A-Raf and B-Raf Are Dispensable for Normal Endochondral Bone Development, and Parathyroid Hormone-Related Peptide Suppresses Extracellular Signal-Regulated Kinase Activation in Hypertrophic Chondrocytes[▽]

Sylvain Provot,^{1,2} Gregory Nachtrab,¹ Jennifer Paruch,¹ Adele Pin Chen,^{3†}
Alcino Silva,³ and Henry M. Kronenberg^{1*}

*Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, 50 Blossom Street, Boston, Massachusetts 02114¹;
Department of Anatomy, University of California, San Francisco, 513 Parnassus Avenue, Box 0452, San Francisco,
California 94143²; and Departments of Neurobiology, Psychiatry, and Psychology and Brain Research Institute,
University of California, Los Angeles, Room 2357, Gonda Building, Box 951761,
695 Charles Young Drive South, Los Angeles, California³*

Received 9 April 2007/Returned for modification 16 May 2007/Accepted 17 October 2007

Parathyroid hormone-related peptide (PTHrP) and the parathyroid hormone-PTHrP receptor increase chondrocyte proliferation and delay chondrocyte maturation in endochondral bone development at least partly through cyclic AMP (cAMP)-dependent signaling pathways. Because data suggest that the ability of cAMP to stimulate cell proliferation involves the mitogen-activated protein kinase kinase kinase B-Raf, we hypothesized that B-Raf might mediate the proliferative action of PTHrP in chondrocytes. Though B-Raf is expressed in proliferative chondrocytes, its conditional removal from cartilage did not affect chondrocyte proliferation and maturation or PTHrP-induced chondrocyte proliferation and PTHrP-delayed maturation. Similar results were obtained by conditionally removing B-Raf from osteoblasts. Because A-raf and B-raf are expressed similarly in cartilage, we speculated that they may fulfill redundant functions in this tissue. Surprisingly, mice with chondrocytes deficient in both A-Raf and B-Raf exhibited normal endochondral bone development. Activated extracellular signal-regulated kinase (ERK) was detected primarily in hypertrophic chondrocytes, where C-raf is expressed, and the suppression of ERK activation in these cells by PTHrP or a MEK inhibitor coincided with a delay in chondrocyte maturation. Taken together, these results demonstrate that B-Raf and A-Raf are dispensable for endochondral bone development and they indicate that the main role of ERK in cartilage is to stimulate not cell proliferation, but rather chondrocyte maturation.

Endochondral ossification accounts for the formation of most of the bones in the skeleton. It involves a two-stage mechanism whereby chondrocytes form a matrix template through an organized structure called the growth plate, which is progressively replaced by bone (16, 17, 33). During this process, growth plate chondrocytes undergo well-ordered and controlled phases of cell proliferation, differentiation, and apoptosis. Chondrocytes initially form from condensed mesenchymal cells and proliferate to expand the cartilage mold longitudinally. Immature proliferative chondrocytes express type II collagen (Col2a1) and form columns of flattened cells (the columnar layer). Chondrocytes then exit the cell cycle, and postmitotic cells differentiate into prehypertrophic chondrocytes expressing Indian hedgehog (Ihh), which then further mature into type X collagen (Col10a1)-expressing hypertrophic chondrocytes. These cells eventually become late hypertrophic chondrocytes and express osteopontin (OP), a marker

also detected in bone. This differentiation process is followed by the death of the hypertrophic chondrocytes, blood vessel invasion, and finally, the replacement of the cartilaginous matrix by bone (the primary spongiosa), marked by the expression of Osterix (Osx), type I collagen (Col1a1), and OP.

Several lines of evidence show that parathyroid hormone-related peptide (PTHrP) signaling critically controls the rate of proliferation and differentiation in the growth plate. In fetal development, PTHrP is produced by the most distal perichondrium and chondrocytes at the articular surface and the parathyroid hormone (PTH)-PTHrP receptor (PPR) is synthesized in the proliferative and prehypertrophic zones of the growth plate (18, 45). Mice lacking either PTHrP or its receptor exhibit dwarfism of their long bones due to premature chondrocyte maturation (14, 18). Conversely, ectopic expression of either PTHrP or an activated form of PPR results in a dramatic blockade of cartilage maturation (37, 46). Thus, a major action of PTHrP is to maintain chondrocytes in the proliferative pool. PTHrP is also needed for maximal rates of proliferation in the proliferative pool, particularly early in endochondral bone development (15).

The PPR is a member of the B subfamily of G protein-coupled receptors that can activate multiple G proteins (38, 44): Gs, which activates the adenylyl cyclase/protein kinase A (PKA) pathway; the Gq/G11 family, which activates the phos-

* Corresponding author. Mailing address: Massachusetts General Hospital-Harvard Medical School, Endocrine Unit, 50 Blossom Street, Thier 1101, Boston, MA 02114-2696. Phone: (617) 726-3966. Fax: (617) 726-7543. E-mail: hkronenberg@partners.org.

† Present address: Key Laboratory of Systems Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200031 China.

[▽] Published ahead of print on 29 October 2007.

pholipase C/protein kinase C pathway; Gi, which inhibits adenylyl cyclase and exerts other actions as well; and G12/G13 (41). Through the activation of these pathways, PPR generates multiple intracellular secondary messengers, including cyclic AMP (cAMP), inositol triphosphate, diacylglycerol, and cytosolic Ca^{2+} , which then mediate various cellular responses. We have previously reported that the phospholipase C pathway downstream of PPR slows the proliferation and hastens the differentiation of chondrocytes, actions that oppose the dominant effect of PPR and that involve cAMP-dependent signaling pathways (1, 10).

The effect of cAMP on cell division has long been mysterious: this remarkable regulator seems both to activate and to inhibit cell proliferation, depending on the cell type. Important recent data reviewed by Stork and Schmitt (43) establish a model that may explain the mechanisms that foster such opposite effects. In many cell types, the capacity of cAMP to induce or inhibit proliferation depends on its capacity for regulating extracellular signal-regulated kinases (ERKs) positively or negatively, respectively. The ERKs are also named mitogen-activated protein kinases (MAPKs) and are activated by phosphorylation by the MAPK kinases MEK1 and MEK2. These kinases are themselves activated by phosphorylation by MAPK kinase kinases. There are three different Raf MAPK kinase kinases in mammals (31): A-Raf, B-Raf, and C-Raf. A line of evidence suggests that the regulation of ERKs, through the cAMP/PKA pathway, depends on the presence or absence of B-Raf: while a cAMP/PKA signal activates B-Raf, it inactivates C-Raf. The consequence is that, in cells expressing only C-Raf, ERK is inactivated and proliferation is inhibited whereas, in B-Raf-expressing cells, ERK is activated and proliferation is induced. Interestingly, this model has proven to be valid for osteoblastic cells (8), which are thought to derive from the same osteochondroprogenitor as chondrocytes. Furthermore, several publications have presented results of in vitro experiments supporting a role for the Raf/MEK/ERK pathway in chondrocyte proliferation (28, 40, 51). Therefore, we undertook to determine whether B-Raf mediates PTHrP/cAMP/PKA-induced chondrocyte proliferation in the growth plate and, more generally, to determine the role of B-Raf in cartilage. In the mouse, knocking out B-Raf leads to embryonic lethality (47, 49). The embryos die between embryonic day 10 (E10) and E12.5. They present vascular defects caused by the excessive death of endothelial cells, as well as growth retardation. Because the B-Raf universal-knockout (universal-KO) embryos die before endochondral bone development starts, we conditionally removed B-Raf from cartilage by using a *B-raf* flox allele (4) and *Col2a1* promoter-driven Cre, ensuring specific expression of the recombinase in chondrocytes (30).

MATERIALS AND METHODS

Mice and genotyping. To generate B-Raf conditional-knockout (CKO) animals that lacked B-Raf in cartilage, hemizygous *Col2-Cre* transgenic mice (in which the expression of Cre recombinase is under the control of a mouse *Col2a1* promoter) (30) were bred with animals homozygous for a *B-raf* flox allele (*B-raf*^{flox/flox} mice have exon 14 of the *B-raf* locus flanked by *loxP* sites) (4). After appropriate breeding, *Col2-Cre B-raf*^{flox/flox} mutant CKO mice (referred to hereinafter simply as B-Raf CKO or CKO mice) were generated and identified by PCR, as described by Chen and collaborators (4). The *Col2-Cre* transgene was identified as previously described (30). All results presented in this report have been obtained with the mouse lines listed above, but similar results were ob-

tained with another *Col2-Cre* transgenic line, in which Cre is under the control of a rat *Col2a1* promoter (39). The removal of B-Raf in osteoblastic cells was achieved using either *Col1a1*-driven Cre (*Col1-Cre*) (21) or *Osterix*-driven Cre (*Osx-Cre*) (35). CKO animals generated with *Col1-Cre* (*Col1-CKO*) and *Osx-Cre* (*Osx-CKO*) were identified as previously described (21, 35).

To generate B-Raf CKO animals that also carried a transgene for a constitutively active form of PPR expressed under the control of the *Col2a1* promoter (*Col2-caPPR*) (37), we mated mice hemizygous for both *Col2-Cre* and *Col2-caPPR* transgenes and heterozygous for the *B-raf* flox allele with mice hemizygous for the *Col2-caPPR* transgene and homozygous for the *B-raf* flox allele. In order to evaluate the role of B-Raf downstream of PPR in cartilage, B-Raf CKO mice homozygous for the *Col2-caPPR* transgene were compared to mice that still expressed B-Raf and were also homozygous for the *Col2-caPPR* transgene (see Results). *Col2-caPPR* mice were genotyped by Southern blotting as previously described (37). In order to evaluate the role of B-Raf downstream of PPR in bone, mice hemizygous for both *Col1-caPPR* (the CL2 line described in reference 3) and *Col1-Cre* transgenes and heterozygous for the *B-raf* flox allele were mated with mice homozygous for the *B-raf* flox allele. The offspring were genotyped as previously described (reference 3; also see above).

