

The Ras-GTPase activity of neurofibromin restrains ERK-dependent FGFR signaling during endochondral bone formation

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The severe defects in growth plate development caused by chondrocyte extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) gain or loss-of-function suggest that tight spatial and temporal regulation of mitogen-activated protein kinase signaling is necessary to achieve harmonious growth plate elongation and structure. We provide here evidence that neurofibromin, via its Ras guanosine triphosphatase-activating activity, controls ERK1/2-dependent fibroblast growth factor receptor (FGFR) signaling in chondrocytes. We show first that neurofibromin is expressed in FGFR-positive prehypertrophic and hypertrophic chondrocytes during growth plate endochondral ossification. Using mice lacking *neurofibromin 1* (*Nf1*) in type II collagen-expressing cells, (*Nf1*^{col2} mutant mice), we then show that lack of neurofibromin in post-mitotic chondrocytes triggers a number of phenotypes reminiscent of the ones observed in mice characterized by FGFR gain-of-function mutations. Those include dwarfism, constitutive ERK1/2 activation, strongly reduced *Ihh* expression and decreased chondrocyte proliferation and maturation, increased chondrocytic expression of *Rankl*, *matrix metalloproteinase 9* (*Mmp9*) and *Mmp13* and enhanced growth plate osteoclastogenesis, as well as increased sensitivity to caspase-9 mediated apoptosis. Using wildtype (WT) and *Nf1*^{col2} chondrocyte cultures *in vitro*, we show that FGF2 pulse-stimulation triggers rapid ERK1/2 phosphorylation in both genotypes, but that return to the basal level is delayed in *Nf1*^{col2} chondrocytes. Importantly, *in vivo* ERK1/2 inhibition by daily injection of a recombinant form of C-type natriuretic peptide to post-natal pups for 18 days was able to correct the short stature of *Nf1*^{col2} mice. Together, these results underscore the requirement of neurofibromin and ERK1/2 for normal endochondral bone formation and support the notion that neurofibromin, by restraining RAS-ERK1/2 signaling, is a negative regulator of FGFR signaling in differentiating chondrocytes.

INTRODUCTION

In vertebrates, long bones are formed through endochondral ossification, a highly coordinated process regulated by paracrine and hormonal factors (1,2). This process starts with the condensation of undifferentiated mesenchymal cells that differentiate into chondrocytes, forming the first bone anlagen. Following directional division, these immature chondrocytes form columnar structures which are responsible for overall skeletal longitudinal growth. Subsequent to this proliferation

step, chondrocytes exit from the cell cycle and differentiate into hypertrophic chondrocytes that are characterized by their enlarged cell size. Chondrocyte proliferation and bone collar formation during limb development is under the control of Indian Hedgehog (IHH), a cytokine secreted by prehypertrophic chondrocytes (3–6). IHH induces the expression of parathyroid hormone-related protein (PTHrP), another cytokine that keeps proliferating chondrocytes from premature maturation and hypertrophy. Chondrocyte maturation and hypertrophy is also regulated by local and hormonal factors,

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including IHH, PTHrP and thyroid hormone (7,8), and is characterized by a switch from the synthesis of collagen type II to collagen type X, and by the expression of genes involved in growth plate catabolism, mineralization and vascularization. Alkaline phosphatase and osteopontin (OPN) contribute to the calcification of the hypertrophic zone (9–12). Matrix metalloproteinase-9 and 13 (MMP-9 and –13) are responsible for matrix degradation and growth factor activation (13), whereas vascular endothelial growth factor promotes capillary invasion into the cartilaginous matrix and eventually the recruitment of monocytes and osteoblasts to the osteochondral border. Receptor activator of nuclear factor kappa-B ligand (RANKL) is secreted by hypertrophic chondrocytes to stimulate the formation of osteoclasts and to promote resorption of the cartilaginous calcified matrix (14–17). This highly coordinated process of endochondral bone formation gives long bones their shape and structure, with two distal cartilaginous growth plates at the epiphyses and a bony region (diaphysis) extending in between, until growth plate closure (18).

The fibroblast growth factors (FGFs) and their receptors (FGFRs) play an important role during skeletal development. Activating mutations in *FGFR1* and *FGFR2* cause syndromes characterized by craniosynostosis (Pfeiffer, Crouzon and Apert syndromes) (19–21), whereas *FGFR3* activating mutations are typically associated with achondroplastic (thanatophoric dysplasia) and hypochondroplastic dwarfism (22–24) as well as craniosynostosis (Muenke syndrome). Because *FGFR1*-activating mutation (P252R) causes craniosynostosis, FGFR1 function has been associated primarily with flat bone growth and skull formation. However, FGFR1 is expressed in the growth plate during development and displays a distinct spatial distribution with FGFR3. Activating missense mutations in FGFR1 cause osteoglophonic dysplasia, a ‘crossover’ disorder that has skeletal phenotypes associated with *FGFR1*, *FGFR2* and *FGFR3* mutations, including dwarfism (25). FGFR1 may thus contribute to endochondral bone formation; however, this has been very little explored.

FGFR signaling is controlled by extracellular ligands of the FGF and heparin sulfate proteoglycan family, by the cellular location of the receptor following maturation (cell surface or ER), by intracellular crosstalk with other signaling pathways, such as the bone morphogenetic protein (BMP) pathway (26) and by the phosphorylation and ubiquitination status of the receptor which regulate its activity and degradation (27–29). FGFRs transmit signals through the activation of STAT1 and Ras-mitogen-activated protein kinase (MAPK) signaling (30). MAPK activity induced downstream of FGFR3, in particular, is a negative regulator of bone growth. Indeed, constitutive activation of MEK1 in chondrocytes causes STAT1-independent achondroplasia (ACH)-like dwarfism in mice and rescues the overgrowth of FGFR3-deficient mice (31). Accordingly, inhibition of ERK by genetic overexpression of C-type natriuretic peptide (CNP) in chondrocytes counteracts the dwarfism of a mouse model of ACH induced by activated FGFR3 in cartilage (32,33). Lastly, lack of *Spred2*, an inhibitor of FGF-induced MAPK signaling that binds to Ras and inhibits phosphorylation of Raf-1, causes an ACH-like dwarfism phenotype (34).

