (yellow and blue lines). Data are means ± SD, n = 4, $p < 0.05$.

(Ihh) and PTHR1, both markers of prehypertrophic cells, were upregulated and located closer to the epiphysis than in the control (Figures 3D and 3E). Quantitative PCR of mRNA isolated from Zfp521 cKO and Zfp521$^{fl/fl}$ Cre$^-$ mice growth plate chondrocytes confirmed the ISH results (Figures 3F–3J).

Zfp521 Regulates Chondrocyte Proliferation

Cell proliferation was decreased in the Zfp521 cKO growth plate (40% ± 5% BrdU-positive cells in Zfp521$^{fl/fl}$ Cre$^-$ mice, 28% ± 3% in the cKO mice, $p < 0.005$) (Figures 4A and 4B). In ATDC5 cells, depleting Zfp521 with shRNA (Figure S2A)

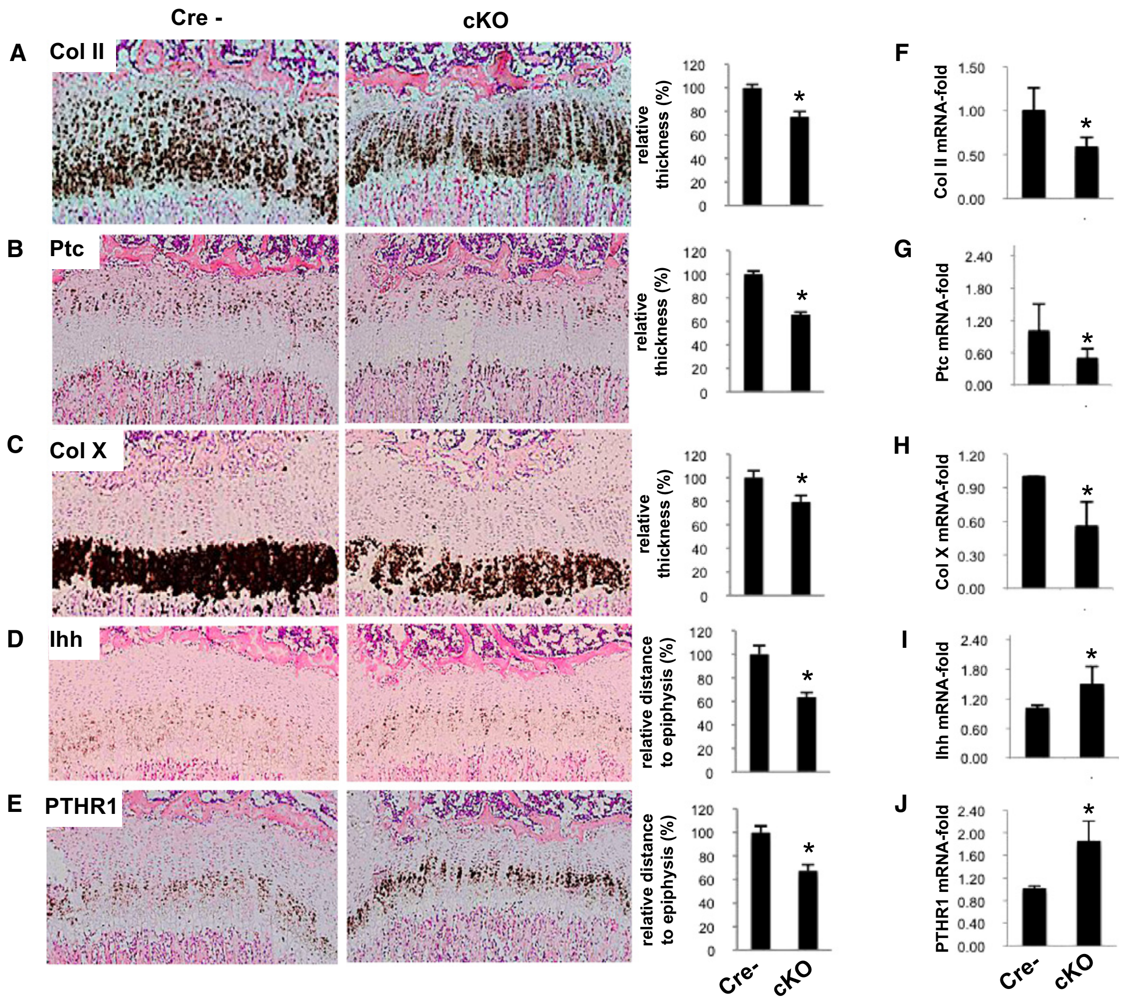

Figure 3. ISH of Differentiation Markers

(A–E) ISH (left panels) including quantification (right panels) of proximal tibiae from 2-week-old Zfp521 cKO and floxed control (Cre$^-$) mice (10×). (A) The Collagen type II (Col II) signal in the cKO growth plate was thinner (25% ± 2% reduction), demonstrating the overall thinning of the growth plate. The signals for Ptc (B) and Collagen type X (Col X) (C) revealed the narrower proliferative (34.3% ± 3% reduction) and hypertrophic (20.7% ± 3% reduction) zones, respectively. Prehypertrophy markers Ihh (D) and PTHR1 (E) demonstrated the closer proximity of the prehypertrophic zone to the epiphysis (35.9% ± 2% and 32.9% ± 3% reduced distance between markers and top of the growth plate, respectively). Quantification of the granular area fraction covered by Col X, PTHR1, and Ihh signal, as described in Experimental Procedures, revealed changes of 6-fold reduction, 3-fold increase, and 1.7-fold increase, respectively, in the Zfp521 cKO growth plate. Data are means ± SD.

(F–J) qPCR of mRNA isolated from floxed and mutant growth plate chondrocytes are presented.

increased the doubling time and decreased both micromass size and BrdU incorporation (Figures S2B–S2D), whereas forced expression of Zfp521 increased BrdU incorporation by about 50% (Figures S2E and S2F). Neither transient overexpression of Zfp521 in fibroblastic NIH 3T3 cells, nor stable depletion of Zfp521 in osteoblastic MC3T3-E1 cells altered BrdU incorporation (Figures S2G and S2H), demonstrating that the effect of Zfp521 on cell proliferation is chondrocyte specific.

Cyclin D1 promotes chondrocyte proliferation, and Cyclin D1-deficient mice exhibit a thin proliferative zone (Beier et al., 2001). We found decreased proliferation in primary chondrocytes from Zfp521 cKO mice (data not shown), confirming our in vivo results, and decreased expression of Cyclin D1 (Figure 4C). Similarly, depleting Zfp521 reduced Cyclin D1 expression in ATDC5 cells (Figure 4D), whereas forced expression of Zfp521 increased both mRNA and protein levels of Cyclin D1 (Figure 4E). These results suggest that Zfp521 promotes Cyclin D1 expression, contributing to keeping growth plate chondrocytes in a proliferative state, although we found that Zfp521 did not affect the activity of a Cyclin D1-luciferase reporter gene in ATDC5 cells (data not shown), suggesting that the changes in Cyclin D1 expression that occur when Zfp521 is depleted or overexpressed are not the result of a direct effect on the Cyclin D1 promoter.

Zfp521 Slows the Progression of Chondrocytes to Hypertrophy

Because growth plate development requires a balance between chondrocyte proliferation and progression to hypertrophy, we next examined the effect of Zfp521 on alkaline phosphatase (ALP) activity, an early hypertrophy marker. Overexpressing a tetracycline (tet)-inducible Zfp521 in ATDC5 cells from day 0 until day 16 of culture, i.e., before the transition to hypertrophy, repressed the formation of Alcian blue-positive cartilage nodules (Figure 4F upper panel), suggesting impaired chondrogenic differentiation. Overexpression from day 16 until day 30, i.e., at the time of hypertrophic transition and beyond, induced a significant decrease in ALP activity (Figure 4F lower panel). Thus, Zfp521 appears to antagonize hypertrophic differentiation as well as promoting chondrocyte proliferation.

Zfp521 Antagonizes Runx2 Activity in Chondrocytes

Runx2 is a key promoter of chondrocyte hypertrophy. Therefore, we assayed the expression of Runx2 and its target genes in cultured primary rib chondrocytes from Zfp521 cKO and Zfp521$^{fl/fl}$ Cre^{-} mice. The expression of Runx2, Ihh, osteopontin (OPN), ALP, and MMP-13 all increased in the absence of Zfp521 (Figure 4G). Consistent with the ISH results, and despite the fact that it is also considered a Runx2 target gene, expression of the matrix component Col X was decreased by about 50%, suggesting that Zfp521 also regulates *Col X* gene expression by Runx2-independent mechanisms.

We next examined the effect of Zfp521 on Runx2 expression in MCT cells, an immortalized chondrocyte cell line that rapidly becomes hypertrophic at nonpermissive temperatures (Zheng et al., 2003). As expected, inducing hypertrophic differentiation increased the expression of Runx2 significantly (data not shown). Transiently transfecting MCT cells with Zfp521 prior to inducing hypertrophy reduced Runx2 as much as treating with PTHrP and Forskolin (Figure 4H), confirming the ability of Zfp521 to suppress Runx2 expression.