The generation and genotyping of PTHrP-p57 KO mice have been described elsewhere (19). A-Raf KO mice with a C57BL/6 background (32) were kindly provided by Catrin Pritchard (University of Leicester, Leicester, United Kingdom). The *A-raf* gene is present on the X chromosome, and thus, males heterozygous for the *A-raf* null mutation (Y/X^-) have A-Raf knocked out. Mice with cartilage deficient in A-Raf and B-Raf were generated by mating males heterozygous for the *B-raf* flox allele and hemizygous for the *Col2-Cre* transgene with females homozygous for the *B-raf* flox allele and heterozygous for the *A-raf* null allele (X^+/X^-). Mice with osteoblastic cells deficient in A-Raf and B-Raf were generated by a similar mating protocol but using males heterozygous for the *B-raf* flox allele and hemizygous for the *Osx-Cre* transgene instead. Mice were genotyped for the *A-raf* null mutation as previously described (20).

Skeletal preparations and histological analysis. Newborn mice were sacrificed and eviscerated, and their skin was peeled off before they were fixed and stained for cartilage for 24 h with a solution of 80% ethyl alcohol and 20% glacial acetic acid that contained 15 mg of Alcian blue 8GX (Sigma; catalog no. A-3157)/100 ml. Samples were then dehydrated for 5 to 7 days with 100% ethyl alcohol and stained for mineralized matrix overnight in a solution of 1% KOH that contained 10 mg of alizarin red S (Sigma; catalog no. A-5533)/100 ml. Samples were placed in a 1% KOH–20% glycerol clearing solution until the soft tissues become transparent and the skeleton visible. Then they were placed in successive solutions of 0.5% KOH with increasing percentages of glycerol (20, 40, 60, and 80%). Samples were stored in 100% glycerol.

To perform histological analyses of the long bones, both hind limbs and forelimbs were obtained from E14.5 to E18.5 mouse embryos (delivered by caesarean section) and from newborn, postnatal day 5 (P5), and 2- and 8-week-old mice. All bones were fixed in 10% formalin–phosphate-buffered saline (pH 7.4) for 36 h at room temperature. Only postnatal bones from mice at P5, 2 weeks, and 8 weeks of age were decalcified at room temperature in 20% EDTA– H_2O (pH 7.5) solution for 3, 7 to 8, and 14 days, respectively. The limbs were then embedded into paraffin blocks prepared by standard histological procedures. Sections (5 to 6 μm thick) were cut from several levels of the blocks and stained with hematoxylin and eosin.

ISH analysis, immunohistochemistry, bromodeoxyuridine (BrdU) assay, and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. Nonradioactive in situ hybridization (ISH) analysis was done on paraffin sections with digoxigenin-labeled probes, as previously described (24). Radioactive (^{35}S -labeled-ribo probe) ISH analysis was performed as previously described (6). *B-raf* cDNA was supplied by Alain Eychene (Curie Institute, Orsay, France). *C-raf* cDNA was supplied by Manuela Baccarini (Vienna Bio-center, Institute of Microbiology and Genetics, Vienna, Austria). *A-raf* cDNA was supplied by Catrin Pritchard (University of Leicester, Leicester, United Kingdom). Further details of the probes employed for ISH are available upon request.

Immunohistochemistry analysis for phosphorylated ERK (phospho-ERK) was performed as follows: after deparaffination and rehydration, slides were incubated in a solution of 90% methanol–10% dimethyl sulfoxide for 20 min at room temperature, washed, incubated in a solution of 0.3% H_2O_2 in phosphate-buffered saline for 20 min, and washed again. Blocking, incubation with antibodies, and detection were performed using the TSA biotin system (catalog no. NEL700) as recommended by the manufacturer (PerkinElmer). The primary antibody directed against phospho-ERK (Cell Signaling; catalog no. 4377S) was diluted 1:100, and the secondary biotinylated swine anti-rabbit antibody (Dako; reference no. E0431) was diluted at 1:300. Immunohistochemistry analysis for phos-

phorylated histone H3 (used at 1:500 [Upstate; catalog no. 06-570]) was performed using EnVision kit (reference no. K4010) according to the instructions of the manufacturer (Dako).

The BrdU assay was performed using a BrdU staining kit (catalog no. 93-3944) according to the instructions of the manufacturer (Zymed). At least three animals of each genotype (two sections of proximal tibia per animal) were analyzed to quantify the proliferation rate at a given stage. The proliferation rate (given as a percentage) corresponds to the ratio of BrdU-positive cells to the total number of cells counted in the proliferative zone of the growth plate (excluding quiescent hypertrophic chondrocytes).

The TUNEL assay was performed using an in situ cell death detection kit (catalog no. 11684817910) under the conditions specified by the manufacturer (Roche).

Western blot analysis of primary chondrocytes. Primary chondrocytes were isolated from newborn B-Raf CKO and wild-type (WT) littermates and littermates heterozygous for a CKO transgene, referred to herein as conditional heterozygotes (CHETs). Forelimbs and hind limbs were dissected, skin and muscles were carefully removed, the distal epiphyses of radii, ulnas, tibiae, and femurs plus the proximal epiphyses of tibiae were isolated in Hanks balanced salt solution medium (GIBCO BRL), and samples from each animal were pooled. Epiphyses were digested in 0.25% trypsin-EDTA for 30 min at 37°C and then with 195 U of collagenase type II (Worthington)/ml in Hanks balanced salt solution for 2 h at 37°C. Cell suspensions were homogenized by pipetting, harvested without remaining cartilaginous pieces, and centrifuged at 400 × g for 10 min. The collagenase was inactivated by fetal bovine serum (FBS; HyClone), and chondrocytes were resuspended after an additional centrifugation in Dulbecco's modified Eagle medium (GIBCO BRL) supplemented with 10% FBS and 1% penicillin-streptomycin. Chondrocytes were plated (3 million/10-cm dish) and cultured for 6 days in Dulbecco's modified Eagle medium-10% FBS. Cell extracts were prepared in an NP-40 buffer (137 mM NaCl, 20 mM Tris-HCl [pH 8], 10% glycerol, 1% NP-40, 2 mM EDTA) supplemented with a proteinase inhibitor cocktail (Roche; catalog no. 1836170). The concentration of soluble proteins was quantified using the Bradford reagent (Bio-Rad). Total protein extracts (40 µg/lane) were run on sodium dodecyl sulfate-4 to 15% polyacrylamide gradient gels (Bio-Rad) and transferred onto nitrocellulose membranes (Bio-Rad) by a standard Western blot procedure. Membranes were incubated with primary and secondary antibodies, and signals were detected as previously described (2). The antibodies and concentrations used were as follows: mouse anti-B-Raf (F7 [Santa Cruz; catalog no. sc-5284]) used at 1:5,000, mouse anti-C-Raf (E-10 [Santa Cruz; catalog no. sc-7267]) used at 1:1,000, rabbit anti-A-Raf (C-20 [Santa Cruz; catalog no. sc-408]) used at 1:1,000, goat antiactin (I-19 [Santa Cruz; catalog no. sc-1616]) used at 1:300, goat anti-mouse immunoglobulin G-horseradish peroxidase (IgG-HRP [Santa Cruz; catalog no. sc-2005]) used at 1:10,000, rabbit anti-goat IgG-HRP (Santa Cruz; catalog no. sc-2768) used at 1:10,000, and anti-rabbit IgG-HRP (Cell Signaling; reference no. 7074) used at 1:3,000.

Culture of metatarsal explants. Metatarsal explants were obtained from E15.5 mouse hind limbs (from WT and CKO mice) and cultured as described previously (11). The second, third, and fourth metatarsals from each hind limb were dissected, cultured for 24 h, and then treated with either a PPR agonist, [Nle8,21,Tyr34] rat PTH(1-34)NH₂ [PTH(1-34)], produced by the Massachusetts General Hospital Biopolymer Core Facility, or vehicle, α -minimal essential medium-0.25% heat-inactivated FBS, used to dilute the agonist and to culture the explants, for an extra 24 h or for the last hour or last 2 h of culture (see Fig. 7). The PPR agonist was used at a final concentration of 10⁻⁷ M. In some experiments, metatarsals were treated with either the MEK1-MEK2 inhibitor U0126 (Promega; reference no. V1121), used at 20 µM, or vehicle (dimethyl sulfoxide used to dilute U0126) for the indicated periods of time (see Fig. 8). The culture medium was supplemented with ascorbic acid (50 µg/ml) and β -glycerophosphate (1 mM) to allow the mineralization of the cartilage and bone matrix. The three metatarsals obtained from one hind limb and treated with the PPR agonist or U0126 were systematically compared to the three metatarsals obtained from the second hind limb of the same embryo. Representative metatarsals are shown. The same results were obtained in at least three independent experiments.

RESULTS

B-Raf expression is restricted to immature proliferative chondrocytes during endochondral ossification. B-Raf is the major Raf isoform in the brain and is expressed in a wide variety of cell types, but expression in chondrocytes has not

FIG. 1. B-Raf is expressed in immature proliferative chondrocytes. Nonradioactive ISH analysis of E16.5 proximal tibia with the indicated chondrogenic markers. Note that *B-raf* mRNA is detected in the proliferative layer (Pro). Its expression declines in prehypertrophic (PH) chondrocytes, marked by *Ihh*, and is not detectable in mature hypertrophic (H) chondrocytes, marked by *Col10a1*, unlike *Col2a1*, which is expressed in the whole cartilaginous growth plate. Note that *B-raf* mRNA is also detected in bone (primary spongiosa [PS]).

been evaluated (31, 47). To assess the function of B-Raf in cartilage, we first examined whether *B-raf* is expressed in chondrocytes and whether its expression is restricted to proliferative chondrocytes by ISH analysis of developing mouse limbs using a probe specific for *B-raf* (Fig. 1). In the growth plates of E16.5 mouse embryos, marked by the expression of *Col2a1*, we found that *B-raf* mRNA was expressed relatively uniformly in the layer of round proliferative chondrocytes; its expression slightly increased in the flat proliferative chondrocytes, which had a slightly higher proliferation rate than round chondrocytes. In contrast, *B-raf* mRNA expression decreased in prehypertrophic chondrocytes, marked by *Ihh* mRNA expression, and was not detectable in mature quiescent hypertrophic chondrocytes, marked by *Col10a1* mRNA expression (Fig. 1). While *B-raf* mRNA was observed in proliferative chondrocytes at all stages examined, the level of expression was significantly higher at early stages of chondrogenesis (E14.5 to E15.5), when chondrocytes actively proliferate, than at later stages (P0 to P14), when their proliferation rate decreases (data not shown). These data demonstrate that *B-raf* expression is restricted to proliferative immature chondrocytes. It is therefore possible that the presence of B-Raf may account for the pro-

liferative action of the PTHrP/cAMP/PKA pathway in those cells, as suggested by the model described above in the introduction.