The product of the *neurofibromin 1* (*NF1*) gene, neurofibromin, is a large cytoplasmic protein with a small central region

that shares homology with guanosine triphosphatase (GTPase)-activating family proteins (GAP) (35). Through its GAP domain, it negatively regulates p21-Ras in multiple cell types (36). Therefore, loss of *NF1* function is associated with constitutive activation of Ras and downstream signaling, including ERK1/2. We have shown previously that neurofibromin is expressed in growth plate chondrocytes and that conditional-mutant mice lacking *Nf1* in chondrocytes (*Nf1^{Col2}* mice) have a reduced stature postnatally (37). The observations that FGFRs signal through ERK1/2, that neurofibromin negatively regulates ERK1/2, and that mice with constitutive activation of FGFR1 or FGFR3 share a number of endochondral bone formation phenotypes with *Nf1^{Col2}* mice (38–44), led us to hypothesize that neurofibromin may be an important regulator of FGFR signaling, controlling the dynamics of growth plate elongation, maturation and catabolism during development. We show here that neurofibromin is required for multiple steps of endochondral bone formation during limb development, and provide evidence that the GTPase activity of neurofibromin restrains FGFR-Ras-ERK1/2 signaling in post-mitotic differentiated chondrocytes to allow proper chondrocyte proliferation, maturation and growth plate catabolism.

RESULTS

Neurofibromin expression is enriched in differentiated chondrocytes

We have previously reported the expression of neurofibromin in the bone mesenchymal lineage, including mesenchymal osteochondroprogenitor cells, osteoblasts and chondrocytes (37). To further delineate neurofibromin expression within the growth plate and in the context of chondrocyte differentiation, the expression pattern of neurofibromin was investigated on tissue sections by immunocytochemistry and in differentiating primary chondrocytes *in vitro* by quantitative RT-PCR. Examination of neurofibromin immunoreactivity in the femoral growth plate of newborn mice (P0) indicated that neurofibromin is not expressed in resting and proliferating chondrocytes, whereas it is highly expressed in prehypertrophic and hypertrophic chondrocytes (Fig. 1A), where FGFR3 (45–48) and FGFR1 are expressed (Fig. 1B). To examine the dynamics of *Nf1* expression during chondrocyte differentiation, primary rib chondrocytes were extracted by sequential digestions, cultured in high-density micromass conditions, differentiated using ascorbic acid (49) and RNAs were collected at different time points following ascorbic acid induction. Quantitative RT-PCR analyses showed that the expression of *Col10a1*, a marker gene expressed by differentiated chondrocytes, increased with time, thus attesting for proper *in vitro* chondrocyte differentiation (Fig. 1C). *Nf1* mRNA expression increased during chondrocyte differentiation (Fig. 1D), with a pattern similar to the one of *Fgfr1* expression (Fig. 1E). *Nf1* expression was also up-regulated during chondrogenic differentiation of the clonal mesenchymal cell line C3H10T1/2 cells induced by rBMP2 (200 ng/ml, data not shown). In contrast, *Fgfr3* expression was rather decreased during chondrocyte differentiation (Fig. 1F). *Nf1* is thus expressed at late stages of chondrocyte differentiation,

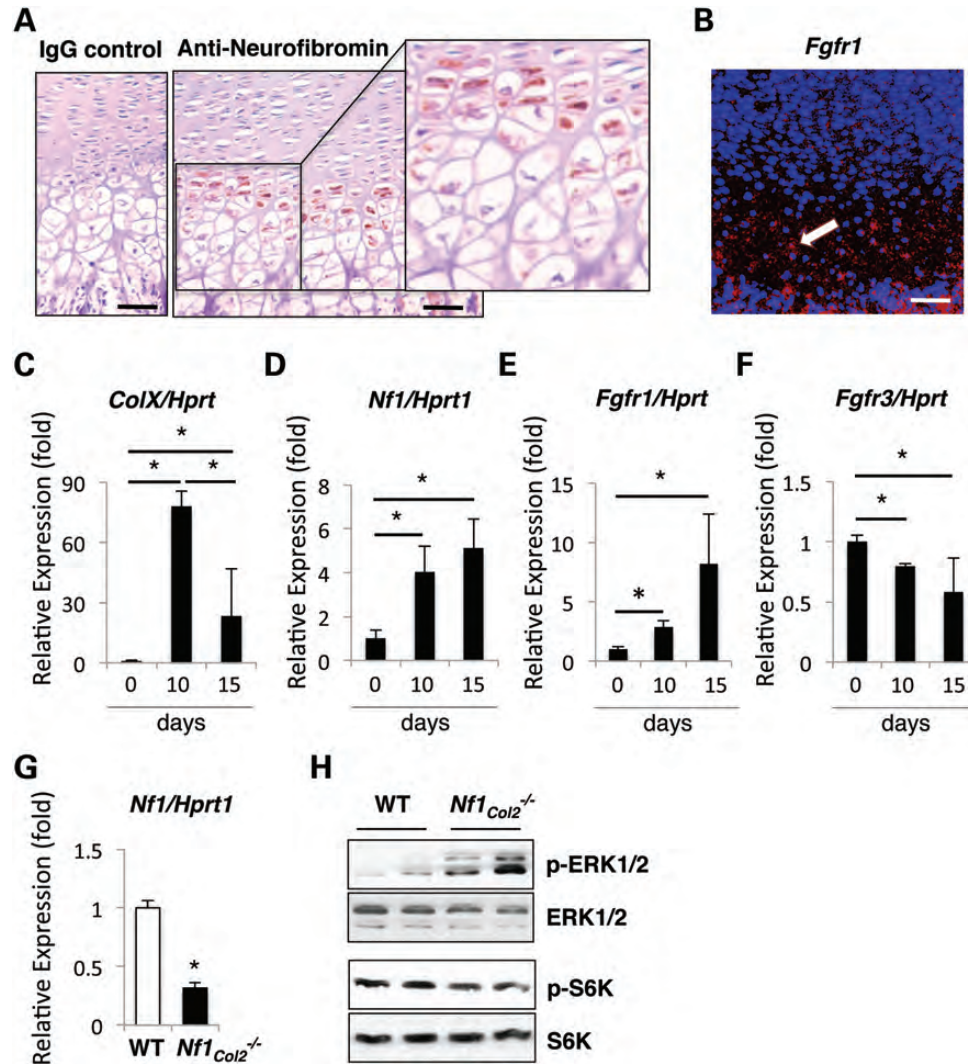


Figure 1. Neurofibromin is expressed in differentiated growth plate chondrocytes. (A) Neurofibromin immunoreactivity in mouse tibiae prehypertrophic and hypertrophic chondrocytes (P0) (Left: Control Isotype IgG, right: anti-Neurofibromin antibody). Scale bar: 100 μ m. (B) *In situ* hybridization for *Fgfr1* (red signal) and Hoechst nuclear staining (blue signal). Scale bar: 100 μ m. Arrow points to hypertrophic *Fgfr1*-positive chondrocytes. (C–E) mRNA expression level of *Col10a1* (C), *Nf1* (D), *Fgfr1* (E) and *Fgfr3* (F) in primary chondrocytes high density pellets during differentiation *in vitro*, measured by qPCR (* $P < 0.01$, $n = 3$). (G) *Nf1* mRNA expression is reduced in the cartilage of *Nf1*^{Col2-/-} mice (* $P < 0.01$, qPCR, $n = 3$). (H) ERK1/2 activation, assessed by western blot analysis, is increased in primary chondrocytes extracted from *Nf1*^{Col2-/-} mice compared to WT mice, whereas phosphorylated-p70S6K is not changed ($n = 2–3$).

suggesting a role of neurofibromin in chondrocyte maturation and hypertrophy.