We have previously shown that Zfp521 associates with Runx2 and antagonizes its transcriptional activity in osteoblasts (Wu et al., 2009). Endogenous Zfp521 also immunoprecipitated with Runx2 from ATDC5 and MCT cells (Figure 4I). Therefore, we examined whether Zfp521 also regulated Runx2 activity in hypertrophic chondrocytes and found that Zfp521 dose-dependently repressed Runx2-induced activation of the 6xOSE2-luc reporter plasmid in MCT cells during temperature-induced hypertrophic differentiation, (Figure 4J), indicating that Zfp521 also controls Runx2 transcriptional activity in differentiating chondrocytes.

Repression of Chondrocyte Runx2 Activity by Zfp521 Requires HDAC4

Several histone deacetylases (HDACs) antagonize Runx2 (Kang et al., 2005; Schroeder et al., 2004; Westendorf et al., 2002), and it has been reported that HDAC4 binds and antagonizes Runx2 in prehypertrophic chondrocytes (Vega et al., 2004) and that PTHrP induces the nuclear translocation of HDAC4 (Kozhemyakina et al., 2009). Therefore, we looked for a possible connection between Zfp521 and HDAC4 and found that the two proteins form an endogenous complex in ATDC5 cells (Figure 4K) and that depleting HDAC4 blunted the Zfp521-induced Runx2 repression (Figure 4L).

Zfp521 Regulates Chondrocyte Apoptosis

Apoptosis of hypertrophic chondrocytes is another key event in growth plate development. The reduced number of hypertrophic cells suggested that Zfp521 might antagonize chondrocyte apoptosis. As hypothesized, the number of apoptotic hypertrophic chondrocytes increased about 3-fold in the Zfp521 cKO growth plate (Figure 5A). Induction of apoptosis culminates in increased caspase activity, especially Caspase-3 (Porter and Janicke, 1999), and indeed, caspase activity was increased in Zfp521 cKO primary chondrocytes (Figure 5B top panels). Similarly, caspase activity and activated Caspase-3 protein in ATDC5 cells were increased by depleting Zfp521 (Figure 5B middle panels and Figure 5C) and decreased by overexpressing Zfp521 (Figures 5B [bottom panels] and 5D). Finally, we found that expression of the anti-apoptotic protein Bcl-2, which enhances survival of terminally differentiated chondrocytes (Amling et al., 1997), was significantly decreased in primary chondrocytes from Zfp521 cKO mice (Figure 5E). Thus, our results suggest that Zfp521 antagonizes chondrocyte apoptosis.

PTHrP Regulates Zfp521 Expression in Differentiating Chondrocytes

Our results consistently demonstrated that Zfp521 regulates growth plate chondrocyte proliferation, differentiation, and apoptosis. The reduced proliferation and early transition to hypertrophy in Zfp521 cKO growth plates are reminiscent of the effects of decreased PTHrP signaling in chondrocytes (Amizuka et al., 1994; Kobayashi et al., 2002; Lanske et al., 1999). Therefore, we examined whether Zfp521 played a role in the responses of chondrocytes to PTHrP.

Consistent with the in vivo localization of Zfp521 in prehypertrophic chondrocytes (Figure 1H), the expression of endogenous Zfp521 paralleled the temporal and spatial expression of endogenous PTHR1 in ATDC5 micromass and pellet cultures (Figure S3), with peaks of expression coinciding during the hypertrophic transition of ATDC5 cells and the highest responsiveness to PTHrP (Shukunami et al., 1996). Transient overexpression of Zfp521 in ATDC5 micromass cultures caused a significant decrease in the number of Alcian blue-positive nodules after 2 weeks, comparable to the decrease in cultures treated with PTHrP (1-34) (Figure 6A). The inhibitory effects of PTHrP (1-34) and Zfp521 on nodule formation were not additive, suggesting that PTHrP and Zfp521 share the same signaling pathway.

Given the similar expression patterns of Zfp521 and PTHR1 in chondrocytes in vivo and in vitro, and their comparable inhibitory effects on chondrocyte differentiation, we hypothesized that Zfp521 could be an effector downstream of PTHrP. Indeed, PTHrP (1-34) increased Zfp521 expression dose dependently (Figure 6B) and time dependently (Figures 6C and 6D). Forskolin had a similar effect, and the selective PKA inhibitor H-89 prevented the induction of Zfp521 expression by both PTHrP (1-34) and forskolin (Figure 6E), indicating that the $G_{\alpha s}$/cAMP/PKA signaling pathway couples PTHrP to Zfp521 expression.

In contrast to the effect of PTHrP (1-34), PTHrP (7-34), which selectively activates PLC-γ signaling (Nutt et al., 1990), downregulated Zfp521 mRNA and protein levels in a similar time-dependent manner (Figures 6F and 6G). Stimulating protein kinase C directly with 12-O-tetradecanoylphorbol-13-acetate (TPA) achieved a similar reduction (Figure 6H), suggesting that the PTHrP-induced PLC-γ signaling pathway might oppose cAMP/PKA in the regulation of Zfp521 downstream of PTHR1.

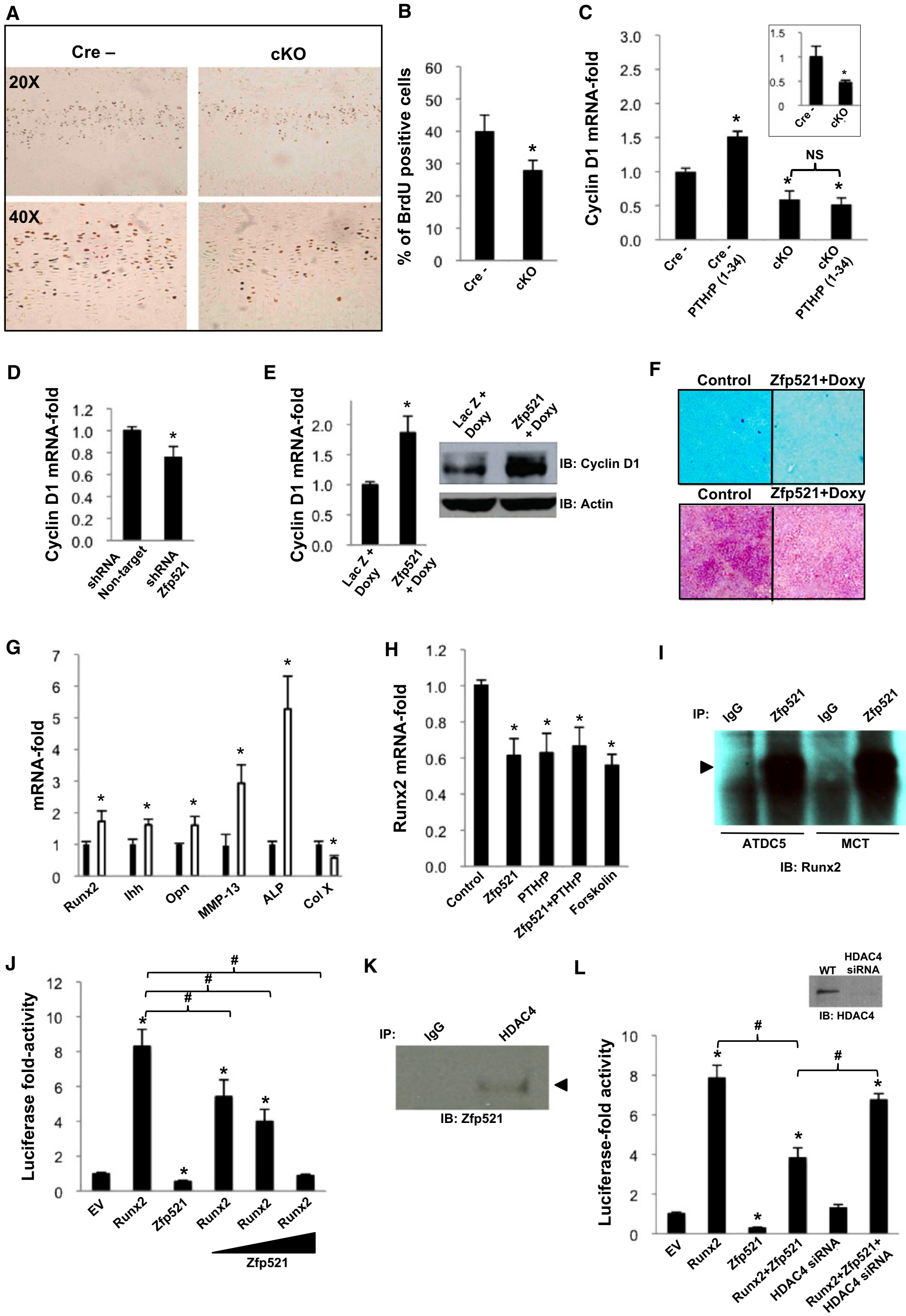

Figure 4. Zfp521 Regulates Chondrocyte Proliferation and the Expression of Hypertrophy and Terminal Differentiation Markers

(A) Two-week-old floxed control and Zfp521 cKO mice were injected with BrdU labeling solution (1 ml/100 g body weight) 20 and 4 hr before sacrifice, and proximal tibia were immunostained for BrdU.