Conditional knockout of B-Raf in cartilage does not impair skeletogenesis. To test the possibility that B-Raf plays a role downstream of PTHrP in inducing chondrocyte proliferation, we conditionally removed B-Raf from chondrocytes by the *cre-lox* approach, since the B-Raf universal-KO mice die too early to study endochondral bone development. To remove B-Raf, we used mice carrying the *B-raf* flox allele (*B-raf*^{flox/flox}), in which exon 14 (the first exon of the kinase domain) of the *B-raf* locus has been flanked by *loxP* sites. It has been shown previously that the deletion of this exon in the brain leads to a total absence of produced protein (4). No truncated protein was detected using an antibody directed against the N-terminal part of B-Raf protein (4); thus, no dominant negative form of B-Raf protein was formed. We mated these mice with transgenic mice expressing the Cre recombinase under the control of the *Col2a1* promoter (Col2-Cre) to allow the expression of Cre in cartilage (30). Among all offspring analyzed, B-Raf CKO animals (Col2-Cre; *B-raf*^{flox/flox} mice) were obtained at expected Mendelian ratios. B-Raf CKO postnatal animals did not exhibit any gross anatomical abnormalities; they grew to normal size and had a normal life span. To verify that B-Raf protein was efficiently removed from cartilage, we performed a Western blot analysis of primary chondrocytes obtained from WT, CHET, and CKO animals. Figure 2A shows that only traces of B-Raf protein could be detected in CKO animals. Because it is difficult to obtain a pure population of chondrocytes from bones, the remaining B-Raf protein detected in the CKO mice may have resulted from a minority of fibroblasts or osteoblasts trapped during the isolation of chondrocytes. Alternatively, this result may reflect the fact that the *B-raf* allele was not recombined in 100% of the chondrocytes, as is often the case in CKO experiments. In any event, the dramatic reduction in B-Raf levels in cartilage was sufficient to study the role of B-Raf in this tissue.

Alcian blue and alizarin red staining of newborn animals showed no evidence of skeletal abnormalities, as all the bones were formed, had normal sizes, and were mineralized similarly to WT bones (Fig. 2B and C). Histological analyses of both hind limbs and forelimbs of E14.5 to E18.5, newborn, P5, and 2- and 8-week-old animals were performed. In all bones, growth plate chondrocytes were morphologically normal and perfectly organized into the classical layers of round and flat immature chondrocytes and prehypertrophic and hypertrophic chondrocytes (Fig. 2D and data not shown). No difference in the size of each layer in CKO bones compared to WT bones was observed, suggesting that chondrocyte maturation, differentiation, and apoptosis in CKO growth plates were not affected. Consistent with this histological analysis, our ISH analysis showed comparable distributions and levels of mRNA expression for the chondrogenic markers *Col2a1* and *Sox9* (which mark immature proliferative chondrocytes) and *Ihh* and *Col10a1* (which mark, respectively, prehypertrophic and hypertrophic chondrocytes) in CKO growth plate chondrocytes and controls in all bones at all stages analyzed (Fig. 2E and data not shown). These results demonstrate that chondrocyte maturation in CKO growth plates was not affected. Because B-Raf has been shown to negatively regulate apoptosis (49), we

also performed a TUNEL assay on CKO growth plates. We observed low numbers of apoptotic chondrocytes in the hypertrophic layers adjacent to the primary spongiosas of the CKO growth plates, similar to the numbers in control growth plates (data not shown). Lastly, we evaluated the effect of B-Raf on chondrocyte proliferation by performing a BrdU assay on growth plates of E14.5, E15.5, E17.5, and E18.5 embryos. At all stages, we observed no significant difference in BrdU incorporation between control and CKO chondrocytes (Fig. 2F and data not shown). Thus, B-Raf is not necessary for normal chondrocyte proliferation in vivo. Taken together, these results demonstrate that B-Raf is dispensable for normal endochondral bone development.

B-Raf is not required for a PTHrP-induced increase in chondrocyte proliferation and PTHrP-delayed chondrocyte maturation. In spite of the lack of an abnormal skeletal phenotype in CKO animals, we wondered whether B-Raf may have subtle effects in the growth plate that could be revealed in settings in which PTHrP signaling had been ectopically activated and, more generally, whether B-Raf has any link with PTHrP signaling in chondrocytes. To answer these questions, we cultured metatarsal explants obtained from an E15.5 WT mouse embryo in vitro in the presence or absence of PPR agonist PTH(1-34) for 24 h and first looked at *B-raf* mRNA expression by ISH. As previously described for this experimental system (10, 19), PTH(1-34) treatment induced an important delay in chondrocyte maturation, as indicated by the massive reduction of *Ihh* and *Col10a1* expression compared to that in metatarsals treated with vehicle (Fig. 3A). PTH(1-34) also induced chondrocyte proliferation, as indicated by the increase in BrdU incorporation compared to that in the control (Fig. 3A). While B-Raf expression was detected uniformly in metatarsals treated with PTH(1-34), we did not observe any significant increase of *B-raf* mRNA levels (Fig. 3A), indicating that PTHrP signaling does not regulate *B-raf* gene expression. A similar result was obtained in vivo with mice that express a constitutively active form of PPR in chondrocytes (Col2-caPPR) (37; data not shown). We then looked for possible consequences of the lack of B-Raf on PTHrP effects on the maturation and proliferation of chondrocytes by treating metatarsal explants obtained from E15.5 CKO mouse embryos with PTH(1-34). Despite the absence of B-Raf, treated metatarsal explants exhibited a proliferation rate and a delay of chondrocyte maturation similar to those observed in WT metatarsal explants (Fig. 3B). We obtained a similar result in vivo, since Col2-caPPR transgenic mice presented considerable levels of inhibition of chondrocyte hypertrophy and delays of endochondral bone development that were similar in the presence and absence of B-Raf (Fig. 3C; note the absence of hypertrophic chondrocytes and primary spongiosa). Taken together, these data demonstrate that B-Raf alone is not required for PTHrP actions in growth plate chondrocytes.

Conditional knockout of B-Raf in bone does not impair skeletogenesis. Because *B-raf* mRNA was detected in bone in addition to proliferative chondrocytes (Fig. 1; note the robust expression in the primary spongiosa), and because the possible involvement of B-Raf in the PTH/cAMP-induced proliferation of osteoblastic cell lines in vitro has been noted previously (8), we wondered whether the removal of B-Raf from osteoblasts could affect bone formation. To test this possibility, we used an

FIG. 2. Conditional removal of B-Raf from cartilage does not affect skeletogenesis. (A) Analysis of B-Raf expression by Western blot evaluation of primary chondrocytes isolated from newborn WT, CHET, and CKO littermates. Note that B-Raf protein expression is dramatically reduced in CKO chondrocytes compared to WT chondrocytes, whereas the expression of the housekeeping protein β -actin is unchanged. (B) Skeletal preparations (subjected to alizarin red and alcian blue staining) from newborn WT and CKO mice. (C) Blown-up image of skeletal preparations of forelimbs (FL) and hind limbs (HL) of WT and CKO newborn littermates. (D) Histological analysis (hematoxylin and eosin staining) of tibias of newborn WT, CHET, and CKO littermates. Note that the widths of each the proliferative (Pro) and hypertrophic (H) layers are comparable for all genotypes. (E) Nonradioactive ISH analysis of proximal tibias of E16.5 mice of the indicated genotypes, with the indicated chondrogenic markers. Note the similar intensities and distributions in WT, CHET, and CKO mice of *Ihh* and *Col10a1*, which mark prehypertrophic and hypertrophic chondrocytes, respectively. (F) BrdU assay of proximal tibias of E18.5 mice of the indicated genotypes. Note the similar distributions of black BrdU-positive cells in WT and CKO growth plates. The proliferation rates of chondrocytes (the numbers of BrdU-positive cells divided by the total numbers of cells counted, expressed as percentages) in E18.5 WT and CKO growth plates are given. The values indicated correspond to the average of two counts of cells from at least three animals of each genotype. Prehypertrophic and hypertrophic chondrocytes identified histologically were excluded from these counts.

Osx-Cre line that expresses the Cre recombinase under the control of the *Osterix* promoter, which drives Cre expression in early osteoblasts (25, 35). We observed that mice with osteoblasts deficient in B-Raf (Osx-CKO mice) had trabecular and cortical bones comparable to those of WT littermates at all stages analyzed (Fig. 4A and data not shown). The same result was obtained using the type I collagen promoter to drive the expression of Cre in mature osteoblasts (25; data not shown).

We verified that the recombinase worked efficiently in osteoblasts using reporter genes for both types of Cre transgenic mice (data not shown). However, we could not assess specifically the efficiency of B-Raf deletion in osteoblasts. Thus, we

cannot exclude the possibility that enough B-Raf protein remained expressed to fulfill its functions in bone.