Neurofibromin restrains ERK1/2 signaling in chondrocytes

Stimulation of FGFR signaling by FGF ligands or following activating mutation in FGFR3 leads to RAS-ERK pathway activation in chondrocytes (31,32,50,51). Similarly, as neurofibromin is a negative regulator of RAS activity, inactivating mutations in *Nf1* lead to constitutive RAS-ERK activation in multiple cell types (52–54); however, such activation and its functional consequence have not been investigated in chondrocytes. To determine whether neurofibromin activity regulates ERK1/2 activity in chondrocytes, we used mice lacking *Nf1* specifically in this lineage, generated by crossing transgenic

Col2a1-Cre deleter mice with *Nf1*^{flox/flox} mice (37,55). Long-bone epiphyses extracted from newborn *Nf1*^{Col2-/-} mice showed a 80% reduction in *Nf1* mRNA expression compared with wildtype (WT) littermates (Fig. 1G), despite the presence of contaminating *Col2a1*-negative tissues/cells in tissue preparations from mutant *Nf1*^{Col2-/-} (*Nf1*^{flox/flox}; *Col2a1-Cre*) pups. Primary rib chondrocytes extracted from 4-day-old WT and mutant *Nf1*^{Col2-/-} pups were then prepared, cultured for 3 days in the presence of ascorbic acid, and assessed for ERK1/2 phosphorylation by western blot analyses. As observed in other lineages, a marked increase in ERK1/2 phosphorylation was detected in *Nf1*-deficient chondrocyte cultures compared with WT chondrocytes cultures, whereas p70S6K phosphorylation was not affected (Fig. 1H). Increased phospho-ERK immunoreactivity was also observed in

prehypertrophic and hypertrophic chondrocytes from 10-day-old $Nf1^{Col2-/-}$ mice compared with WT littermates (see Fig. 5E). These results confirm efficient recombination of the floxed $Nf1$ allele by the mouse $Col2a1-Cre$ transgene *in vivo* and *in vitro*, and show that it leads to reduced $Nf1$ expression and ERK1/2 activation in $Nf1^{-/-}$ chondrocytes.

***Nf1* in prehypertrophic chondrocytes is required for proper chondrocyte proliferation**

The activation of ERK1/2 in $Nf1^{-/-}$ chondrocytes and in chondrocytes expressing an activated form of FGFR3, along with the overlapping growth plate expression of neurofibromin, FGFR1 and FGFR3 during endochondral bone formation, led us to hypothesize that neurofibromin is a negative regulator of FGFR signaling in mature chondrocytes. To test this hypothesis, we analyzed in detail the progression of bone growth in $Nf1^{Col2-/-}$ mice during embryonic and post-natal development, and then related the observed phenotypes to known developmental and molecular defects associated with FGFR-mutant mice.

We have previously shown that $Nf1^{Col2-/-}$ mice, at birth, are morphologically indistinguishable from WT littermates and have comparable body weight. However, size difference appears and becomes significant 5 days after birth and increases progressively thereafter (37). Hematoxylin and eosin (H&E) staining and subsequent growth plate histological analyses at E14 and E15 did not reveal significant difference in the formation of primary ossification centers between WT and $Nf1^{Col2-/-}$ embryos (Fig. 2A), as observed in mice lacking *Fgfr1* in chondrocytes (56) and despite growth plate expression of neurofibromin at this developmental stage (57). No obvious difference in vascular invasion between genotypes could be detected either upon CD31 staining of E16.5 tibiae (Fig. 2B). However, starting at P0, the length of both proliferating and hypertrophic zones was significantly shorter in $Nf1^{Col2-/-}$ mice compared with WT mice, although gross body size differences could not be detected. Shortening of the proliferating zone worsened with time and was most pronounced at 3 weeks of age, at which time point the proliferating zone of $Nf1^{Col2-/-}$ mice was < 60% of WT mice (Fig. 2C and D). The shortening of the hypertrophic zone, in contrast, was most severe at P0 (Fig. 2C and E). The formation of secondary ossification centers (SOCs), initiated with vascular invasion into hypertrophic chondrocytes in the middle of the distal epiphyses, was delayed in $Nf1^{Col2-/-}$ mice. At 1 week of age, SOCs were not detected in the proximal tibiae of $Nf1^{Col2-/-}$ mice, whereas they were already present in WT tibiae, as evidenced by the presence of hypertrophic chondrocytes and red blood cells (Fig. 2C). Similar phenotypes and delay in the formation of SOCs were observed in other bones from $Nf1^{Col2-/-}$ mice (data not shown), as well as in mice with FGFR3 activating mutation (58,59). Ablation of *Nf1* in chondrocytes thus causes dysregulation of endochondral ossification and results in postnatal growth retardation.

The progressive postnatal reduction in size of the proliferating zone in $Nf1^{Col2-/-}$ mice suggested that neurofibromin regulates chondrocyte proliferation. To address this hypothesis, the effect of *Nf1* deficiency on chondrocyte proliferation was assessed by *in vivo* bromodeoxyuridine (BrdU) labeling. At

P0, no significant difference was observed in the labeling index of tibial proliferative chondrocytes between $Nf1^{Col2-/-}$ mice and WT littermates (Fig. 3A). In contrast, chondrocyte proliferation was significantly decreased postnatally at P14 in the tibiae from $Nf1^{Col2-/-}$ mice compared with WT littermates (Fig. 3B). Since neurofibromin is not detected in proliferative chondrocytes (Fig. 1A), we reasoned that a factor(s) released by post-mitotic $Nf1^{-/-}$ prehypertrophic and/or hypertrophic chondrocytes may indirectly affect the proliferative activity of adjacent *Nf1*-negative chondrocytes, in a paracrine manner. In addition, since activation of FGFR3 represses chondrocyte proliferation through its inhibition of *Ihh* signaling (44,60,61), we reasoned that *Nf1* deficiency in prehypertrophic chondrocytes may reduce *Ihh* expression, thus possibly leading to the observed reduced chondrocyte proliferation. To address this hypothesis, gene expression studies were performed using long-bone cartilage tissues excised from $Nf1^{Col2-/-}$ and WT littermates, and high cell density micromass cultures of primary chondrocytes prepared from WT and $Nf1^{Col2-/-}$ ribs, differentiated or not *in vitro* with ascorbic acid (62–64). In agreement with the reduced proliferative index observed in the growth plates of $Nf1^{Col2-/-}$ mice *in vivo*, *Ihh* expression was significantly decreased in growth plate cartilage from $Nf1^{Col2-/-}$ mice compared with WT littermates (Fig. 3C). In micromass cultures, *Ihh* expression was strongly decreased as well, in both immature and differentiated cultures (Fig. 3D). *Pthrp* expression was very low in both chondrocyte cultures and cartilage of both WT and mutant mice (data not shown). These data suggest that suboptimal *Ihh* levels in the growth plates of $Nf1^{Col2-/-}$ mice may contribute to the observed reduction in chondrocyte proliferation. This phenotype is also in agreement with the increase in chondrocyte proliferation, differentiation and *Ihh* signaling observed in mice deficient for FGFR3 or FGF18 (65), and with the decrease in *Ihh* observed upon constitutive activation of FGFR3 (44). Despite these supporting evidence, a genetic *Ihh* gain-of-function experiment in $Nf1^{Col2-/-}$ mice will be necessary to definitively prove the contribution of reduced *Ihh* signaling to the chondrocyte proliferation phenotype of $Nf1^{Col2-/-}$ mice.