Zfp521 expression was also reduced in primary rib chondrocytes from PTHrP$^{-/-}$ mice (Figure 6I) and increased in primary rib chondrocytes from mice that overexpress the mutated human PTHR1 that constitutively activates adenylyl cyclase (Schipani et al., 1997) (Figure 6J). Together with the results from the ATDC5 cultures, these data firmly establish that PTHrP induces the expression of Zfp521 in chondrocytes.

Zfp521 Contributes to the Effects of PTHrP on Chondrocytes

PTHrP induces Cyclin D1 expression and proliferation (Beier et al., 1999, 2001) and downregulates Runx2 expression (Guo et al., 2006; Li et al., 2004) (Figure 7A), whereas deleting *Zfp521* decreased proliferation and Cyclin D1 expression and increased Runx2 expression. The induction of Zfp521 expression by PTHrP suggested that Zfp521 mediated at least part of PTHrP's effects on chondrocyte proliferation and Runx2 expression. As hypothesized, we found that the loss of Zfp521 prevented the PTHrP (1-34)-induced upregulation of Cyclin D1 mRNA (Figure 4C). We also found that although PTHrP (1-34) downregulated the expression of Runx2 in ATDC5 cells at the beginning of the hypertrophic conversion (Figure 7A) and in primary chondrocytes from Zfp521$^{fl/fl}$ Cre^{-} mice (Figure 7B) as expected, it failed to reduce Runx2 expression when Zfp521 was absent (Figures 7B and 7C). Finally, the absence of Zfp521 also increased constitutive Ihh expression in primary chondrocytes and prevented PTHrP's downregulation of Ihh (Figure 7D), identifying another downstream response to PTHrP that requires Zfp521 and indicating the potential importance of Zfp521 in the negative feedback loop that controls hypertrophy.

To evaluate the effect of *Zfp521* deletion on PTHrP's control of hypertrophy, metatarsals from 2-week-old Zfp521 cKO and control Zfp521$^{fl/fl}$ Cre^{-} mice were cultured for 10 days with or without PTHrP (1-34), and expression of Col X, a hypertrophic marker that is downregulated by PTHrP, was examined by ISH (Figure 7E). PTHrP effectively repressed the expression of Col X mRNA in controls, but deletion of *Zfp521* blunted the effect of PTHrP on Col X expression, providing more evidence of the role of Zfp521 downstream of PTHrP in controlling the transition to hypertrophy in the growth plate.

Zfp521 Represses PTHR1 Expression in Prehypertrophic Chondrocytes

In addition to Zfp521 expression being regulated by PTHrP downstream of PTHR1, we observed that PTHR1 expression was markedly increased in the Zfp521 cKO growth plate (Figures 3E and 3J), suggesting that Zfp521 downregulates PTHR1 expression in prehypertrophic chondrocytes. We confirmed this by showing that depleting Zfp521 in ATDC5 cells increased PTHR1 protein (Figure S4A), whereas overexpressing Zfp521 in ATDC5 cells and MCT cells reduced PTHR1 protein (Figures S4B and S4C). These data suggest that Zfp521 mediates a negative feedback loop that downregulates PTHR1 expression in prehypertrophic chondrocytes, thereby further contributing to the regulation of PTHrP effects on the growth plate.

DISCUSSION

Postnatal longitudinal bone growth depends on chondrocyte proliferation and differentiation in the growth plate, a process that is tightly controlled by PTHrP and Ihh (Kronenberg, 2003). Zfp521 is a 180 kDa 30 Krüppel-like zinc finger protein that regulates the differentiation of neurons, hematopoietic cells, and osteoblasts (Bond et al., 2004; Warming et al., 2003; Wu et al., 2009). We now report that Zfp521 is also expressed in growth plate chondrocytes and plays a role in the regulation of their proliferation, differentiation, and apoptosis in response to PTHrP. Targeted deletion of *Zfp521* in chondrocytes led to postnatal growth retardation with reduced thickness of all growth plate zones. Zfp521 cKO chondrocytes exhibited decreased proliferation, early hypertrophic transition, reduced matrix production, and enhanced apoptosis. Deletion of *Zfp521* also decreased the expression of Cyclin D1 and increased the expression of Runx2 and several Runx2 target genes. Also, Bcl-2 expression decreased and caspase-3 activation increased when Zfp521 was absent. The decreased

(B) Zfp521 cKO growth plates contained significantly fewer BrdU-positive chondrocytes than controls.

(C) Rib chondrocytes and growth plate chondrocytes (inset) isolated from floxed control and cKO mice were cultured with or without PTHrP (1-34) (100 nM) for 48 hr. The lack of Zfp521 decreased Cyclin D1 expression in untreated chondrocytes and abolished the PTHrP (1-34)-induced increase in Cyclin D1 expression. NS, not significant.

(D) Zfp521 shRNA (seq 4) reduced Cyclin D1 expression in ATDC5 cells cultured in micromass for 10 days.

(E) Doxycycline-induced overexpression of Zfp521 in micromass cultures of ATDC5 cells stably transfected with tet-inducible Zfp521 increased Cyclin D1 mRNA (graph) and protein (western blot).

(F) tet-inducible Zfp521-overexpressing ATDC5 cells and LacZ control cells were cultured in the presence of 0.5 μg/ml doxycycline for 16 days and stained with Alcian blue (upper panel) or from days 16 to 30 and stained for ALP activity (lower panel). Overexpressing Zfp521 repressed both the formation of Alcian blue-positive cartilage nodules and ALP activity.

(G) Primary rib chondrocytes isolated from floxed controls (black bars) and Zfp521 cKO mice (white bars) were cultured for 3 days in maintenance medium, and the expression of Runx2 and its target genes was analyzed by qPCR. The expression of Runx2 and all target genes except *Col X* were increased in the Zfp521 cKO chondrocytes. Col X expression was reduced by ~50%.

(H) Overexpressing Zfp521 in MCT cells reduced Runx2 expression to the same extent as PTHrP (1-34) (100 nM) and forskolin (10 μM), and the effects were not additive.

(I) Co-immunoprecipitation of endogenous Zfp521 and Runx2 in ATDC5 and MCT cells.

(J) 6xOSE2-luc reporter assay showing Zfp521 dose-dependently repressing the inductive activity of Runx2 in MCT cells.

(K) Co-immunoprecipitation of endogenous Zfp521 and HDAC4 in ATDC5 cells.

(L) 6xOSE2-luc reporter assay demonstrating that Zfp521 requires HDAC4 to effectively repress Runx2 transcriptional activity in MCT cells. Inset, HDAC4 knockdown efficiency by siRNA transiently transfected into MCT cells before hypertrophic induction. (B–E, G, H, J, and L) Data are means ± SD, n = 3, *p < 0.05, significant difference from control, #p < 0.05 significant difference from indicated condition.