We also wondered whether the absence of B-Raf in osteoblasts could influence the effects of the constitutive activation of the PPR in bone. As described previously, mice expressing a constitutively active form of the PPR under the control of the *type I collagen* promoter (Col1-caPPR) exhibit a dramatic increase in trabecular bone volume and reduced cortical thickness (3). In tibias from 2-week-old mice, this phenotype was characterized in part by the presence of trabecula-like bone in the mid-diaphyseal endocortical region and porosity of the cortical bone of transgenic bones, whereas tibias from control

FIG. 3. B-Raf is not required for a PTHrP-induced increase in chondrocyte proliferation and delayed chondrocyte maturation. (A) ISH with the indicated chondrogenic markers and BrdU assay of metatarsal explants obtained from WT E15.5 hind limbs treated with PPR agonist [+PTH(1-34)] at 10^{-7} M or vehicle (control) for 24 h. Note that *B-raf* mRNA expression is not increased upon the activation of PTHrP signaling whereas chondrogenic maturation is repressed and proliferation is increased, as indicated by the repression of *Ihh* and *Col10a1* mRNA expression and the increase of BrdU incorporation, respectively. (B) ISH with the indicated chondrogenic markers and BrdU assay of metatarsal explants obtained from B-Raf CKO E15.5 hind limbs treated with PPR agonist or vehicle (control) for 24 h. Note that chondrogenic maturation is repressed and proliferation is increased upon the activation of PTHrP signaling, in spite of the lack of B-Raf. (C) Histological analysis (hematoxylin and eosin staining) of tibias of E16.5 littermates of the indicated genotypes. Note the tremendous delay of endochondral bone development observed in mice homozygous (transgene/transgene [Tg/Tg]) for Col2-caPPR; the delay is characterized by the extension of the proliferative chondrocyte layer and the loss of the hypertrophic chondrocyte layer, with only a few maturing chondrocytes found on the side of the bone (asterisks). This ca-PPR-induced delay is not affected in the absence of B-Raf.

littermates did not present any trabecula-like bone in the mid-diaphysis and had nonporous cortical bone (Fig. 4B). An equivalent increase in trabecula-like bone and an increase in cortical bone porosity induced by Col1-caPPR in the absence

of B-Raf from osteoblasts were observed (Fig. 4B, Col1-CKO and Col1-caPPR). Taken together, these results indicate that B-Raf is dispensable for bone formation and that B-Raf alone is not responsible for the effects of the PPR in bone.

A-Raf is expressed in immature proliferative chondrocytes, whereas C-Raf is highly expressed in mature hypertrophic chondrocytes. Based on our Western blot analysis, the absence of a detectable mutant phenotype of B-Raf CKO animals cannot be explained simply by the inefficient removal of B-Raf in chondrocytes (Fig. 2A). To try to understand the absence of an abnormal phenotype, we thus looked at the expression of the two other members of the Raf family in the growth plate in order to see whether their presence could compensate for the lack of B-Raf.

Similar to a previous study of chicken tibia in vivo (13), we found that *C-raf* mRNA was highly expressed in hypertrophic chondrocytes in E16.5 WT mouse tibia, but not in immature proliferative chondrocytes, in which only extremely low levels of expression, if any, could be detected (Fig. 5A). Because C-Raf is not expressed where B-Raf is expressed in cartilage, it is very unlikely that C-Raf plays the same role as B-Raf in this tissue. Further, we verified that the *C-raf* gene expression was not ectopically up-regulated in proliferative chondrocytes in the absence of B-Raf. In CKO growth plates, *C-raf* mRNA expression in proliferative chondrocytes was not increased and was strictly identical to that observed in WT animals (Fig. 5A), further suggesting that C-Raf most likely does not compensate for the lack of B-Raf in chondrocytes. We also looked at C-Raf protein expression in WT and CKO primary chondrocytes and verified that the levels of expression were comparable (data not shown).

In contrast to *C-raf* mRNA expression, we found that *A-raf* mRNA expression is restricted to immature proliferative chondrocytes (Fig. 5B). Thus, *A-raf* mRNA expression was remarkably similar to that of *B-raf* mRNA in cartilage. We then wondered whether A-Raf expression was increased in the absence of B-Raf in chondrocytes. We found that, in fact, *A-raf* mRNA and protein were expressed at comparable levels in WT and CKO growth plates (Fig. 5B and C, respectively). Despite the absence of any obvious increase in A-Raf expression, the remarkably similar patterns of expression of A-Raf

FIG. 4. B-Raf conditional knockout in osteoblasts does not affect bone formation or the effects of PPR in bone. (A) Histological analysis of tibias of 2-week-old littermate mice expressing or not expressing B-Raf in bone. Note that mice with an Osterix-Cre-mediated conditional knockout of B-Raf in early osteoblasts (Osx-CKO) present amounts of trabecular and cortical bones (stained in pink) similar to those in control animals. (B) Histological analysis of tibias of 2-week-old littermate mice expressing or not expressing B-Raf in bone in the presence of caPPR in bone. The mid-diaphysis region of a control tibia is characterized by the absence of trabecular bone (no pink) and the presence of a homogenous compact cortical bone. The misexpression of Col1-caPPR in bone in mice hemizygous for the Col1-caPPR transgene (transgene/WT [Tg/+]) dramatically increases bone formation in the trabecular region and forms a porous cortical bone. The phenotype observed in mice expressing the Col1-caPPR transgene but the conditional knockout of B-Raf in osteoblasts (B-raf Col1-CKO) is identical to that observed in the presence of B-Raf (B-Raf Col1-CHET).

FIG. 5. A-Raf is expressed in immature proliferative chondrocytes, and C-Raf is expressed in mature hypertrophic chondrocytes. (A) Radioactive ISH analysis of *C-raf* mRNA expression in proximal tibias of E16.5 WT and CKO animals. Note that *C-raf* mRNA is expressed almost exclusively in mature hypertrophic (H) chondrocytes in the growth plate, with only traces of expression in proliferative chondrocytes. Its expression is unchanged in CKO growth plates that lack B-Raf in chondrocytes. (B) Nonradioactive ISH analysis of A-Raf expression in proximal tibias of E18.5 WT and CKO animals. Note that *A-raf* mRNA expression is restricted to immature proliferative chondrocytes and, thus, A-Raf expression is identical to that of B-Raf. A-Raf expression is not significantly increased in CKO growth plates that lack B-Raf in chondrocytes. Pro, proliferative layer. (C) Western blot analysis of A-Raf protein expression in primary chondrocytes of newborn WT and CKO animals. Note that similar to the level of expression of the housekeeping protein β -actin, the level of A-Raf protein expression is unchanged in CKO chondrocytes that do not express B-Raf.

and B-Raf suggest that A-Raf, unlike C-Raf, may compensate for the lack of B-Raf in cartilage.

Mice with cartilage deficient in both A-Raf and B-Raf have normal endochondral bone development. Because we thought that A-Raf, unlike C-Raf, might compensate for the role of B-Raf in immature proliferative chondrocytes, we analyzed the skeletons of mice with cartilage deficient in both A-Raf and B-Raf. A-Raf KO mice have neurological and intestinal defects and die between days 7 and 21 after birth due to a failure to thrive (32). A-Raf KO mice appear normal at birth but rapidly appear wasted and growth retarded compared to WT littermates only a few days after birth (32; data not shown). Mice with cartilage deficient in both A-Raf and B-Raf (A-Raf KO-B-Raf CKO mice) were generated by mating Col2-Cre; *B-raf*^{fllox/fllox}; *A-raf*^{f^y/X⁺} male mice with *B-raf*^{fllox/fllox}; *A-raf*^{f^y/X⁻}

female mice. Because A-Raf KO animals exhibit growth retardation after birth, likely as a consequence of the spectrum of neurological and intestinal problems, we focused our analysis of the skeleton on developing embryos and newborn animals. Double-KO mice exhibit a normal skeleton at birth and long bones indistinguishable from those of WT littermates (Fig. 6A and B). Our histological analyses of growth plate chondrocytes did not reveal any detectable mutant phenotype at any stage analyzed (Fig. 6C and data not shown). Consistent with this result, ISH showed identical distributions of all chondrogenic markers tested at all stages analyzed in double-KO growth plates and those of WT littermates (Fig. 6D and data not shown). Interestingly, *C-raf* mRNA expression was restricted to hypertrophic chondrocytes and was not up-regulated in A-Raf and B-Raf double-KO chondrocytes (Fig. 6D). This finding indicates that C-Raf was not mobilized in immature proliferative chondrocytes to compensate for the lack of Raf protein in these cells.

Because *A-raf* mRNA, like *B-raf* mRNA, is expressed in both immature chondrocytes and osteoblasts (Fig. 5B), we also analyzed the bone phenotype of mice lacking both A-Raf and B-Raf in osteoblasts. These mice were generated by mating *Osx-Cre*; *B-raf*^{fllox/fllox}; *A-raf*^{f^y/X⁺} male mice with *B-raf*^{fllox/fllox}; *A-raf*^{f^y/X⁻} female mice. We did not observe any significant defect in the histologic appearance of bone in these animals either (data not shown). Taken together, these results demonstrate that A-Raf and B-Raf are dispensable for normal endochondral bone development. They also suggest either that only traces of C-Raf expression can compensate for the lack of Raf molecules in immature proliferative chondrocytes or, alternatively, that the Raf/ERK pathway is not involved in chondrocyte proliferation at all.

ERK activation is associated with hypertrophic chondrocytes and is inhibited by PTHrP signals. In order to evaluate the role of the Raf/ERK pathway in endochondral bone development and to assess its potential function downstream of PTHrP signaling, we looked at ERK activation in WT and Col2-caPPR-expressing chondrocytes by immunohistochemistry analysis using an antibody directed against phospho-ERK. Among WT E17.5 growth plate chondrocytes, activated ERK was detected almost exclusively in hypertrophic chondrocytes (Fig. 7A). Phospho-ERK was not detected in immature proliferative chondrocytes, with the exception of a few chondrocytes located at the articular surface. Further, no signal was detected in immature chondrocytes proliferating even more actively at earlier stages (E15.5 to E16.5) (data not shown). Interestingly, mice hemizygous for the Col2-caPPR transgene presented reduced phospho-ERK staining in hypertrophic chondrocytes and ectopic activation of ERK in immature proliferative chondrocytes (Fig. 7A). ERK activation in proliferative chondrocytes was dose dependent, since mice homozygous for the Col2-caPPR transgene showed greater numbers of immature proliferative chondrocytes positive for phospho-ERK than mice hemizygous for this transgene (Fig. 7A). In homozygous mice, chondrocyte maturation was severely repressed, and thus, not surprisingly, only a weak signal was detected in the few cells that partly escaped the caPPR-induced repression of maturation (Fig. 7A, bottom far-right panel). The same results were obtained with WT and transgenic animals by using an antibody directed against the acti-

FIG. 6. A-Raf-B-Raf double-KO animals present normal endochondral bone development. (A) Skeletal preparations from newborn WT and A-Raf-B-Raf double-KO mice (A-Raf KO-B-Raf CKO mice developed by using Col2-Cre to remove B-Raf from chondrocytes). (B) Detailed view of forelimbs (FL) and hind limbs (HL) of the mice corresponding to the samples presented in panel A. (C) Histological analysis of tibias of newborn WT and A-Raf-B-Raf double-KO littermates. Note the absence of histological differences between samples from the two genotypes. (D) Nonradioactive ISH analysis of proximal tibias of newborn mice of the indicated genotypes with the indicated chondrogenic markers. Note the similar intensities and distributions in WT and double-KO mice of *Ihh* and *Col10a1*, which mark prehypertrophic and hypertrophic chondrocytes, respectively. Note also that *C-raf* mRNA is not up-regulated in double-KO mice and is still restricted to hypertrophic chondrocytes.

vated phosphorylated form of MEK, which acts immediately downstream of Raf and upstream of ERK (data not shown). Collectively, these results show that the endogenous activation of the Raf/ERK pathway in normal mice occurs with the hypertrophic maturation of chondrocytes rather than with proliferation. Moreover, they indicate that whereas the ectopic induction of PPR signals in cartilage partially represses the activation of the Raf/ERK pathway in hypertrophic chondrocytes, the same signals are able to stimulate this pathway in immature proliferative chondrocytes. The physiologic consequences of the activation of ERK in proliferative chondrocytes are unclear.