***Nf1* is required for proper chondrocyte hypertrophy and apoptosis**

The shortening of the hypertrophic zone observed in $Nf1^{Col2-/-}$ mice can conceivably be caused by a decreased pool of proliferating chondrocytes, defective or premature chondrocyte differentiation, an increase in chondrocyte apoptosis, or by premature/overt catabolism of growth plate cartilage. The results of our gene expression studies indicated that *Opn* expression was increased in differentiated (*Col10a1*-positive) mutant chondrocyte cultures and growth plates (Fig. 3C and D), as observed in chondrocytes treated with FGF9 (66) or in mice with FGFR3 activating mutation (58,67). *Col10a1* expression, however, was decreased in chondrocyte cultures and growth plates from $Nf1^{Col2-/-}$ pups compared with WT littermates (Fig. 3C and D), as observed in mice with constitutive FGFR3 activation (44,67), but converse to FGF18^{-/-} and FGFR3^{-/-} mice (65). The decreased expression of *Ihh* and *Col10a1* observed in the absence of *Nf1* is thus suggestive of impaired or delayed differentiation.

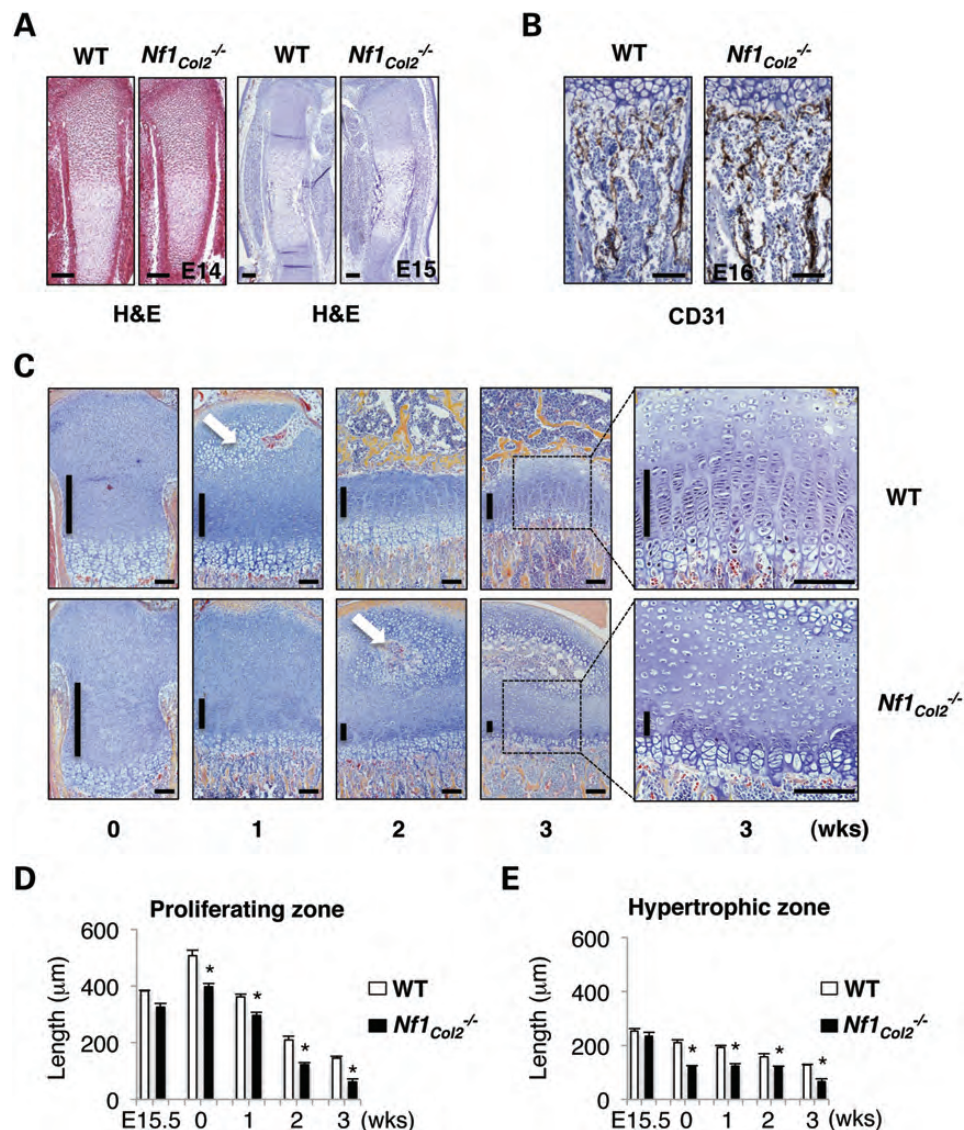


Figure 2. Defective endochondral bone formation in *Nf1^{Col2}^{-/-}* mice. (A) No significant difference in the formation of the primary ossification center is observed between WT and *Nf1^{Col2}^{-/-}* tibial growth plates at E14 and E15. Scale bar: 100 μm, H&E staining. (B) No significant difference in vascular invasion was detected in tibial primary spongiosae from *Nf1^{Col2}^{-/-}* and WT mice at E16, as measured by CD31 immunohistochemistry (blood vessels, brown staining). Scale bar: 200 μm. (C) Sections of tibial growth plates at birth and at weekly intervals thereafter. The length of the proliferating zone is shown by the vertical black lines. Scale bar: 100 μm, H&E staining. (D and E) Length quantification of the proliferating (D) and hypertrophic (E) zones at E15.5, P0, 1, 2 and 3 weeks of age (*:*P* < 0.01, *n* = 3). The length of both proliferating and hypertrophic zones was significantly shorter in *Nf1^{Col2}^{-/-}* mice compared with WT mice, starting from birth.