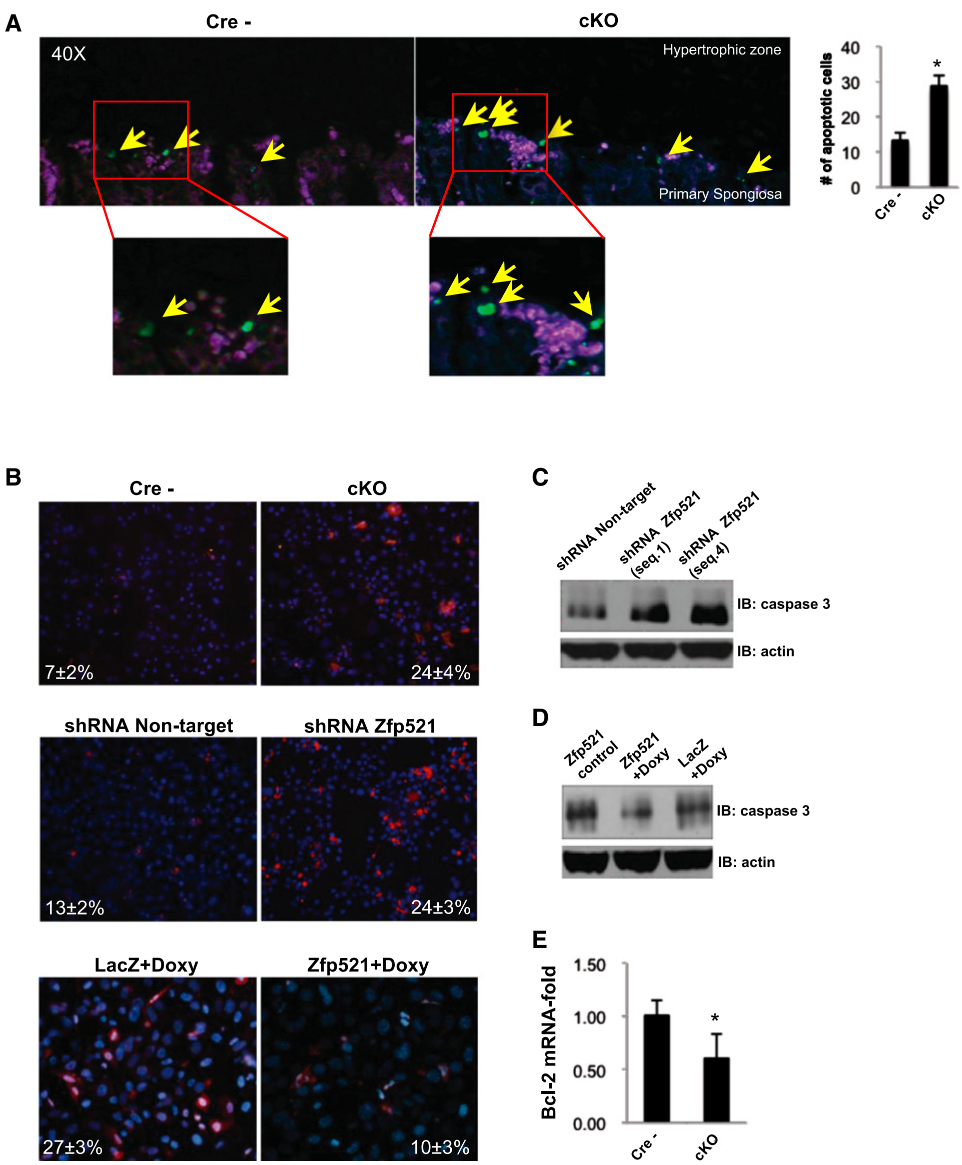

Figure 5. Zfp521 Regulates Chondrocyte Apoptosis

(A) TUNEL assay in 3-week-old proximal tibias from Cre- and cKO mice demonstrating an in vivo increased (~3-fold) chondrocyte apoptosis in the absence of Zfp521. Apoptotic cells are indicated (arrows).

(B) Primary rib chondrocytes isolated from Zfp521 cKO mice (top panels), Zfp521 shRNA-expressing ATDC5 cells (seq 4) (middle panels), and tet-inducible Zfp521-overexpressing ATDC5 cells (bottom panels), and respective controls were stained for active caspase with FLICA (red). Nuclei were counterstained with Hoechst stain (blue). Caspase activity was increased by deleting/depleting Zfp521 and decreased by overexpressing Zfp521. The percentage of caspase-positive cells is indicated.

(C) Western blot of total cell lysates showed increased activated caspase-3 in Zfp521 shRNA-expressing ATDC5 cells (two different sequences) cultured for 12 days in chondrogenic conditions.

(D) Activated caspase-3 was decreased in doxycycline (0.5 μg/ml)-treated tet-inducible Zfp521-overexpressing ATDC5 cells.

(E) Bcl-2 mRNA expression was decreased in primary rib chondrocytes isolated from Zfp521 cKO mice. Data are means ± SD, n = 3, *$p < 0.05$, significant difference from floxed control.

proliferation and consequent reduction in the number of cells entering the differentiation program are likely key contributors to the reduced thickness of all the zones of the Zfp521 cKO growth plate. In addition the reduced matrix production and increased apoptosis would also contribute to the thinning of the growth plate.

PTHrP is an essential regulator of chondrocyte proliferation, differentiation, and life span in the growth plate during longitudinal bone growth (Kobayashi et al., 2002; Kronenberg, 2006). It controls the expression of at least three key regulatory proteins: Cyclin D1 is increased to maintain proliferation of early-stage chondrocytes (Beier et al., 1999, 2001); Runx2

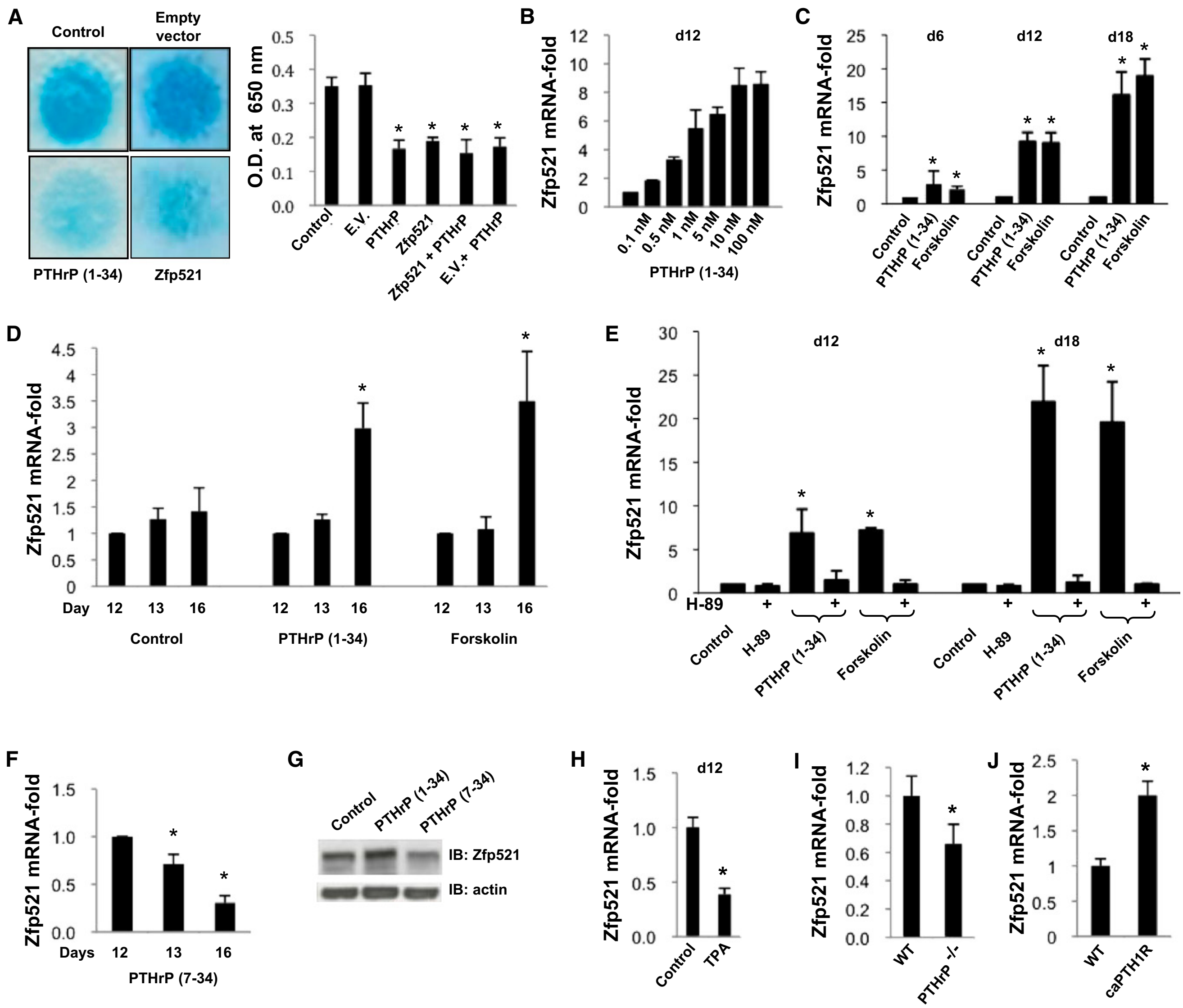

Figure 6. PTHrP Regulates Zfp521 Expression in Differentiating Chondrocytes

(A) Transiently transfected Zfp521 reduced the formation of Alcian blue-stained cartilage nodules in 12 day ATDC5 cell micromass cultures to the same extent as 100 nM PTHrP (1-34) (E.V., empty vector). Alcian blue staining was quantified spectrophotometrically after guanidine extraction.

(B) Continuous treatment of ATDC5 cells with PTHrP for 12 days dose dependently increased Zfp521 mRNA expression.

(C and D) PTHrP (1-34) (100 nM) and forskolin (10 μM) increased Zfp521 mRNA expression in a time-dependent manner in ATDC5 cells treated either continuously for 6, 12, or 18 days (C), or at the beginning of hypertrophic conversion (days 12 to 16) (D).

(E) The PKA inhibitor H-89 (10 μM) prevented the PTHrP- and forskolin-induced increase in Zfp521 mRNA expression in ATDC5 cells.

(F and G) Treatment of ATDC5 cells with 100 nM PTHrP (7-34) at the beginning of hypertrophic transition downregulated Zfp521 mRNA (F), and protein (G) levels at day 16.

(H) Treating ATDC5 cells with TPA (1 μM) continuously for 12 days to activate PKC downregulated the expression of Zfp521 mRNA.

(I and J) Zfp521 mRNA was decreased in primary rib chondrocytes isolated from PTHrP$^{-/-}$ mice (I) and increased in primary rib chondrocytes from mice that overexpress constitutively active PTHR1 (caPTHR1) (J), confirming that PTHrP/PTHR1 signaling induces Zfp521 expression. Data are means ± SD, n = 5, *$p < 0.05$, significant difference from control.

(and secondarily Ihh) expression is decreased to delay the onset of the hypertrophic transition and initiation of matrix mineralization (Guo et al., 2006); and Bcl-2 is increased to delay apoptosis of hypertrophic chondrocytes (Amling et al., 1997).