Despite the absence of an abnormal growth plate phenotype in B-Raf CKO animals, we tested the possibility that the increase in ERK activation in proliferative chondrocytes observed upon the constitutive activation of PPR in cartilage may be mediated in part by B-Raf. The levels of activation of ERK in proliferative chondrocytes expressing B-Raf and those not expressing B-Raf in hemizygous Col2-caPPR mice were in fact comparable (Fig. 7B) ($44\% \pm 11\%$ of proliferative chondrocytes were phospho-ERK positive in the presence of B-Raf and $53\% \pm 5\%$ in the absence of B-Raf; $P = 0.15$). Thus, B-Raf is not necessary for the ERK activation induced by the constitutive activation of PPR in chondrocytes.

In order to assess the requirement for PTHrP signals to elicit ERK activation, we also looked at phospho-ERK expression in PTHrP KO mice. As described previously, these mice present accelerated chondrocyte maturation characterized by the shortening of the growth plate (18) (Fig. 7C). Surprisingly, PTHrP KO growth plates showed a remarkable ectopic activation of ERK in many chondrocytes located in the immature proliferative layer and at the articular surface, in addition to

modest activation in hypertrophic chondrocytes (Fig. 7C). This result demonstrates that PTHrP is not required for ERK activity in cartilage. However, the activation of ERK in immature chondrocytes in the absence of PTHrP is superficially paradoxical, since the constitutively active PPR also leads to ERK activation in proliferative chondrocytes. These findings certainly suggest that changes in the activation of the PPR may have indirect effects on ERK activation. Perhaps, for example, the absence of PTHrP may change the expression of signaling molecules expressed in the prehypertrophic region or change the receptivity or responsiveness of proliferative chondrocytes to certain signals. To test this possibility, we looked at phospho-ERK expression in growth plate chondrocytes that do not express PTHrP but have a compensatory pathway maintaining the proliferation of chondrocytes. The abolition of the cell cycle inhibitor p57 partially inhibits the accelerated maturation of chondrocytes in PTHrP KO mice, as indicated in part by the elongation of the growth plates in PTHrP-p57 double-KO mice compared to those in PTHrP KO mice (19) (Fig. 7C). In these double-KO mice, the pattern of phospho-ERK was similar to that in control littermates (Fig. 7C). Thus, the ectopic activation of ERK observed in PTHrP KO chondrocytes was not due to the reversal of a direct repressive effect of PTHrP but likely reflects changed relationships or receptivity of these cells. This result emphasizes how difficult it is to interpret correctly the causes of particular levels of ERK activation in cartilage, which likely result from the interaction of multiple signals.

The apparent suppression of ERK phosphorylation in hypertrophic chondrocytes in the Col2-caPPR growth plates is not easy to interpret in physiological terms. In Col2-caPPR mice, the constitutively active receptor is expressed at high levels throughout the proliferative layer and the caPPR protein

FIG. 7. ERK activation is independent of A-Raf and B-Raf, is restricted to mature hypertrophic chondrocytes, and is suppressed by PTHrP signals. (A) Immunohistochemistry analysis to detect phospho-ERK (activated ERK) on sections of proximal tibias of E17.5 WT mice, mice hemizygous for a transgene encoding a constitutively active version of PPR in cartilage (Col2-caPPR transgene/WT [Tg/+]), or mice homozygous for this transgene (Col2-caPPR Tg/Tg). The rectangles in blue and in red correspond to the regions of proliferative (Pro) and hypertrophic (H) chondrocytes, respectively, which are shown at higher magnification in the middle and lower panels, respectively. Note that in WT growth plates, phospho-ERK is detected essentially in mature hypertrophic chondrocytes, whereas ERK is activated in a dose-dependent manner in proliferative chondrocytes of transgenic animals. Note also that the signal appears weaker in hypertrophic chondrocytes of transgenic animals than in those of wild-type animals. In homozygous transgenic mice, only a few mature chondrocytes are present on one side of the tibia and these cells present only a weak phospho-ERK signal. (B) Immunohistochemistry analysis for the detection of phospho-ERK on sections of proximal tibias of E18.5 control mice or mice hemizygous (Tg/+) for the Col2-caPPR transgene and expressing B-Raf (B-raf WT) or not (B-raf CKO) in cartilage (Col2-Cre-mediated conditional knockout). Note that ERK is found activated in transgenic animals even in the absence of B-Raf in chondrocytes. The rectangles correspond to the regions of the growth plates shown at higher magnification in the lower panels. (C) Immunohistochemistry analysis for the detection of phospho-ERK on sections of metatarsal explants obtained from WT E15.5 hind limbs treated with PPR agonist [1-34] or vehicle (control) for the indicated times. A higher-magnification image of the hypertrophic chondrocytes is shown for each condition. Note that PTHrP completely abolishes ERK activation after 1 h of treatment. This effect is transient, since some phospho-ERK signal is detected after 2 h of treatment. Explants treated for 24 h do not undergo chondrogenic maturation and thus do not present any phospho-ERK signal. (D) Immunohistochemistry analysis for the detection of phospho-ERK on sections of proximal tibias from newborn WT and A-Raf-B-Raf double-KO mice (A-Raf KO-B-Raf CKO mice developed by using Col2-Cre to remove B-Raf from chondrocytes). Note that the activation of ERK is not only restricted to hypertrophic chondrocytes but also independent of A-Raf and B-Raf.

may continue to be active in the hypertrophic chondrocytes. This pattern of PPR activation is quite different from the normal situation, in which PPR is expressed at low levels in the proliferative layer and at high levels only in prehypertrophic and early hypertrophic chondrocytes (18, 45). Moreover, similar to what we saw in PTHrP KO chondrocytes, the ectopic activation of ERK in Col2-caPPR growth plates may result not from a direct action of PPR but in fact from complicated indirect non-cell-autonomous effects that are likely generated upon the widespread activation of PPR signaling. To study PPR activation in the setting of normal PPR expression, we treated metatarsal explants briefly with PTHrP. PTHrP treatment of metatarsals for 1 h completely suppressed ERK activation in hypertrophic chondrocytes but did not activate ERK in immature proliferative chondrocytes (Fig. 7D). The repres-

sion of ERK activation in hypertrophic chondrocytes was only transient, since hypertrophic chondrocytes showed partial recovery of the phospho-ERK signal after 2 h of treatment (Fig. 7D). After 24 h, no phospho-ERK signal was detected at all, consistent with the suppression of chondrocyte maturation over this time period (Fig. 7D). Thus, these results support the idea that PPR downstream signals do not stimulate ERK activity *in vivo* but rather may repress it, concomitantly with the repression of chondrocyte maturation.

The preponderant presence of activated ERK in hypertrophic chondrocytes suggests that the activation of ERK is independent of *A-raf* and *B-raf*, both expressed in immature proliferative chondrocytes. We verified this possibility by looking at phospho-ERK in growth plate chondrocytes lacking both A-Raf (A-Raf KO) and B-Raf (Col2-CKO) (Fig.

FIG. 8. Suppression of ERK activation delays hypertrophic chondrocyte maturation. (A) Metatarsals from E15.5 WT hind limbs were treated with the MEK1-MEK2 inhibitor U0126 or vehicle for the indicated time and photographed while in culture. The dark region visible at day 5 in the vehicle-treated metatarsal preparations corresponds to mineralized matrix (opaque to light) produced by mature chondrocytes. Note that U0126-treated metatarsals do not present any mineralized region, even after 6 days in culture. (B) E15.5 WT metatarsals treated for 2 or 6 days with U0126 or vehicle were processed for ISH with markers that cover different stages of chondrocyte maturation. The chondrogenic markers *Ihh*, *Col10a1*, and *OP* mark prehypertrophic, hypertrophic, and late-hypertrophic chondrocytes, respectively. Note that *Ihh* expression is affected only mildly after 6 days of treatment, whereas that of *Col10a1* and *OP* is severely suppressed. Adjacent sections were subjected to immunohistochemistry analysis for phospho-histone H3 (P-H3; a mitotic marker) and phospho-ERK (P-ERK). Note that the numbers of mitotic cells in U0126- and vehicle-treated samples were comparable, although ERK activation was efficiently suppressed. A blown-up image of the regions of hypertrophic chondrocytes is shown for phospho-ERK signals.

7E). The activity of ERK was identical in WT and double-KO growth plate chondrocytes, demonstrating that A-Raf and B-Raf are not required for ERK activation in these cells. This result strongly suggests that C-Raf may play an important role in regulating ERK activation in hypertrophic chondrocytes.