Both FGFR3 activation (68) and phosphate (63) trigger hypertrophic chondrocyte apoptosis via the activation of ERK. Therefore, we hypothesized that lack of *Nf1* in hypertrophic chondrocytes, by inducing ERK activation, may promote apoptosis and explain, in part, the shortening of the hypertrophic zone observed in *Nf1^{Col2}^{-/-}* pups. Since chondrocyte apoptosis is activated by a caspase-9 mediated mitochondrial pathway (63), we measured caspase-9 activation by western blotting, using high cell density primary chondrocytes micromass cultures from WT and *Nf1^{Col2}^{-/-}* mice. Treatment of ascorbic acid-differentiated chondrocytes with 25 mM phosphate for 2 h did not affect cleavage of caspase 9 in WT cells but clearly induced caspase-9 cleavage into its active form in *Nf1^{-/-}* chondrocytes (Fig. 3E). These results indicate

that loss of *Nf1* function in hypertrophic chondrocytes promotes their sensitivity to apoptosis.

***Nf1* is required for proper growth plate catabolism**

MMP9 and 13 are expressed by hypertrophic chondrocytes and work synergistically to degrade type I and II collagens (69). In addition, the expression of these two genes in chondrocytes is increased by FGF2 treatment (16,70). We thus examined *Mmp9* and *Mmp13* expression in chondrocyte cultures from WT and *Nf1^{Col2}^{-/-}* mice to address whether shortening of the hypertrophic zone in *Nf1^{Col2}^{-/-}* mice could stem from an MMP-dependent increase or premature growth plate catabolism caused by unrestrained FGFR signaling. In

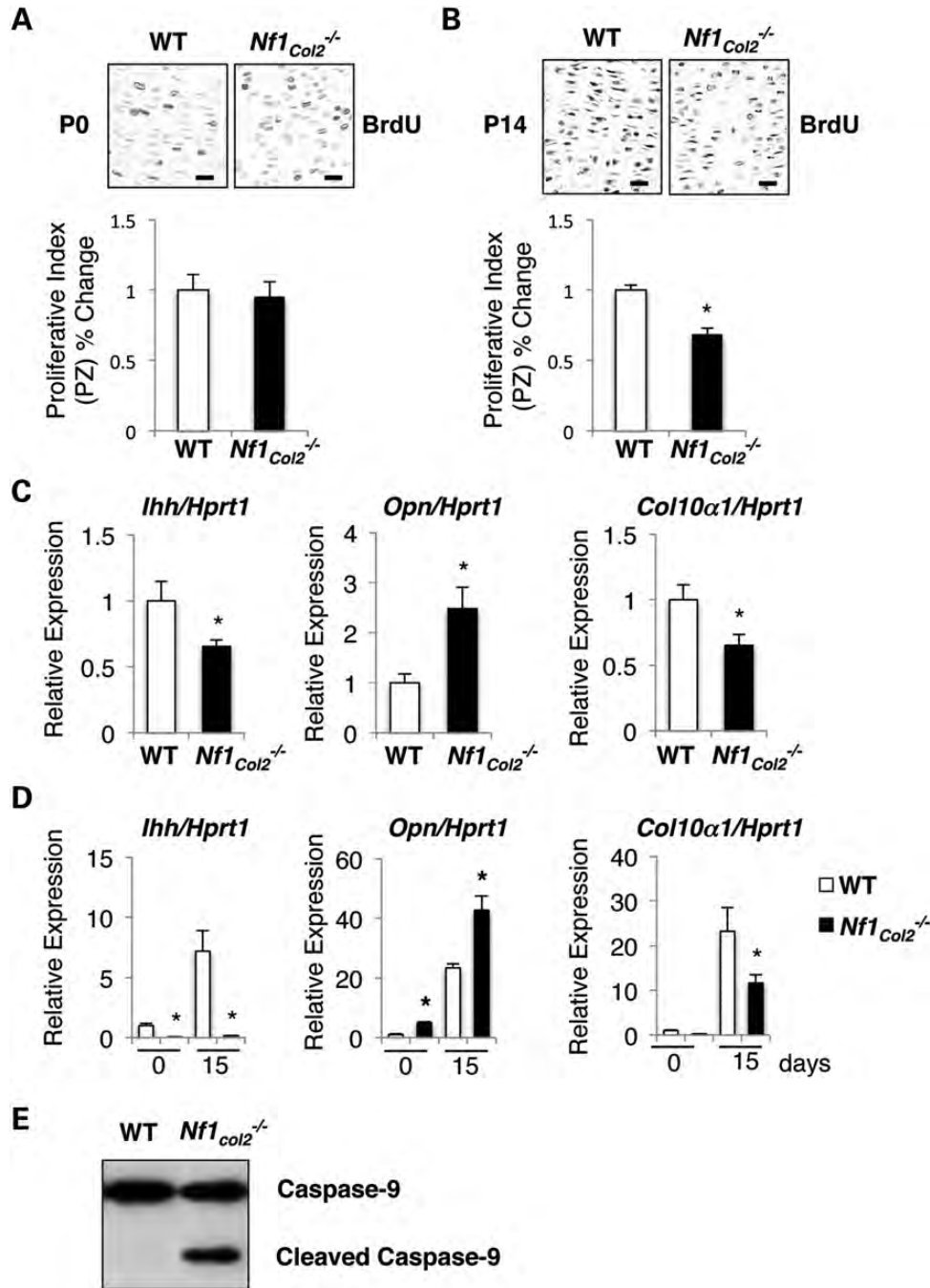


Figure 3. Decreased post-natal chondrocyte proliferation, abnormal chondrocyte gene expression and increased sensitivity to apoptosis in *Nf1*^{-/-} mice. (**A** and **B**) Tibial proximal growth plate BrdU immunohistochemistry and quantification of the proliferative index at P0 (**A**) and P14 (**B**) (PZ, proliferating zone, scale bar: 50 μ m, $*P < 0.01$, $n = 5$), showing reduced chondrocyte proliferation at post-natal stage only (scale bar: 50 μ m, $*P < 0.01$, $n = 5$). (**C** and **D**) *Ihh*, *Opn* and *Col10a1* mRNA expression measured in long-bone epiphyseal cartilage from P0 WT or *Nf1*^{-/-} mice (**C**, $*P < 0.01$, $n = 4$) and in micromass cultured primary chondrocytes at proliferative stage (d0) and differentiated stage (d15, following ascorbic acid treatment) (**D**, $*P < 0.01$, $n = 3$). *Ihh* and *Col10a1* expressions are decreased, whereas *Opn* expression is increased in the cartilage and chondrocyte cultures from *Nf1*^{-/-} mice compared with WT littermates. (**E**) Total and cleaved caspase-9 in differentiated primary chondrocytes from WT or *Nf1*^{-/-} mice ($n = 2$, western blotting). *Nf1*^{-/-} chondrocytes are more sensitive to apoptosis than WT chondrocytes upon phosphate treatment.