Our results indicate that PTHrP also regulates the expression of Zfp521 and that Zfp521 mediates some of the important downstream effects of PTHrP in the growth plate. First, several effects of overexpressing Zfp521 in chondrocytes resemble changes induced by PTHrP, whereas the Zfp521 cKO growth plate resembles the growth plates of the PTHrP KO and chondrocyte-specific PTHR1 KO mice (Amizuka et al., 1994; Kobayashi et al., 2002). Second, PTHrP induced the expression of Zfp521 in chondrocytes in a dose- and time-dependent manner, similar to our observation in osteoblasts (Matsubara et al., 2009;

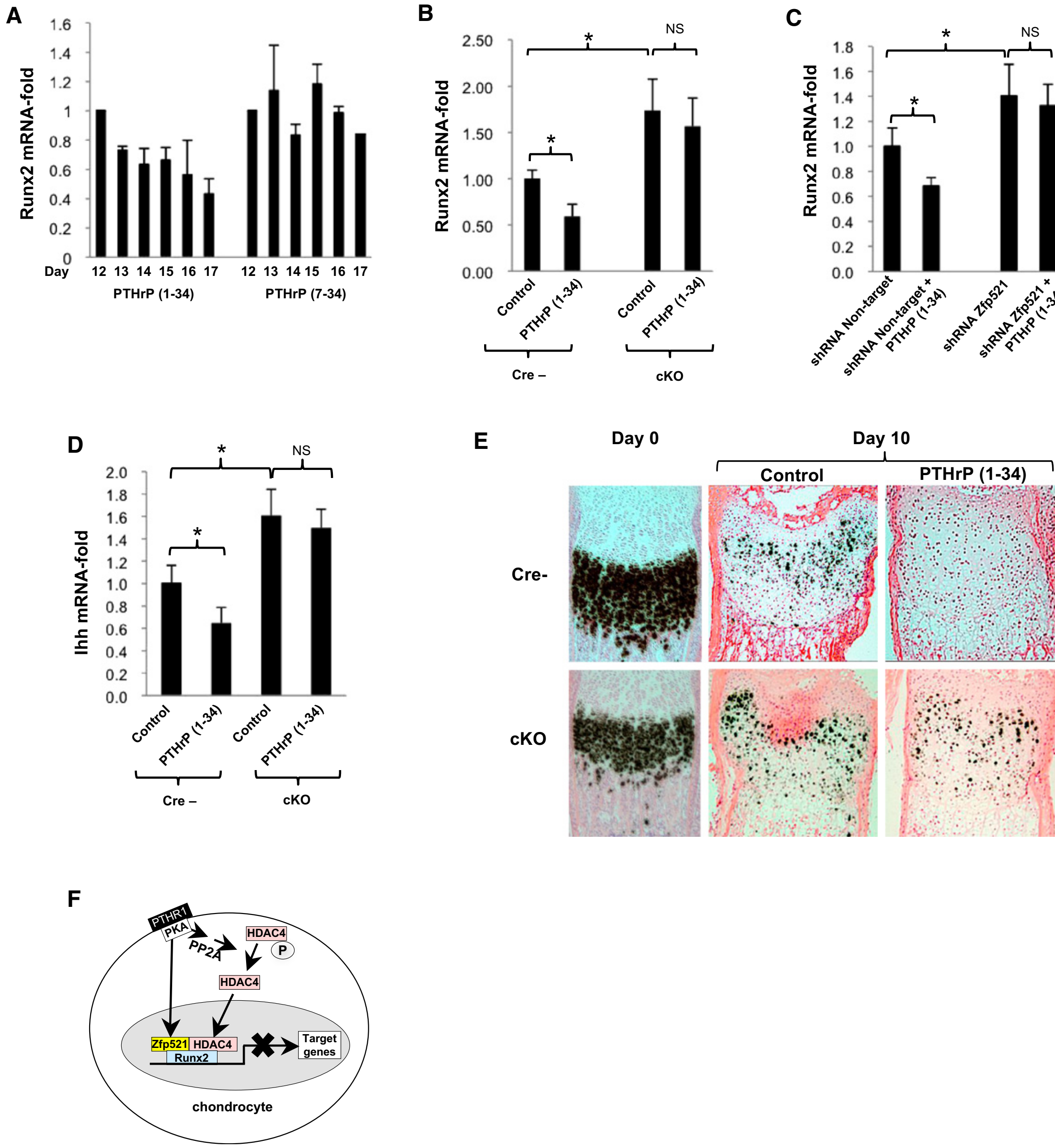

Figure 7. Zfp521 Mediates Some Effects of PTHrP on Chondrocytes

(A) Micromass cultures of ATDC5 cells were exposed to 100 nM PTHrP (1-34) or PTHrP (7-34) at the onset of hypertrophic conversion (days 12 to 16). PTHrP (1-34) induced a progressive decrease in Runx2 mRNA expression, but PTHrP (7-34) had no effect.

(B) Primary rib chondrocytes were isolated from 2-week-old Zfp521 cKO and floxed control mice and treated for 48 hr with 100 nM PTHrP (1-34). PTHrP failed to repress Runx2 mRNA expression in the Zfp521 cKO cells. NS, not significant.

(C) Micromass cultures of ATDC5 cells stably expressing Zfp521 (seq 4) or nontarget shRNAs were treated with 100 nM PTHrP (1-34) for 48 hr. Depleting Zfp521 prevented the PTHrP-induced decrease in Runx2 expression. NS, not significant.

(D) Ihh expression was measured in primary chondrocytes from 2-week-old Zfp521 cKO and floxed control mice treated for 48 hr with 100 nM PTHrP (1-34). PTHrP failed to repress Ihh mRNA expression in the Zfp521 cKO cells. NS, not significant.

(E) Metatarsals from 2 weeks old Zfp521 cKO and floxed control mice were cultured with or without PTHrP (1-34) for 10 days and assayed for Col X mRNA by ISH. The absence of Zfp521 blunted the repressive effect of PTHrP on Col X expression. Note the presence of the growth plate phenotype on mutant metatarsals both at days 0 and 10, and the alterations of the growth plate morphology of both wild-type and mutant mice at day 10 due to in vitro assay conditions.

(F) Proposed model of action of Zfp521 in prehypertrophic chondrocytes. In chondrocytes, activation of the parathyroid hormone (PTH) receptor 1 (PTHR1) causes a PKA-mediated increase in Zfp521 expression and activates protein phosphatase 2A, which dephosphorylates HDAC4 (Kozhemyakina et al., 2009), causing its nuclear accumulation. In the nucleus, Zfp521 forms a complex with HDAC4 and Runx2, leading to a repression of Runx2-mediated target gene activation. (A–D) Data are means ± SD, n = 3, *$p < 0.05$.

Wu et al., 2009). Zfp521 expression in primary chondrocytes was markedly decreased by deleting *PTHrP* and increased by expressing the mutated PTHR1 that constitutively activates adenylyl cyclase. Third, PTHrP failed to induce Cyclin D1 expression and to repress Runx2, Ihh, and Col X expression in chondrocytes when Zfp521 was absent, and Bcl2 expression, which is induced by PTHrP in chondrocytes (Amling et al., 1997), was also reduced in the absence of Zfp521. Thus, *Zfp521* is a PTHrP target gene that appears to be a key effector of PTHrP in mechanisms that modulate chondrocyte proliferation, differentiation, and apoptosis in the growth plate.

The decreased cell proliferation is chondrocyte specific because it was not seen in fibroblastic or osteoblastic cells, and it is consistent with reports that Zfp521 acts to prevent differentiation and increases early precursor pools within the hematopoietic lineage (Bond et al., 2004; Warming et al., 2003). Therefore, Zfp521 may function as a repressor of exit from the cell cycle and progression to more mature cells. Zfp521 may contribute to the regulation of chondrocyte proliferation by at least two mechanisms. First, Zfp521 promotes basal Cyclin D1 expression in primary chondrocytes, which is required for normal proliferation of growth plate chondrocytes, and mediates the PTHrP-induced increase in Cyclin D1 expression, although we found that Zfp521 did not affect the activity of a Cyclin D1-luciferase reporter gene in ATDC5 cells (data not shown), suggesting that the changes in Cyclin D1 expression that occur when Zfp521 is depleted or overexpressed are not the result of a direct effect on the Cyclin D1 promoter. The mechanism could involve modulating the coupling of PTHrP to CREB (Beier et al., 2001) or possibly AP-1 factors, which we find bind to Zfp521 (data not shown), or other factors (e.g., Ebf1) that have been reported to interact with Zfp521. However, Cyclin D1 expression and chondrocyte proliferation are also induced by Ihh (Long et al., 2001), and we cannot rule out a role for Zfp521 downstream of Ihh. Second, the inability of PTHrP to antagonize Runx2 expression in the absence of Zfp521 and the resulting elevated Runx2 expression could also contribute to the reduced size of the proliferative zone because overexpressing Runx2 in proliferating and prehypertrophic chondrocytes reduces proliferation and the thickness of the proliferative zone (Takeda et al., 2001).