Suppression of ERK activation delays chondrocyte maturation in bone explants. The fact that PTHrP signals repress ERK activation, along with chondrocyte maturation, is consistent with the idea that ERK activation in growth plate chondrocytes may favor chondrocyte maturation rather than proliferation. To test this possibility, we treated metatarsal explants with the MEK1-MEK2 inhibitor U0126, which has been used extensively and successfully in different systems to suppress specifically ERK1 and ERK2 activation. Bone explants cultured over a period of 6 days normally grow longitudinally, and chondrocytes mature progressively such that they produce a mineralized matrix easily detectable under a microscope as a dark area opaque to light. Metatarsals treated with vehicle exhibited a mineralized matrix already after 5 days in culture, whereas those treated with U0126 did not present any mineralized region even after 6 days in culture (Fig. 8A). This finding indicates that U0126 induced an important delay of chondrocyte maturation. To confirm this result, we performed ISH on sections of metatarsal explants treated with U0126 or vehicle to look at the expression of *Ihh*, *Col10a1*, and *OP*, which, respectively, mark prehypertrophic, hypertrophic, and late-hypertrophic chondrocytes and thus span all the different steps of chondrocyte maturation. U0126 treatment for 2 days did not significantly affect *Ihh* mRNA expression but strongly re-

pressed *Col10a1* and completely suppressed *OP* mRNA expression (Fig. 8B). These observations indicate that ERK activation is required for chondrocyte maturation and that it ensures the transition both between prehypertrophic and hypertrophic chondrocytes and between hypertrophic and late-hypertrophic chondrocytes. Conversely, the transition from immature to prehypertrophic chondrocytes did not seem to be affected by 2 days of U0126 treatment. This result is consistent with activated ERKs being restricted essentially to prehypertrophic and hypertrophic chondrocytes. ISH analysis of samples treated for 6 days confirmed the dramatic delay in chondrocyte maturation, as indicated by the reduction of the length between the domains of *Col2a1* and *Ihh* mRNA expression and the striking suppression of *Col10a1* and *OP* mRNA expression (Fig. 8B). Conversely, immunohistochemistry analysis for phospho-histone H3 (a mitotic marker) revealed that U0126 did not affect chondrocyte proliferation after 2 days and even 6 days of treatment (Fig. 8B). After 2 days of treatment, $5.3\% \pm 0.7\%$ of cells in the proliferative layer of U0126-treated bones were mitotic, a value comparable to the $5.7\% \pm 1.3\%$ found in vehicle-treated samples ($P = 0.29$). After 6 days of treatment, $4.8\% \pm 1.8\%$ of cells in U0126-treated bones were mitotic, a value not statistically different from the $5.2\% \pm 0.6\%$ found for vehicle-treated samples ($P = 0.35$). We verified that U0126 efficiently suppressed ERK activation in analyzed metatarsals: phospho-ERK signals detected in *Col10a1*-expressing cells in vehicle-treated samples completely disappeared upon U0126 treatment (Fig. 8B). Taken together, these results strongly suggest that the physiological role of ERK in growth

plate chondrocytes is primarily to stimulate chondrocyte maturation.

DISCUSSION

In this study, we initially assessed the role of the proto-oncogene *B-raf* in endochondral bone development and tested the possibility that it may play a role in PTHrP signaling in inducing chondrocyte proliferation. We analyzed the tissue distributions of the expression of all three *raf* genes in growth plate chondrocytes *in vivo* and showed for the first time that *A-raf* and *B-raf* genes are similarly expressed in immature proliferative chondrocytes whereas *C-raf* is expressed only in mature hypertrophic chondrocytes. In spite of the expression of B-Raf in chondrocytes, we demonstrated that the removal of B-Raf from these cells did not affect endochondral bone development and that B-Raf alone was not required for PTHrP-induced chondrocyte proliferation. Because A-Raf and B-Raf are similarly expressed and thus may compensate for each other in chondrocytes, we evaluated endochondral bone development in the absence of both A-Raf and B-Raf. Surprisingly, we did not observe any histological abnormalities in double-KO growth plates, which also exhibited patterns of *C-raf* mRNA expression and ERK activation identical to those of WT growth plates. Lastly, we observed that the suppression of ERK activation in hypertrophic chondrocytes by PTHrP signaling or by a specific inhibitor of MEK is associated with the repression of chondrocyte maturation. Taken together, these results demonstrate that A-Raf and B-Raf are dispensable for normal endochondral bone development, and these findings are consistent with the idea that Raf signaling in chondrocytes may principally regulate hypertrophic chondrocyte maturation through the action of C-Raf.

PTHrP and cAMP/PKA signaling in proliferative chondrocytes. The hypothesis that B-Raf may mediate the proliferative actions of the PPR originated from a model described previously in which the cAMP/PKA pathway induces cell proliferation in the presence, but not in the absence, of B-Raf in many cell types (43). In this model, cAMP/PKA activates the small GTPase Rap1, which is capable of binding to and activating B-Raf, whereas Rap1 binds to and inactivates C-Raf. The consequence of this distinction is that only in B-Raf-positive cells is ERK activated and cell proliferation induced in response to cAMP generation. However, the extent of the involvement of Rap1 in the cAMP-induced activation of B-Raf and ERK remains controversial, since this role for Rap1 is not observed in all cell types and in all settings (7, 31). Interestingly, an *in vitro* study revealed that the capacity of PTH and cAMP to activate ERKs and to induce bone cell lines to proliferate depends on the presence or the absence of B-Raf (8). In these cells, the effect of cAMP is dependent on Rap1. It was thus interesting to test the validity of this model *in vivo* for chondrocytes, cells that are thought to derive from the same osteochondroprogenitor as osteoblasts. Because much of PTHrP action in growth plate chondrocytes is mediated by the activation of cAMP production through the activation of Gs α (1, 10), we hypothesized that PTHrP might lead to a B-Raf-mediated increase in chondrocyte proliferation. We demonstrate here that, in fact, B-Raf is not required for PTHrP induction of chondrocyte proliferation. More broadly, we

present evidence that B-Raf is dispensable for both cartilage and bone formation and that the lack of B-Raf has no detectable effect on the actions of PPR in cartilage and bone. For cartilage, the results suggest either that the PTHrP/cAMP/PKA pathway induces chondrocyte proliferation through the activation of ERK independently of B-Raf or that PTHrP-induced chondrocyte proliferation is completely independent of ERK activation. We tested the first possibility by removing A-Raf from cartilage, *A-raf* being the only gene significantly expressed in proliferative chondrocytes that may compensate for the lack of B-Raf. However, mice deficient in A-Raf and B-Raf still exhibited normal cartilage and bone formation, indicating that either very little C-Raf expression is sufficient to ensure ERK activation or, more likely, that ERK activation is not required for PTHrP-induced chondrocyte proliferation. Interestingly, we observed that the ectopic expression of a constitutively active form of PPR in cartilage activated ERK in proliferative chondrocytes *in vivo*. However, this result was not reproduced *in vitro* when PTHrP was added to cultured metatarsals. This contrast suggests that the action of the caPPR *in vivo* reflects either the unusually high number of activated PPRs in chondrocytes in that model or indirect consequences of complex, non-cell-autonomous effects of the activated PPR unrelated to cell proliferation. The latter possibility is supported by the observation of ectopic ERK activation in PTHrP KO mice and by the fact that ERK most likely does not play any role in chondrocyte proliferation (see below). Whereas it is improbable that PTHrP signals normally activate ERK in proliferative chondrocytes, our results are consistent with the idea that PPR activation represses ERK activation in prehypertrophic and hypertrophic chondrocytes. Indeed, ERK activation is increased *in vivo* in these cells in the absence of PTHrP signaling and is decreased both *in vivo* and *in vitro* when PTHrP signaling is activated. A similar result has been obtained *in vitro* using a differentiated osteoblastic MC4 cell line (5). Interestingly, however, ERK activation is increased in undifferentiated MC4 cells by PTHrP, indicating that PTHrP can mediate opposing effects, depending on the differentiation states of the cells, at least *in vitro* (5). The fact that PTHrP activates ERK in undifferentiated MC4 cells and other undifferentiated osteoblastic cell lines (5, 8), but not in immature chondrocytes present in metatarsals cultured *in vitro*, likely reflects the tissue specificity of PTHrP effects. Taken together, our results argue against the implication of the Raf/ERK signaling pathway in PTHrP-induced chondrocyte proliferation.

Role of the Raf/MEK/ERK pathway in endochondral bone development. A-Raf, B-Raf, and C-Raf are the three MAPK kinase kinases that lead to ERK activation. Both B-Raf (47, 49) and C-Raf (12, 22, 47) KO mice die around midgestation, preventing the analysis of their skeletons. Mutant mice that express a truncated form of C-Raf with no dominant negative activity but with kinase activity reduced by 90% compared to that of WT C-Raf have been generated, and some mutant embryos have survived until E18.5 (48). Interestingly, these mice present some growth retardation and a delay in bone mineralization. However, it is not clear whether the growth retardation reflects a role for C-Raf in endochondral ossification or if it is the indirect consequence of placental defects. A-Raf KO mice die in the first 2 weeks after birth, most likely as a consequence of neurological and gastrointestinal problems

(32). KO mice appear smaller than WT mice only 2 to 3 days after birth but are born normal, suggesting that A-Raf alone does not play a major role in endochondral bone development. We show here that B-Raf is specifically expressed in immature proliferative chondrocytes but is dispensable in endochondral bone development. Both the proliferation rates and the hypertrophic chondrocyte differentiation patterns were identical in CKO and WT animals at all stages analyzed. These results are consistent with the recent demonstration that B-Raf plays a major role in placental development but that it is dispensable for embryonic development (9). We also observed the expression of *B-raf* in bone (primary spongiosa), suggesting that this gene may play a role in this tissue. Interestingly, several mutations of *B-raf* which lead to cardiofaciocutaneous syndrome in humans have been found recently (27, 36). This syndrome is characterized by heart defects, mental retardation, and facial characteristics suggesting growth abnormalities of the bones of the face. Thus, it was conceivable that B-Raf might play a more important role in bone than in cartilage. However, our results show that the conditional removal of B-Raf in bone does not lead to any detectable phenotype.