agreement with the aforementioned observations, we detected a significant increase in the expression of both genes in ascorbic acid-differentiated *Nf1*^{-/-} chondrocytes compared with WT controls (Fig. 4A). These results are also in agreement with the immunoreactivity of MMP9 in the last row of

hypertrophic chondrocytes [Supplementary Material, Fig. S1 and (17,69)], with the decrease in *Mmp9* expression observed in mice lacking *Fgfr1* in chondrocytes (56), with the increased hypertrophic chondrocyte zone in *Mmp9*- and *13*-deficient mice (69), and suggest that neurofibromin, by limiting the

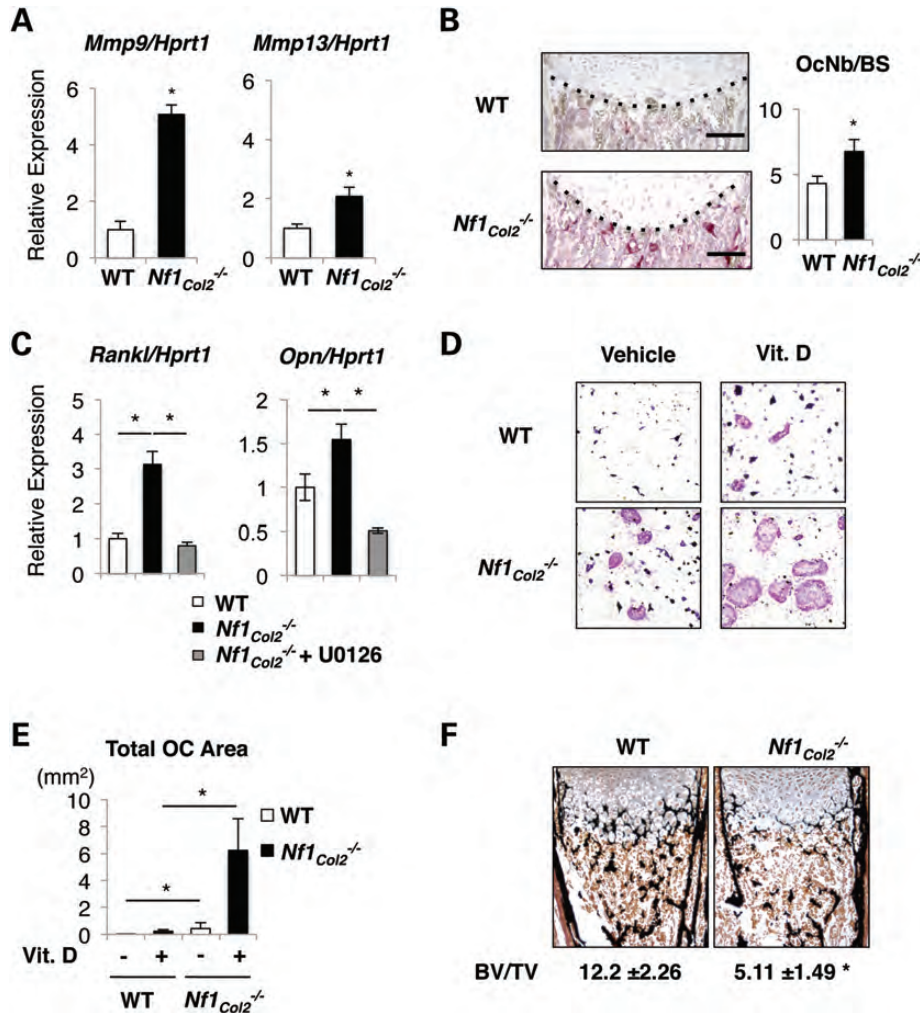


Figure 4. Enhanced growth plate catabolism in *Nf1^{Col2}^{-/-}* mice. (A) Increased expression of *Mmp9* and *Mmp13* mRNA expression in *Nf1^{-/-}* primary chondrocytes *in vitro* compared with WT chondrocytes (**P* < 0.05, *n* = 5). (B) TRAP staining and quantification of the number of TRAP-positive (red) osteoclasts per osteochondral surface in the proximal tibiae in WT and *Nf1^{Col2}^{-/-}* mice at P0 (**P* < 0.01, *n* = 5). Scale bar: 100 μ m. The number of osteoclasts is increased in the chondro-osseous area of *Nf1^{Col2}^{-/-}* pups compared with WT. (C) *Rankl* and *Opn* mRNA expression in differentiated primary chondrocytes with or without MEK inhibitor (U0126, 10 μ M, 6 h) (**P* < 0.01, *n* = 3). *Rankl* and *Opn* expression is significantly increased in primary chondrocytes from *Nf1^{Col2}^{-/-}* mice compared with WT mice, and this increase is attenuated by U0126. (D and E) Representative pictures of TRAP-stained chondrocyte-monocyte co-cultures using WT splenocytes and primary chondrocytes from WT and *Nf1^{Col2}^{-/-}* mice (D) and quantification of osteoclast area per plate in these co-cultures (E, **P* < 0.01, *n* = 4). Osteoclastogenic activity is significantly increased in co-cultures containing *Nf1^{-/-}* chondrocytes compared with WT chondrocytes. (F) Decreased tibial trabecular bone volume over total volume (BV/TV, **P* < 0.01, *n* = 5) in P0 *Nf1^{Col2}^{-/-}* pups compared with WT littermates (Von Kossa/Van Gieson staining).

expression of *Mmp9* and *13*, inhibits growth plate extracellular matrix degradation.

Replacement of growth plate cartilage by the bone matrix is also dependent on matrix degradation by osteoclasts/chondroclasts, as demonstrated by the elongation of the hypertrophic zone and the presence of cartilaginous remnants in mice treated with pharmacological inhibitors of osteoclast activity (bisphosphonates) and in osteopetrotic mutant mice that lack osteoclasts (71). Osteoclast differentiation is dependent on osteoclastogenic cytokines, including RANKL and osteoprotegerin (OPG), produced by bone cells, including osteoblasts and osteocytes. The contribution of chondrocytes to osteoclastogenesis has been less investigated, but recent evidence indicates that matrix embedded cells, including

chondrocytes and osteocytes, are essential sources of RANKL in bones (15,72). We have shown that lack of *Nf1* in both immature and mature osteoblasts promotes *Rankl* expression and thus osteoclastogenesis (37,73), and other have reported that ERK1/2 inactivation in chondrocytes and osteoblasts decreases *Rankl* expression and osteoclastogenesis (74). Thus, as ERK1/2 is constitutively active in *Nf1^{-/-}* chondrocytes, we reasoned that increased *Rankl* expression and enhanced osteoclast formation and activity could cause excessive resorption and shortening of the hypertrophic zone in *Nf1^{Col2}^{-/-}* pups. To address this hypothesis, mature osteoclasts were identified by tartrate-resistant acid phosphatase (TRAP) staining in bone of newborn WT and *Nf1^{Col2}^{-/-}* mice and their number was quantified at the chondro-osseous border. A