The repression of Runx2 expression by PTHrP, mediated by cAMP/PKA, is a critical event in the normal regulation of the chondrocyte hypertrophic transition (Iwamoto et al., 2003; Li et al., 2004). Mice that lack Runx2 or overexpress a dominant-negative form of Runx2 exhibit no hypertrophic conversion (Inada et al., 1999; Ueta et al., 2001), whereas targeted overexpression of Runx2 in prehypertrophic chondrocytes accelerates hypertrophic differentiation (Takeda et al., 2001). We confirmed that PTHrP-induced activation of the cAMP/PKA pathway in chondrocytes represses Runx2 expression and showed that it also increases Zfp521 expression. More importantly, we found that PTHrP failed to repress Runx2 expression in chondrocytes when Zfp521 was absent. Consistent with the inhibitory effect of Zfp521 on Runx2 transcriptional activity (Wu et al., 2009), hypertrophic conversion was accelerated in the Zfp521 cKO mice, with increased expression of ALP, Runx2, and the Runx2 target genes *Ihh*, *MMP-13*, and *OPN*, all markers of chondrocyte differentiation, strongly suggesting that Zfp521 contributes to the PTHrP-induced repression of Runx2 expression and activity in chondrocytes, and the resulting delay of hypertrophy.

HDACs inhibit gene expression by deacetylating both histones (class I HDACs) and transcription factors (class II HDACs) (Haberland et al., 2009). We found that the repression of Runx2 activity by Zfp521 requires HDAC4. This class II HDAC represses the transcriptional activity of Runx2 and MEF2C, another key regulatory factor in prehypertrophic chondrocytes, and HDAC4 null mice display premature chondrocyte hypertrophy (Vega et al., 2004), much like the effect of deleting Zfp521 (this study) or the constitutive expression of Runx2 or MEF2C in chondrocytes (Arnold et al., 2007). PTHrP induces the nuclear translocation and repression of gene expression by HDAC4 (Kozhemyakina et al., 2009). Our findings (1) that PTHrP also induces Zfp521 expression and (2) that Zfp521 inhibits Runx2 transcriptional activity in an HDAC4-dependent manner add another key element to this important growth plate regulatory mechanism.

The last steps in the progression of chondrocyte differentiation in the growth plate are the apoptosis of hypertrophic cells and the replacement of cartilage by bone. PTHrP slows the onset of chondrocyte apoptosis (Amizuka et al., 1996, 2004; Yamanaka et al., 2003), consistent with our report that PTHrP signaling increases the expression of the anti-apoptotic molecule Bcl-2 (Amling et al., 1997). Conversely, mice with Col II promoter-driven Runx2 overexpression exhibit accelerated hypertrophic differentiation and premature cartilage mineralization (Ueta et al., 2001), and overexpression of Runx2 in primary chondrocytes increased matrix calcification (Enomoto et al., 2000). Consistent with the increased Runx2, we found that apoptosis of terminally differentiated chondrocytes was increased in the Zfp521 cKO growth plates, whereas Bcl-2 expression was decreased and Caspase-3 activation was increased in Zfp521 cKO primary chondrocytes. This increased cell death presumably contributes to the thinner hypertrophic zone of the Zfp521 cKO growth plate, together with the reduced proliferation and production of matrix proteins and the accelerated hypertrophic conversion.

Although the growth plate phenotype of the Zfp521 cKO mice resembles that of the Col II-Cre: PTHR1$^{fl/fl}$ mice, it is less severe, suggesting that not all PTHrP-induced regulatory mechanisms depend on Zfp521 or that Zfp521 also modulates PTHrP-independent mechanisms that regulate growth plate development. A major difference is the time of presentation. Zfp521 cKO newborns were little different from control littermates, in contrast to the Col II-Cre: PTHR1$^{fl/fl}$ mice, in which the shortening of the growth plate was well established by E17.5 (Kobayashi et al., 2002). The lack of an embryonic phenotype in chondrocyte-specific *Zfp521* deletion was unexpected, given the clear co-expression of Zfp521 and PTHR1 in prehypertrophic cells as early at E17.5. However, deregulation or deletion of other regulatory molecules, e.g., constitutively active FGFR3 mutants (Chen et al., 1999) and deletion of the *growth hormone (GH) receptor* (Lupu et al., 2001), led to phenotypes that appear only postnatally. Most relevant for this study, deleting *Cyclin D1* (Beier et al., 2001) and overexpressing Runx2 (Takeda et al., 2001) both cause a postnatal thinning of the growth plate similar to that in the Zfp521 cKO mice, consistent with functional interaction of the molecules. The absence of a phenotype seen in the Runx2-overexpressing mice in late embryonic and perinatal stages, even though endogenous Runx2 expression is highest at E14.5 (Inada et al., 1999; Takeda et al., 2001), suggests that different Runx2-dependent mechanisms regulate growth plate chondrocyte development in embryonic and postnatal growth plates and that growth plate chondrocyte differentiation may be more sensitive to high levels of Runx2 postnatally. Compensation during embryogenesis for the loss of Zfp521 by the related Zfp423, which is also expressed in chondrocytes, could also contribute to the late onset of the phenotype.

In conclusion, our results demonstrate that the 30 Krüppel-like zinc finger protein Zfp521 contributes significantly to the regulation of proliferation, hypertrophy, and apoptosis of growth plate chondrocytes, thereby playing an important role in longitudinal bone growth. Zfp521 accomplishes this in part by mediating PTHrP-induced inhibition of Runx2 expression in prehypertrophic chondrocytes in an HDAC4-dependent mechanism (Figure 7F). Zfp521 may also modulate other regulatory mechanisms that control chondrocyte proliferation and apoptosis. In the absence of Zfp521, the organization of the growth plate is disturbed, and PTHrP responses are altered. Thus, Zfp521 is an important regulator of growth plate differentiation and endochondral bone development that is required for key downstream responses to PTHrP.

EXPERIMENTAL PROCEDURES

Cell and Organ Cultures

ATDC5 cells were expanded and cultured in micromass as previously described (Shukunami et al., 1996). The medium was changed every other day and supplemented with PTHrP (1-34), PTHrP (7-34), forskolin, H-89, and TPA as indicated. MCT cells were cultured as previously described (Zheng et al., 2003). PTHrP (1-34) or forskolin was added to the cultures at the beginning of the hypertrophic differentiation (nonpermissive temperature). Primary chondrocytes were isolated from rib cages of mice, cultured as previously described (Lefebvre et al., 1994), and treated with PTHrP (1-34) or PTHrP (7-34) for 48 hr. Metatarsals from 2-week-old mice hind limbs were cultured in α-MEM medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 1% L-glutamine with or without PTHrP (1-34) for 10 days in 37°C/5% CO_2, changing medium every other day. MC3T3-E1 cells were cultured as previously described (Wu et al., 2009). NIH 3T3 cells were cultured in DMEM supplemented with 10% BCS, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Gene Reporter Assay

MCT cells (2×10^5 cells/well in six-well plates) were cultured at 32°C (permissive temperature) until 70% confluent. Cells were transfected according to manufacturer's instructions using FuGene6 (Roche) in a 3:1 ratio with the 6xOSE2-Luc reporter plasmid along with combinations of Flag-Runx2 (0.5 μg), HA-Zfp521 (1 μg), HDAC4 siRNA (0.5 μg), and GAPDH siRNA (0.5 μg) as indicated and adjusting the total amount of transfected DNA with empty vector (pcDNA 3.1). Zfp521 dose response was assayed using Zfp521:Runx2 ratios of 1:1, 3:1, and 5:1. pCMV-Renilla-luciferase (Promega) (17 ng) was used for normalization. Transfected cells were cultured for 48 hr at 37°C/5% CO_2 (nonpermissive temperature), and luciferase activity was determined using the dual-luciferase reporter assay kit (Promega) following manufacturer's instructions. Results were calculated as fold activation over empty vector control.

Coimmunoprecipitation Assay

Co-immunoprecipitation was performed essentially as described elsewhere (Purev et al., 2009) using 2 μg mouse monoclonal anti-HDAC4 (Santa Cruz), 2 μg rabbit polyclonal anti-Zfp521 (Wu et al., 2009), or 2 μg anti-mouse IgG antibody (Promega) and Protein A/G-agarose bead slurry (Santa Cruz). Beads were collected by centrifugation and washed three to four times in mRIPA. The immune complexes were boiled in 2× SDS-PAGE sample buffer and subjected to immunoblot analysis.

tet-Inducible Zfp521 Overexpression System

ATDC5 cells stably transfected with tet-inducible Zfp521 were generated using the T-Rex inducible system (Invitrogen) according to manufacturer's instructions. Doxycycline (0.5 μg/ml) was added to culture medium to induce Zfp521 overexpression. ATDC5 cells stably transfected with tet-inducible LacZ were used as control. mRNA was assessed by qPCR.

Zfp521 shRNA-Expressing Cells

Two ATDC5 cell lines that expressed Zfp521 shRNAs were created using MISSION® shRNA lentiviral transduction particles (Sigma-Aldrich) encoding five different shRNA sequences (Broad Institute, Cambridge, MA, USA) (protocol available at www.sigmaaldrich.com). ATDC5 cells were transduced at 60% confluence (MOI 2 and 5) according to manufacturer's instructions and selected with puromycin (4 μg/m). Knockdown efficiency was evaluated by measuring Zfp521 expression in 12 day micromass cultures by qPCR. Cells expressing either nontarget shRNA or TurboGFP control shRNAs were prepared similarly. MC3T3-E1 cells stably expressing nontarget shRNA or Zfp521 shRNA (sequence [seq] 4) were established similarly.