We initially hypothesized that the lack of a detectable mutant phenotype in cartilage of B-Raf CKO animals likely resulted from genetic redundancies. The targeted disruption of *raf* genes in mice demonstrated that the functions of these genes are not fully redundant since null mutations of each gene resulted in distinct phenotypes; yet, many tissues express all three *raf* genes and are not affected when only one is deleted. Moreover, the double knockout of *B-raf* and *C-raf* clearly shows that these two genes have redundant functions during early gestation (47). We observed that the expression pattern of *A-raf* mRNA was similar to that of *B-raf* mRNA in immature proliferative chondrocytes whereas *C-raf* mRNA expression was restricted to hypertrophic chondrocytes. Thus, the expression pattern of *C-raf* mRNA does not suggest that C-Raf functions redundantly with B-Raf in chondrocytes. Conversely, because the pattern of *A-raf* mRNA expression in cartilage was very similar to that of *B-raf* mRNA expression, we speculated that the A-Raf and B-Raf may play redundant roles in this tissue. However, our study showed that endochondral bone development was not affected after the removal of A-Raf and B-Raf and that no phospho-ERK (or phospho-MEK) signal was detected in proliferative chondrocytes of WT growth plates. These findings suggest that the Raf/ERK pathway may be dispensable for chondrocyte proliferation. Alternatively, it is possible that only traces of C-Raf are necessary to ensure ERK activation and that ERK activation in proliferative chondrocytes is too transient and too weak to be detected with phospho-ERK antiserum, but this possibility seems less likely. The fact that metatarsal explants treated with a MEK inhibitor presented numbers of mitotic cells similar to those presented by untreated explants argues against a role of ERK in the proliferation of growth plate chondrocytes. Conversely, the rather strong phospho-ERK and phospho-MEK signals detected in hypertrophic chondrocytes suggest a role for the Raf/ERK pathway in hypertrophic maturation. The suppression of these signals upon the activation of PTHrP signaling, concomitant with the inhibition of chondrocyte maturation, raises the possibility that the ERK pathway stimulates hypertrophic differentiation. In fact, the dramatic delay in chondro-

cyte maturation observed with metatarsal explants treated with a MEK inhibitor indicates that this is the case.

The role of MAPK pathways in endochondral bone development has been studied mostly in vitro with established cell lines by using pharmacological inhibitors. It is perhaps not surprising that some studies report a positive, and others a negative, action of ERK on chondrogenesis (reviewed in reference 42). These differences likely result from the diversity of the systems used, including the cell type utilized; the nature, duration, and intensity of the upstream signals; and other factors. Interestingly, however, ERK activation has been implicated in the growth arrest of a chondrogenic cell line induced by fibroblast growth factor in vitro (34). This result is consistent with our data suggesting that the Raf/ERK pathway does not induce chondrocyte proliferation.

Only a few publications have analyzed possible roles of the ERK pathway in endochondral bone development in vivo. The misexpression in transgenic mice of a constitutively active form of MEK1 (caMEK), which specifically activates ERK in chondrocytes, indicates that ERK activation does not affect chondrocyte proliferation but delays hypertrophic chondrocyte differentiation and leads to a dwarf phenotype similar to that in achondroplasia (23). Interestingly, caMEK misexpression can abolish the fibroblast growth factor receptor 3 (FGFR3) null mouse phenotype, suggesting that ERK acts downstream of FGFR3 in chondrocytes. This result is particularly interesting since it suggests that achondroplasia, a disease caused by activating mutations in FGFR3 (26, 29), may result in part from nonphysiological activation of ERK in chondrocytes. This idea is supported by the fact that the misexpression of C-type natriuretic peptide in chondrocytes in transgenic mice represses ERK activity and partly abolishes the skeletal defects of mice with achondroplasia mutations in FGFR3 (50). It is interesting that these findings from gain-of-function studies are partly consistent with our results, since none of the studies revealed any effect of ERK activation or repression on chondrocyte proliferation and since, in fact, both studies suggest a role for the ERK pathway in the control of hypertrophic differentiation. However, our results indicate that ERK activation stimulates chondrocyte maturation, which contrasts with the delay in endochondral bone development observed in mice misexpressing caMEK. This apparent discrepancy may be explained by the different nature of the two approaches employed. In one case, ERK was ectopically activated throughout the entire growth plate, including cells in which it may not normally be activated, and this activation may have led to nonphysiological effects. Although these effects led to important findings regarding achondroplasia and FGFR3 signaling in chondrocytes, the study did not directly address the role of endogenous ERK activation in hypertrophic chondrocytes. In the other case, endogenous ERK activation was suppressed acutely, revealing theoretically the physiological consequences of this activation. This study was limited, however, by the in vitro nature of the experiment and by the action of the ERK inhibitor on both chondrocytes and perichondrial cells; it is possible that changes in ERK signaling in the perichondrium influenced chondrocyte maturation. Although our data indicate that ERK activation stimulates chondrocyte maturation, it would be important to confirm this result by in vivo loss-of-function experiments. We showed that *C-raf* mRNA was very strongly expressed in ma-

ture hypertrophic chondrocytes. It is thus possible that C-Raf may be responsible for ERK activation in endochondral development and that this activation may fulfill an important function in the hypertrophic differentiation of chondrocytes.

If our observations suggest that the endogenous activation of ERK favors hypertrophic differentiation, the fact that the suppression of ERK activation by either a MEK inhibitor or PTHrP signals is associated with the inhibition of chondrocyte maturation also raises the possibility that PTHrP represses chondrocyte hypertrophy perhaps partly by suppressing ERK activation. A possible mechanism may be via the sequestration of C-Raf upon the activation of the cAMP/PKA pathway (43). Alternatively, PTHrP may delay differentiation by mechanisms that have nothing to do with the suppression of ERK activation. The conditional knockout of either C-Raf or ERKs in hypertrophic chondrocytes could test these interesting possibilities.

ACKNOWLEDGMENTS

We thank Manuella Baccarini, Alain Eyche, Catrin Pritchard, and Thomas Gardella for the kind gift of either experimental reagents or DNA constructs. We thank Richard Behringer and Catrin Pritchard for providing us with Col2-Cre mice and A-Raf KO mice, respectively. Lastly, we thank Philip Stork, Catrin Pritchard, and Alain Eyche for helpful discussions.

This work was supported by an NIH grant to H.M.K. (grant number DK56246) and by an NIH grant to Z. Werb (grant number AR046238).