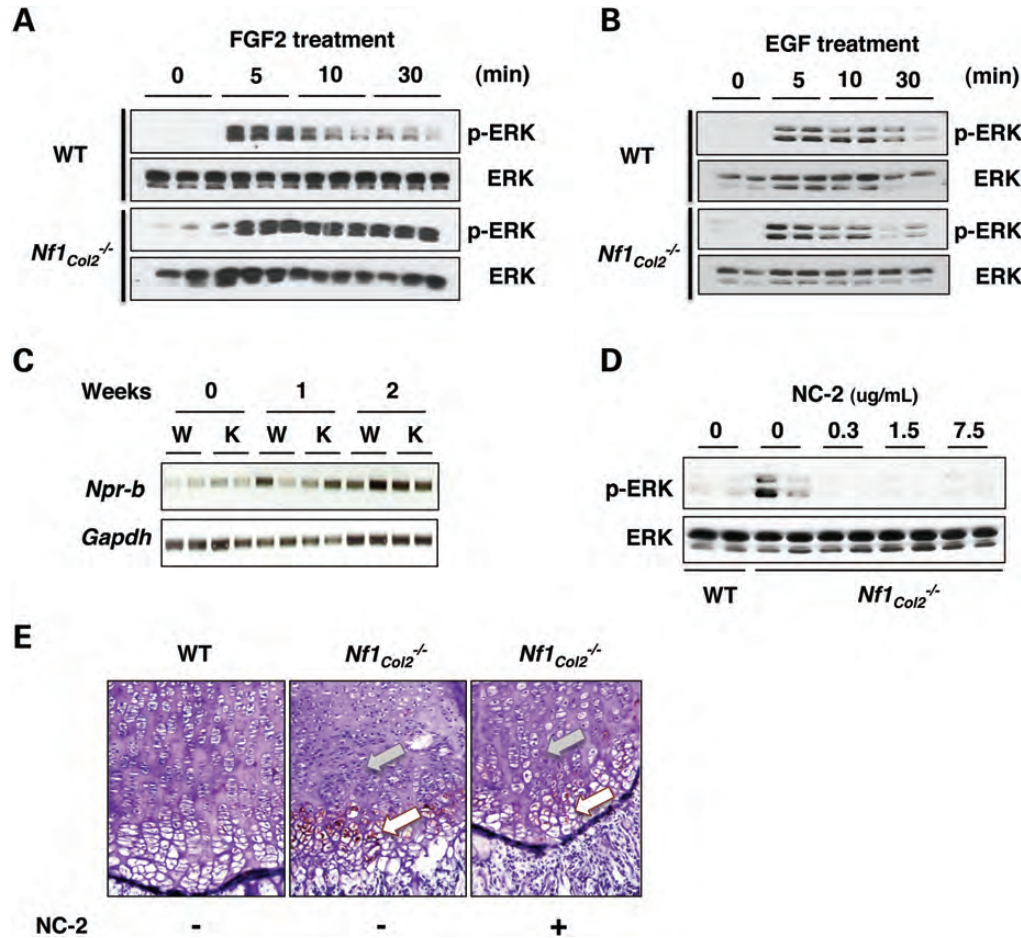


Figure 5. Neurofibromin restrains ERK-dependent FGFR signaling in chondrocytes. (A and B) Confluent primary chondrocytes from WT or *Nf1^{Col2-/-}* mice were serum-starved and harvested following FGF2 (10 ng/ml, 5 min) (A) or EGF (100 ng/ml, 5 min) (B) pulse treatment. Cells were collected at indicated periods of time after treatment. ERK1/2 activation was transient in WT chondrocytes but remained sustained in *Nf1^{Col2-/-}* chondrocytes in response to FGF2 (A), whereas no difference between genotype was observed following EGF treatment (B). (C) *Npr-b* expression in rib primary chondrocytes cultures differentiated for 2 weeks, measured by RT-PCR (W; WT chondrocytes, K; *Nf1^{Col2-/-}* chondrocytes). *Npr-b* is expressed throughout chondrogenic differentiation in primary chondrocytes from WT and *Nf1^{Col2-/-}* mice. (D) Decreased phospho-ERK1/2 status in serum-starved chondrocytes from *Nf1^{Col2-/-}* mice following NC-2 treatment for 30 min, measured by western blot analysis ($n = 2$). (E) Reduced *in vivo* P-ERK immunoreactivity in the tibial growth plate of *Nf1^{Col2-/-}* mice (P10) following daily treatment with NC-2 (300 mg/kg), compared with phosphate buffer saline treatment ($n = 2$). Gray arrows show the changes in the formation of proliferative columns. White arrows show the immunoreactivity (brown) for p-ERK1/2.

clear increase in the number of TRAP-positive multinuclear mature osteoclasts was observed in the tibial chondro-osseous area of *Nf1^{Col2-/-}* mice compared with WT pups (Fig. 4B), similarly to what has been observed in mice characterized by FGFR3 activating mutation (58). In agreement with this phenotype, *Rankl* and *Opn* expression was significantly increased in differentiated *Nf1^{Col2-/-}* primary chondrocytes compared with WT controls, and this increase could be prevented by short-term pre-treatment of the cultures with the ERK1/2 inhibitor U0126 (Fig. 4C). *Opn* expression was also increased in *Nf1^{Col2-/-}* chondrocytes (see Fig. 4C), possibly contributing to the enhanced osteoclastogenesis observed in *Nf1^{Col2-/-}* mice (75,76). In contrast, chondrocytic *Opg* expression was not affected by *Nf1* loss-of-function (data not shown).

To address whether the increased osteoclastogenesis and cartilage resorption observed in *Nf1^{Col2-/-}* mice had a chondrocytic origin, an *ex vivo* chondrocyte-monocyte *in vitro* co-culture

system was used. Primary chondrocyte cultures from WT and *Nf1^{Col2-/-}* pups were prepared and WT spleen monocytes (used as osteoclast progenitors) were purified and plated on the top of the chondrocyte cultures, in the presence or absence of 1,25 OH-vitamin D₃. As shown in Figure 4D and E, osteoclast area, measured as readout of the osteoclastogenic potential of WT and *Nf1^{Col2-/-}* chondrocytes, was significantly increased in co-cultures containing *Nf1^{Col2-/-}* chondrocytes following 5 days of differentiation, even in the absence of vitamin D, compared with cultures containing WT chondrocytes (Fig. 4D and E). Consistent with the increase in osteoclastogenesis observed in *Nf1^{Col2-/-}* pups, the amount of calcified bone in the primary spongiosa of newborn *Nf1^{Col2-/-}* pups was significantly reduced compared with WT littermates (Fig. 4F). These findings indicate that neurofibromin in differentiated chondrocytes restrains osteoclastogenesis and cartilage matrix degradation in an ERK, MMP9/13 and RANKL-dependent manner.