Generation of a Chondrocyte-Specific Zfp521 Conditional Knockout Mouse

The *Zfp521* gene was specifically deleted from chondrocytes by crossing mice with a floxed *Zfp521* gene locus (R.K., K.Y., H.S., E.H., D.C., A.A., W.C.H., N.A.J., N.G.C., S.W., and R.B., unpublished data) with mice that express the Cre recombinase under the control of the Collagen type II (α1) promoter (Long et al., 2001).

Growth Plate Histology and Histomorphometry

Hind limbs from 2-week-old Zfp521 cKO and floxed control mice were dissected and processed for paraffin and methylmethacrylate embedding with and without decalcification, respectively, as previously described (Wu et al., 2009). Paraffin sections were stained with safranin O. von Kossa staining of mineralized matrix was performed on methylmetacrylate sections after plastic removal and analyzed under an inverted microscope (Maeda et al., 2007). Growth plate histomorphometry was performed on safranin O-stained sections of proximal tibia growth plates using Osteomeasure software (Osteometrics–Decatur). A representative field at 40× was used to obtain cell counts, mean polar cellular axes (cell heights), and stereological volumetric estimators obtained from measured areas (volume occupied by ECM and cells, respectively, in the hypertrophic zone), as described by Hunziker et al. (1987) with slight modifications. Briefly, cumulative hypertrophic chondrocyte surface area was measured and subtracted from the total hypertrophic zone surface area of the field obtaining the ECM area component of the zone. The area measurements served as volumetric stereological estimators of respective volumes.

Proliferation Assays

Two-week-old mice were injected intraperitoneally with 200 μl (1 ml/100 g of body weight) of BrdU labeling reagent (Zymed Laboratories) 20 and 4 hr before sacrifice. Incorporated BrdU was localized in proximal tibiae by immunohistochemistry using the Cell Proliferation IHC kit, BrdU detection, with DAB visualization (Chemicon) according to manufacturer's instructions. Zfp521 shRNA-expressing ATDC5 cells and tet-inducible Zfp521 ATDC5 cells were cultured on coverslips for 7 days and exposed to BrdU labeling reagent for 14 hr before fixing in 3.7% formaldehyde for 10 min and BrdU immunolocalization. BrdU incorporation was quantified using the Cell Proliferation ELISA kit, BrdU detection (Chemicon). MC3T3-E1 cells stably expressing nontarget or Zfp521 shRNA and NIH 3T3 cells transiently expressing empty vector (pCMV2) or Zfp521 (pCMV2-Zfp521) were cultured on coverslips for 24 hr and exposed to BrdU labeling reagent for 14 hr before BrdU immunolocalization. Percentage of BrdU-positive cells was determined.

Caspase Activity and TUNEL Assay

10^5 primary rib chondrocytes were cultured on coverslips for 5 days. 10^5 shRNA-expressing ATDC5 cells or 10^5 ATDC5 cells stably transfected with tet-inducible Zfp521 were cultured on coverslips in differentiation medium with or without doxycycline (0.5 μg/ml), respectively, for 1 week. Caspase activity was assessed by fluorescence microscopy using the Sulforhodamine FLICA Apoptosis Detection Kit Caspase Assay (Immunochemistry-Bloomington) according to manufacturer's instructions. TUNEL labeling was done using an In Situ Death Detection Kit, Fluorescein (Roche) on paraffin-embedded proximal tibiae sections, following manufacturer's instructions. The number of apoptotic cells was quantified.

In Situ Hybridization

^{35}S-UTP-labeled riboprobes were synthesized from linearized plasmids using the in vitro transcription kit (Promega) and ^{35}S-UTP (Amersham). ISH using antisense riboprobes was performed on 3.7% formaldehyde-fixed, decalcified, paraffin-embedded proximal tibias of 2-week-old mice and metatarsal bones as previously described (Razzaque et al., 2005). Image J 1.42q software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) was used to calculate growth plate thicknesses and distances, measured in arbitrary units and reported as percent changes, and the granular area fraction covered by the ISH signal of Col X, PTHR1, and Ihh as a measure of the amount of signal, and reported as fold change.

Statistical Analysis

Statistical differences were assessed with the unpaired Student's t test. Data are presented as mean ± SD, and p values less than 0.05 were considered statistically significant.

Additional information about antibodies, transient transfections, quantitative PCR, western blotting, immunohistochemistry, and Alcian blue/alizarin red staining is included in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j.devcel.2010.09.008.

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REFERENCES

Amizuka, N., Warshawsky, H., Henderson, J.E., Goltzman, D., and Karaplis, A.C. (1994). Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. J. Cell Biol. *126*, 1611–1623.

Amizuka, N., Henderson, J.E., Hoshi, K., Warshawsky, H., Ozawa, H., Goltzman, D., and Karaplis, A.C. (1996). Programmed cell death of chondrocytes and aberrant chondrogenesis in mice homozygous for parathyroid hormone-related peptide gene deletion. Endocrinology *137*, 5055–5067.

Amizuka, N., Davidson, D., Liu, H., Valverde-Franco, G., Chai, S., Maeda, T., Ozawa, H., Hammond, V., Ornitz, D.M., Goltzman, D., et al. (2004). Signalling by fibroblast growth factor receptor 3 and parathyroid hormone-related peptide coordinate cartilage and bone development. Bone *34*, 13–25.

Amling, M., Neff, L., Tanaka, S., Inoue, D., Kuida, K., Weir, E., Philbrick, W.M., Broadus, A.E., and Baron, R. (1997). Bcl-2 lies downstream of parathyroid hormone-related peptide in a signaling pathway that regulates chondrocyte maturation during skeletal development. J. Cell Biol. *136*, 205–213.

Arnold, M.A., Kim, Y., Czubryt, M.P., Phan, D., McAnally, J., Qi, X., Shelton, J.M., Richardson, J.A., Bassel-Duby, R., and Olson, E.N. (2007). MEF2C transcription factor controls chondrocyte hypertrophy and bone development. Dev. Cell *12*, 377–389.

Beier, F., Leask, T.A., Haque, S., Chow, C., Taylor, A.C., Lee, R.J., Pestell, R.G., Ballock, R.T., and LuValle, P. (1999). Cell cycle genes in chondrocyte proliferation and differentiation. Matrix Biol. *18*, 109–120.

Beier, F., Ali, Z., Mok, D., Taylor, A.C., Leask, T., Albanese, C., Pestell, R.G., and LuValle, P. (2001). TGFbeta and PTHrP control chondrocyte proliferation by activating cyclin D1 expression. Mol. Biol. Cell *12*, 3852–3863.

Bond, H.M., Mesuraca, M., Carbone, E., Bonelli, P., Agosti, V., Amodio, N., De Rosa, G., Di Nicola, M., Gianni, A.M., Moore, M.A.S., et al. (2004). Early hematopoietic zinc finger protein (EHZF), the human homolog to mouse Evi3, is highly expressed in primitive human hematopoietic cells. Blood *103*, 2062–2070.

Bond, H.M., Mesuraca, M., Amodio, N., Mega, T., Agosti, V., Fanello, D., Pelaggi, D., Bullinger, L., Grieco, M., Moore, M.A., et al. (2008). Early hematopoietic zinc finger protein-zinc finger protein 521: a candidate regulator of diverse immature cells. Int. J. Biochem. Cell Biol. *40*, 848–854.

Chen, L., Adar, R., Yang, X., Monsonego, E.O., Li, C., Hauschka, P.V., Yayon, A., and Deng, C.X. (1999). Gly369Cys mutation in mouse FGFR3 causes achondroplasia by affecting both chondrogenesis and osteogenesis. J. Clin. Invest. *104*, 1517–1525.

Enomoto, H., Enomoto-Iwamoto, M., Iwamoto, M., Nomura, S., Himeno, M., Kitamura, Y., Kishimoto, T., and Komori, T. (2000). Cbfa1 is a positive regulatory factor in chondrocyte maturation. J. Biol. Chem. *275*, 8695–8702.

Guo, J., Chung, U.I., Yang, D., Karsenty, G., Bringhurst, F.R., and Kronenberg, H.M. (2006). PTH/PTHrP receptor delays chondrocyte hypertrophy via both Runx2-dependent and -independent pathways. Dev. Biol. *292*, 116–128.

Haberland, M., Montgomery, R.L., and Olson, E.N. (2009). The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat. Rev. Genet. *10*, 32–42.

Hunziker, E.B., Schenk, R.K., and Cruz-Orive, L.M. (1987). Quantitation of chondrocyte performance in growth-plate cartilage during longitudinal bone growth. J. Bone Joint Surg. Am. *69*, 162–173.

Inada, M., Yasui, T., Nomura, S., Miyake, S., Deguchi, K., Himeno, M., Sato, M., Yamagiwa, H., Kimura, T., Yasui, N., et al. (1999). Maturational disturbance of chondrocytes in Cbfa1-deficient mice. Dev. Dyn. *214*, 279–290.