REFERENCES

- Bastepe, M., L. S. Weinstein, N. Ogata, H. Kawaguchi, H. Juppner, H. M. Kronenberg, and U. I. Chung. 2004. Stimulatory G protein directly regulates hypertrophic differentiation of growth plate cartilage in vivo. *Proc. Natl. Acad. Sci. USA* **101**:14794–14799.
- Benkhalifa, S., S. Provot, E. Nabais, A. Eyche, G. Calothy, and M. P. Felder-Schmittbuhl. 2001. Phosphorylation of MafA is essential for its transcriptional and biological properties. *Mol. Cell. Biol.* **21**:4441–4452.
- Calvi, L. M., N. A. Sims, J. L. Hunzelman, M. C. Knight, A. Giovannetti, J. M. Saxton, H. M. Kronenberg, R. Baron, and E. Schipani. 2001. Activated parathyroid hormone/parathyroid hormone-related protein receptor in osteoblastic cells differentially affects cortical and trabecular bone. *J. Clin. Invest.* **107**:277–286.
- Chen, A. P., M. Ohno, K. P. Giese, R. Kuhn, R. L. Chen, and A. J. Silva. 2006. Forebrain-specific knockout of B-raf kinase leads to deficits in hippocampal long-term potentiation, learning, and memory. *J. Neurosci. Res.* **83**:28–38.
- Chen, C., A. J. Koh, N. S. Datta, J. Zhang, E. T. Keller, G. Xiao, R. T. Franceschi, N. J. D'Silva, and L. K. McCauley. 2004. Impact of the mitogen-activated protein kinase pathway on parathyroid hormone-related protein actions in osteoblasts. *J. Biol. Chem.* **279**:29121–29129.
- Chung, U. I., B. Lanske, K. Lee, E. Li, and H. Kronenberg. 1998. The parathyroid hormone/parathyroid hormone-related peptide receptor coordinates endochondral bone development by directly controlling chondrocyte differentiation. *Proc. Natl. Acad. Sci. USA* **95**:13030–13035.
- Enserink, J. M., A. E. Christensen, J. de Rooij, M. van Triest, F. Schwede, H. G. Genieser, S. O. Doskeland, J. L. Blank, and J. L. Bos. 2002. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nat. Cell Biol.* **4**:901–906.
- Fujita, T., T. Meguro, R. Fukuyama, H. Nakamuta, and M. Koida. 2002. New signaling pathway for parathyroid hormone and cyclic AMP action on extracellular-regulated kinase and cell proliferation in bone cells. Checkpoint of modulation by cyclic AMP. *J. Biol. Chem.* **277**:22191–22200.
- Galabova-Kovacs, G., D. Matzen, D. Piazzolla, K. Meissl, T. Plyushch, A. P. Chen, A. Silva, and M. Baccarini. 2006. Essential role of B-Raf in ERK activation during extraembryonic development. *Proc. Natl. Acad. Sci. USA* **103**:1325–1330.
- Guo, J., U. I. Chung, H. Kondo, F. R. Bringhurst, and H. M. Kronenberg. 2002. The PTH/PTHrP receptor can delay chondrocyte hypertrophy in vivo without activating phospholipase C. *Dev. Cell* **3**:183–194.
- Haaijman, A., M. Karperien, B. Lanske, J. Hendriks, C. W. Lowik, A. L. Bronckers, and E. H. Burger. 1999. Inhibition of terminal chondrocyte differentiation by bone morphogenetic protein 7 (OP-1) in vitro depends on the periarticular region but is independent of parathyroid hormone-related peptide. *Bone* **25**:397–404.
- Huser, M., J. Luckett, A. Chiloehes, K. Mercer, M. Iwobi, S. Gillelt, X. M. Sun, J. Brown, R. Marais, and C. Pritchard. 2001. MEK kinase activity is not necessary for Raf-1 function. *EMBO J.* **20**:1940–1951.
- Kaneko, Y., H. Tanzawa, and K. Sato. 1994. The proto-oncogene C-raf-1 is highly expressed only in the hypertrophic zone of the growth plate. *Calcif. Tissue Int.* **54**:426–430.
- Karaplis, A. C., A. Luz, J. Glowacki, R. T. Bronson, V. L. Tybulewicz, H. M. Kronenberg, and R. C. Mulligan. 1994. Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev.* **8**:277–289.
- Karp, S. J., E. Schipani, B. St.-Jacques, J. Hunzelman, H. Kronenberg, and A. P. McMahon. 2000. Indian hedgehog coordinates endochondral bone growth and morphogenesis via parathyroid hormone related-protein-dependent and -independent pathways. *Development* **127**:543–548.
- Karsenty, G. 2003. The complexities of skeletal biology. *Nature* **423**:316–318.
- Kronenberg, H. M. 2003. Developmental regulation of the growth plate. *Nature* **423**:332–336.
- Lanske, B., A. C. Karaplis, K. Lee, A. Luz, A. Vortkamp, A. Pirro, M. Karperien, L. H. Defize, C. Ho, R. C. Mulligan, A. B. Abou-Samra, H. Juppner, G. V. Segre, and H. M. Kronenberg. 1996. PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* **273**:663–666.
- MacLean, H. E., J. Guo, M. C. Knight, P. Zhang, D. Cobrinik, and H. M. Kronenberg. 2004. The cyclin-dependent kinase inhibitor p57(Kip2) mediates proliferative actions of PTHrP in chondrocytes. *J. Clin. Invest.* **113**:1334–1343.
- Mercer, K., A. Chiloehes, M. Huser, M. Kiernan, R. Marais, and C. Pritchard. 2002. ERK signalling and oncogene transformation are not impaired in cells lacking A-Raf. *Oncogene* **21**:347–355.
- Miao, D., B. He, Y. Jiang, T. Kobayashi, M. A. Soroceanu, J. Zhao, H. Su, X. Tong, N. Amizuka, A. Gupta, H. K. Genant, H. M. Kronenberg, D. Goltzman, and A. C. Karaplis. 2005. Osteoblast-derived PTHrP is a potent endogenous bone anabolic agent that modifies the therapeutic efficacy of administered PTH 1–34. *J. Clin. Invest.* **115**:2402–2411.
- Mikula, M., M. Schreiber, Z. Husak, L. Kucerova, J. Ruth, R. Wieser, K. Zatloukal, H. Beug, E. F. Wagner, and M. Baccarini. 2001. Embryonic lethality and fetal liver apoptosis in mice lacking the c-raf-1 gene. *EMBO J.* **20**:1952–1962.
- Murakami, S., G. Balmes, S. McKinney, Z. Zhang, D. Givol, and B. de Crombrughe. 2004. Constitutive activation of MEK1 in chondrocytes causes Stat1-independent achondroplasia-like dwarfism and rescues the Fgfr3-deficient mouse phenotype. *Genes Dev.* **18**:290–305.
- Murtaugh, L. C., L. Zeng, J. H. Chyung, and A. B. Lassar. 2001. The chick transcriptional repressor Nkx3.2 acts downstream of Shh to promote BMP-dependent axial chondrogenesis. *Dev. Cell* **1**:411–422.
- Nakashima, K., X. Zhou, G. Kunkel, Z. Zhang, J. M. Deng, R. R. Behringer, and B. de Crombrughe. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* **108**:17–29.
- Naski, M. C., J. S. Colvin, J. D. Coffin, and D. M. Ornitz. 1998. Repression of hedgehog signaling and BMP4 expression in growth plate cartilage by fibroblast growth factor receptor 3. *Development* **125**:4977–4988.
- Niihori, T., Y. Aoki, Y. Narumi, G. Neri, H. Cave, A. Verloes, N. Okamoto, R. C. Hennekam, G. Gillesen-Kaesbach, D. Wiczorek, M. I. Kavamura, K. Kurosawa, H. Ohashi, L. Wilson, D. Heron, D. Bonneau, G. Corona, T. Kaname, K. Naritomi, C. Baumann, N. Matsumoto, K. Kato, S. Kure, and Y. Matsubara. 2006. Germline KRAS and BRAF mutations in cardio-facio-cutaneous syndrome. *Nat. Genet.* **38**:294–296.
- Oh, C.-D., S.-H. Chang, Y.-M. Yoon, S.-J. Lee, Y.-S. Lee, S.-S. Kang, and J.-S. Chun. 2000. Opposing role of mitogen-activated protein kinase subtypes, Erk1/2 and p38, in the regulation of chondrogenesis of mesenchymes. *J. Biol. Chem.* **275**:5613–5619.
- Ornitz, D. M. 2005. FGF signaling in the developing endochondral skeleton. *Cytokine Growth Factor Rev.* **16**:205–213.
- Ovchinnikov, D. A., J. M. Deng, G. Ogunrinu, and R. R. Behringer. 2000. Col2a1-directed expression of Cre recombinase in differentiating chondrocytes in transgenic mice. *Genesis* **26**:145–146.
- Peyssonnaud, C., and A. Eyche. 2001. The Raf/MEK/ERK pathway: new concepts of activation. *Biol. Cell* **93**:53–62.
- Pritchard, C. A., L. Bolin, R. Slattery, R. Murray, and M. McMahon. 1996. Post-natal lethality and neurological and gastrointestinal defects in mice with targeted disruption of the A-Raf protein kinase gene. *Curr. Biol.* **6**:614–617.
- Provot, S., and E. Schipani. 2005. Molecular mechanisms of endochondral bone development. *Biochem. Biophys. Res. Commun.* **328**:658–665.
- Rauci, A., E. Laplantine, A. Mansukhani, and C. Basilio. 2004. Activation of the ERK1/2 and p38 mitogen-activated protein kinase pathways mediates fibroblast growth factor-induced growth arrest of chondrocytes. *J. Biol. Chem.* **279**:1747–1756.
- Rodda, S. J., and A. P. McMahon. 2006. Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development* **133**:3231–3244.
- Rodriguez-Viciano, P., O. Tetsu, W. E. Tidyman, A. L. Estep, B. A. Conger,

- M. S. Cruz, F. McCormick, and K. A. Rauen. 2006. Germline mutations in genes within the MAPK pathway cause cardio-facio-cutaneous syndrome. *Science* **311**:1287–1290.
37. Schipani, E., B. Lanske, J. Hunzelman, A. Luz, C. S. Kovacs, K. Lee, A. Pirro, H. M. Kronenberg, and H. Juppner. 1997. Targeted expression of constitutively active receptors for parathyroid hormone and parathyroid hormone-related peptide delays endochondral bone formation and rescues mice that lack parathyroid hormone-related peptide. *Proc. Natl. Acad. Sci. USA* **94**:13689–13694.
38. Schipani, E., and S. Provot. 2003. PTHrP, PTH, and the PTH/PTHrP receptor in endochondral bone development. *Birth Defects Res. C* **69**:352–362.
39. Schipani, E., H. E. Ryan, S. Didrickson, T. Kobayashi, M. Knight, and R. S. Johnson. 2001. Hypoxia in cartilage: HIF-1 α is essential for chondrocyte growth arrest and survival. *Genes Dev.* **15**:2865–2876.
40. Shimoaka, T., T. Ogasawara, A. Yonamine, D. Chikazu, H. Kawano, K. Nakamuura, N. Itoh, and H. Kawaguchi. 2002. Regulation of osteoblast, chondrocyte, and osteoclast functions by fibroblast growth factor (FGF)-18 in comparison with FGF-2 and FGF-10. *J. Biol. Chem.* **277**:7493–7500.
41. Singh, A. T., A. Gilchrist, T. Voyno-Yasenetskaya, J. M. Radeff-Huang, and P. H. Stern. 2005. G α 12/G α 13 subunits of heterotrimeric G proteins mediate parathyroid hormone activation of phospholipase D in UMR-106 osteoblastic cells. *Endocrinology* **146**:2171–2175.
42. Stanton, L. A., T. M. Underhill, and F. Beier. 2003. MAP kinases in chondrocyte differentiation. *Dev. Biol.* **263**:165–175.
43. Stork, P. J., and J. M. Schmitt. 2002. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends Cell Biol.* **12**:258–266.
44. Swarthout, J. T., R. C. D'Alonzo, N. Selvamurugan, and N. C. Partridge. 2002. Parathyroid hormone-dependent signaling pathways regulating genes in bone cells. *Gene* **282**:1–17.
45. Vortkamp, A., K. Lee, B. Lanske, G. V. Segre, H. M. Kronenberg, and C. J. Tabin. 1996. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* **273**:613–622.
46. Weir, E. C., W. M. Philbrick, M. Amling, L. A. Neff, R. Baron, and A. E. Broadus. 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.
47. Wojnowski, L., L. F. Stancato, A. C. Larner, U. R. Rapp, and A. Zimmer. 2000. Overlapping and specific functions of Raf and Craf-1 proto-oncogenes during mouse embryogenesis. *Mech. Dev.* **91**:97–104.
48. Wojnowski, L., L. F. Stancato, A. M. Zimmer, H. Hahn, T. W. Beck, A. C. Larner, U. R. Rapp, and A. Zimmer. 1998. Craf-1 protein kinase is essential for mouse development. *Mech. Dev.* **76**:141–149.
49. Wojnowski, L., A. M. Zimmer, T. W. Beck, H. Hahn, R. Bernal, U. R. Rapp, and A. Zimmer. 1997. Endothelial apoptosis in Raf-deficient mice. *Nat. Genet.* **16**:293–297.
50. Yasoda, A., Y. Komatsu, H. Chusho, T. Miyazawa, A. Ozasa, M. Miura, T. Kurihara, T. Rogi, S. Tanaka, M. Suda, N. Tamura, Y. Ogawa, and K. Nakao. 2004. Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway. *Nat. Med.* **10**:80–86.
51. Yosimichi, G., T. Nakanishi, T. Nishida, T. Hattori, T. Takano-Yamamoto, and M. Takigawa. 2001. CTGF/Hcs24 induces chondrocyte differentiation through a p38 mitogen-activated protein kinase (p38MAPK), and proliferation through a p44/42 MAPK/extracellular-signal regulated kinase (ERK). *Eur. J. Biochem.* **268**:6058–6065.