Iwamoto, M., Kitagaki, J., Tamamura, Y., Gentili, C., Koyama, E., Enomoto, H., Komori, T., Pacifici, M., and Enomoto-Iwamoto, M. (2003). Runx2 expression and action in chondrocytes are regulated by retinoid signaling and parathyroid hormone-related peptide (PTHrP). Osteoarthritis Cartilage *11*, 6–15.

Justice, M.J., Morse, H.C., 3rd, Jenkins, N.A., and Copeland, N.G. (1994). Identification of Evi-3, a novel common site of retroviral integration in mouse AKXD B-cell lymphomas. J. Virol. *68*, 1293–1300.

Kang, J.S., Alliston, T., Delston, R., and Derynck, R. (2005). Repression of Runx2 function by TGF-beta through recruitment of class II histone deacetylases by Smad3. EMBO J. *24*, 2543–2555.

Kim, I.S., Otto, F., Zabel, B., and Mundlos, S. (1999). Regulation of chondrocyte differentiation by *Cbfa1*. Mech. Dev. *80*, 159–170.

Kobayashi, T., Chung, U.I., Schipani, E., Starbuck, M., Karsenty, G., Katagiri, T., Goad, D.L., Lanske, B., and Kronenberg, H.M. (2002). PTHrP and Indian hedgehog control differentiation of growth plate chondrocytes at multiple steps. Development *129*, 2977–2986.

Kozhemyakina, E., Cohen, T., Yao, T.P., and Lassar, A.B. (2009). Parathyroid hormone-related peptide represses chondrocyte hypertrophy through a protein phosphatase 2A/histone deacetylase 4/MEF2 pathway. Mol. Cell. Biol. *29*, 5751–5762.

Kronenberg, H.M. (2003). Developmental regulation of the growth plate. Nature *423*, 332–336.

Kronenberg, H.M. (2006). PTHrP and skeletal development. Ann. N Y Acad. Sci. *1068*, 1–13.

La Rocca, R., Fulciniti, M., Lakshmikanth, T., Mesuraca, M., Ali, T.H., Mazzei, V., Amodio, N., Catalano, L., Rotoli, B., Ouerfelli, O., et al. (2009). Early hematopoietic zinc finger protein prevents tumor cell recognition by natural killer cells. J. Immunol. *182*, 4529–4537.

Lanske, B., Amling, M., Neff, L., Guiducci, J., Baron, R., and Kronenberg, H.M. (1999). Ablation of the PTHrP gene or the PTH/PTHrP receptor gene leads to distinct abnormalities in bone development. J. Clin. Invest. *104*, 399–407.

Lefebvre, V., Garofalo, S., Zhou, G., Metsaranta, M., Vuorio, E., and De Crombrugghe, B. (1994). Characterization of primary cultures of chondrocytes from type II collagen/beta-galactosidase transgenic mice. Matrix Biol. *14*, 329–335.

Li, T.F., Dong, Y., Ionescu, A.M., Rosier, R.N., Zuscik, M.J., Schwarz, E.M., O'Keefe, R.J., and Drissi, H. (2004). Parathyroid hormone-related peptide (PTHrP) inhibits Runx2 expression through the PKA signaling pathway. Exp. Cell Res. *299*, 128–136.

Long, F., Zhang, X.M., Karp, S., Yang, Y., and McMahon, A.P. (2001). Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. Development *128*, 5099–5108.

Lupu, F., Terwilliger, J.D., Lee, K., Segre, G.V., and Efstratiadis, A. (2001). Roles of growth hormone and insulin-like growth factor 1 in mouse postnatal growth. Dev. Biol. *229*, 141–162.

Maeda, Y., Nakamura, E., Nguyen, M.T., Suva, L.J., Swain, F.L., Razzaque, M.S., Mackem, S., and Lanske, B. (2007). Indian Hedgehog produced by postnatal chondrocytes is essential for maintaining a growth plate and trabecular bone. Proc. Natl. Acad. Sci. USA *104*, 6382–6387.

Matsubara, E., Sakai, I., Yamanouchi, J., Fujiwara, H., Yakushijin, Y., Hato, T., Shigemoto, K., and Yasukawa, M. (2009). The role of zinc finger protein 521/early hematopoietic zinc finger protein in erythroid cell differentiation. J. Biol. Chem. *284*, 3480–3487.

Nutt, R.F., Caulfield, M.P., Levy, J.J., Gibbons, S.W., Rosenblatt, M., and McKee, R.L. (1990). Removal of partial agonism from parathyroid hormone (PTH)-related protein-(7-34)NH2 by substitution of PTH amino acids at positions 10 and 11. Endocrinology *127*, 491–493.

Porter, A.G., and Janicke, R.U. (1999). Emerging roles of caspase-3 in apoptosis. Cell Death Differ. *6*, 99–104.

Purev, E., Neff, L., Horne, W.C., and Baron, R. (2009). c-Cbl and Cbl-b Act redundantly to protect osteoclasts from apoptosis and to displace HDAC6 from {beta}-tubulin, stabilizing microtubules and podosomes. Mol. Biol. Cell *20*, 4021–4030.

Razzaque, M.S., Soegiarto, D.W., Chang, D., Long, F., and Lanske, B. (2005). Conditional deletion of Indian hedgehog from collagen type 2alpha1-expressing cells results in abnormal endochondral bone formation. J. Pathol. *207*, 453–461.

Schipani, E., Jensen, G.S., Pincus, J., Nissenson, R.A., Gardella, T.J., and Juppner, H. (1997). Constitutive activation of the cyclic adenosine 3′,5′-monophosphate signaling pathway by parathyroid hormone (PTH)/PTH-related peptide receptors mutated at the two loci for Jansen's metaphyseal chondrodysplasia. Mol. Endocrinol. *11*, 851–858.

Schroeder, T.M., Kahler, R.A., Li, X., and Westendorf, J.J. (2004). Histone deacetylase 3 interacts with Runx2 to repress the osteocalcin promoter and regulate osteoblast differentiation. J. Biol. Chem. *279*, 41998–42007.

Shukunami, C., Shigeno, C., Atsumi, T., Ishizeki, K., Suzuki, F., and Hiraki, Y. (1996). Chondrogenic differentiation of clonal mouse embryonic cell line ATDC5 in vitro: differentiation-dependent gene expression of parathyroid hormone (PTH)/PTH-related peptide receptor. J. Cell Biol. *133*, 457–468.

Takeda, S., Bonnamy, J.P., Owen, M.J., Ducy, P., and Karsenty, G. (2001). Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. Genes Dev. *15*, 467–481.

Ueta, C., Iwamoto, M., Kanatani, N., Yoshida, C., Liu, Y., Enomoto-Iwamoto, M., Ohmori, T., Enomoto, H., Nakata, K., Takada, K., et al. (2001). Skeletal malformations caused by overexpression of Cbfa1 or its dominant negative form in chondrocytes. J. Cell Biol. *153*, 87–100.

Vega, R.B., Matsuda, K., Oh, J., Barbosa, A.C., Yang, X., Meadows, E., McAnally, J., Pomajzl, C., Shelton, J.M., Richardson, J.A., et al. (2004). Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. Cell *119*, 555–566.

Warming, S., Liu, P., Suzuki, T., Akagi, K., Lindtner, S., Pavlakis, G.N., Jenkins, N.A., and Copeland, N.G. (2003). Evi3, a common retroviral integration site in murine B-cell lymphoma, encodes an EBFAZ-related Krüppel-like zinc finger protein. Blood *101*, 1934–1940.

Weir, E.C., Philbrick, W.M., Amling, M., Neff, L.A., Baron, R., and Broadus, A.E. (1996). Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation. Proc. Natl. Acad. Sci. USA *93*, 10240–10245.

Westendorf, J.J., Zaidi, S.K., Cascino, J.E., Kahler, R., van Wijnen, A.J., Lian, J.B., Yoshida, M., Stein, G.S., and Li, X. (2002). Runx2 (Cbfa1, AML-3) interacts with histone deacetylase 6 and represses the p21(CIP1/WAF1) promoter. Mol. Cell. Biol. *22*, 7982–7992.

Wu, M., Hesse, E., Morvan, F., Zhang, J.P., Correa, D., Rowe, G.C., Kiviranta, R., Neff, L., Philbrick, W.M., Horne, W.C., et al. (2009). Zfp521 antagonizes Runx2, delays osteoblast differentiation in vitro, and promotes bone formation in vivo. Bone *44*, 528–536.

Yamanaka, Y., Tanaka, H., Koike, M., Nishimura, R., and Seino, Y. (2003). PTHrP rescues ATDC5 cells from apoptosis induced by FGF receptor 3 mutation. J. Bone Miner. Res. *18*, 1395–1403.

Yamasaki, N., Miyazaki, K., Nagamachi, A., Koller, R., Oda, H., Miyazaki, M., Sasaki, T., Honda, Z.I., Wolff, L., Inaba, T., et al. (2010). Identification of Zfp521/ZNF521 as a cooperative gene for E2A-HLF to develop acute B-lineage leukemia. Oncogene *29*, 1963–1975.

Zheng, Q., Zhou, G., Morello, R., Chen, Y., Garcia-Rojas, X., and Lee, B. (2003). Type X collagen gene regulation by Runx2 contributes directly to its hypertrophic chondrocyte-specific expression in vivo. J. Cell Biol. *162*, 833–842.