Neurofibromin restrains ERK1/2 signaling downstream of FGFR

The phenotypic and gene expression similarities reported above, between mice characterized by FGFR activation in chondrocytes and *Nf1*^{Col2} mice, support the notion that neurofibromin acts as a brake on FGFR signaling in chondrocytes. In such case, we reasoned that ERK1/2 activation upon FGFR stimulation in WT chondrocytes should be controlled and limited in time, whereas it should be prolonged in *Nf1*-deficient chondrocytes. To address this question, primary chondrocytes extracted from P4 WT and mutant *Nf1*^{Col2} pups were prepared, cultured for 7 days and assessed by western analyses for their response to FGF2 pulse-stimulation. Upon FGF2 treatment (10 ng/ml, 5 min), maximal ERK1/2 activation was observed after 5 min of treatment in WT chondrocytes, and activation was progressively dampened at later time points to return close to baseline within 30 min (Fig. 5A). In contrast, in *Nf1*^{-/-} chondrocytes, ERK activation was detectable in non-treated cultures and remained sustained at the 10 and 30 min time points. As a control, ERK1/2 activation in response to epithelial growth factor (EGF, 100 ng/ml) was similar between WT and *Nf1*^{-/-} chondrocytes (Fig. 5B). These results thus indicate that neurofibromin is a negative regulator of ERK1/2 activation downstream of FGFR signaling in differentiated chondrocytes.

The CNP analog NC-2 increases bone growth in *Nf1*^{Col2} mice

Recombinant forms of CNP have shown promising beneficial effects in pre-clinical models of dwarfism characterized by FGFR3 activation (32,33). CNP is a member of the natriuretic peptide family encoded by *Nppc*, which, through activating *cyclic* guanosine monophosphate (cGMP) and PKG, blocks activation of the ERK pathway by inhibiting Raf1 (16,77). If ERK1/2 constitutive activation in *Nf1*^{-/-} chondrocytes contributes to the observed abnormalities in endochondral bone formation observed in *Nf1*^{Col2} mice, we thus reasoned that restraining ERK activation by CNP should correct these defects. To address this hypothesis, we first verified the expression of *Npr-B*, the receptor for CNP, in differentiating primary chondrocytes from WT and *Nf1*^{Col2} chondrocyte cultures. Chondrocytes from both WT and *Nf1*^{Col2} mice showed clear expression of *Npr-B* throughout chondrogenic differentiation (Fig. 5C), as previously described by *in situ* hybridization (33,78,79). *Npr-B* expression was detected at every differentiation stages, with a more pronounced expression level in differentiated chondrocytes (2 weeks post-induction). Importantly, *Nf1* deficiency did not significantly alter the level of expression of *Npr-B*. Next, ERK1/2 activation was assessed in WT and *Nf1*^{-/-} primary chondrocytes treated with increasing doses of NC-2, a stable recombinant form of CNP (see section 'Material and Methods'). At the three different doses tested, NC-2 strongly suppressed the enhanced ERK1/2 phosphorylation typical of *Nf1*^{-/-} chondrocytes *in vitro* (Fig. 5D).

Unlike CNP, which has a half-life of ~3 min, the circulating half-life of NC-2 is ~20 h when injected subcutaneously in mice, making it very useful for *in vivo* studies. Based on our *in vitro* proof of concept data, *Nf1*^{Col2} mice were treated subcutaneously and daily with vehicle or two doses of NC-2

(100 and 300 mg/kg) during their early postnatal growth period (from birth to 18 days of age). As observed *in vitro*, NC-2 treatment (300 mg/kg) for 10 days was able to substantially attenuate P-ERK immunoreactivity in the growth plates of *Nf1*^{Col2} mice, although the effect of treatment on growth plate structure was not yet very visible, except for a striking improvement in the formation of the proliferating chondrocyte columns (Fig. 5E). In agreement with our previous analyses at an earlier age (37), *Nf1*^{Col2} mice displayed, following 18 days of treatment, a 40% reduction in naso-anal length at time of sacrifice compared with WT littermates, whereas *Nf1*^{Col2} mice treated with NC-2 displayed a significantly increased body size at both dose concentrations tested compared with vehicle-treated *Nf1*^{Col2} mice (Fig. 6A). At endpoint, the difference in body length between vehicle and NC-2-treated *Nf1*^{Col2} mice was reduced from 40 to 15%. Growth plate histological analyses indicated that the shortening of the proliferating and hypertrophic zones observed in *Nf1*^{Col2} mice were significantly corrected following daily NC-2 treatment, reaching sizes not significantly different from WT littermates (as quantified in Fig. 6B–D). The columnar disorganization of proliferating chondrocytes notable in *Nf1*^{Col2} mice was also corrected following NC-2 treatment (Fig. 5E and 6B). These results support the notion that *Nf1* controls post-natal endochondral bone formation in an ERK-dependent fashion.

DISCUSSION

FGFRs play a critical role during bone development, as best demonstrated by the various forms of skeletal dysplasias associated with mutations in *Fgfr1* and *Fgfr3* in mice and humans [see (80) for review]. Over the last 10 years, genetic mouse models have been instrumental to the characterization of FGFR downstream signaling pathways, among which the kinases MEK1 and STAT1 play a predominant role (31,40–42,81–83). In this study, we provide evidence that the Ras-GTPase activity of neurofibromin is required for proper chondrocyte proliferation, hypertrophy and growth plate catabolism, and regulates ERK1/2-dependent FGFR signaling in differentiated chondrocytes.

Loss of *Nf1* function in post-mitotic chondrocytes led to the inhibition of chondrocyte proliferation. Both *Fgfr3* and *Ihh* are important genes controlling chondrocyte proliferation during embryonic and postnatal growth (3,4,84). *IHH* promotes chondrocyte proliferation (85–87) and activation of FGFR3 represses chondrocyte proliferation through its inhibition of *IHH* signaling (60,61) as well as *IHH*-independent pathway(s) (88). Because neurofibromin is not detected in proliferative chondrocytes (57) but rather expressed in prehypertrophic chondrocytes (where both FGFR1 and FGFR3 are expressed) and hypertrophic chondrocytes (where FGFR1 is expressed) (56,61), we reasoned that the reduced chondrocyte proliferation observed in *Nf1*^{Col2} mice must stem from alteration in the secretion of paracrine factor(s) by prehypertrophic or hypertrophic *Nf1*^{-/-} chondrocytes. The strongly reduced expression of *Ihh* in *Nf1*^{-/-} chondrocyte cultures and growth plates, as well as the common phenotypic growth plate features between *Nf1*^{Col2} mice and mice lacking *Ihh* in type II collagen-expressing chondrocytes (89) lead us to propose

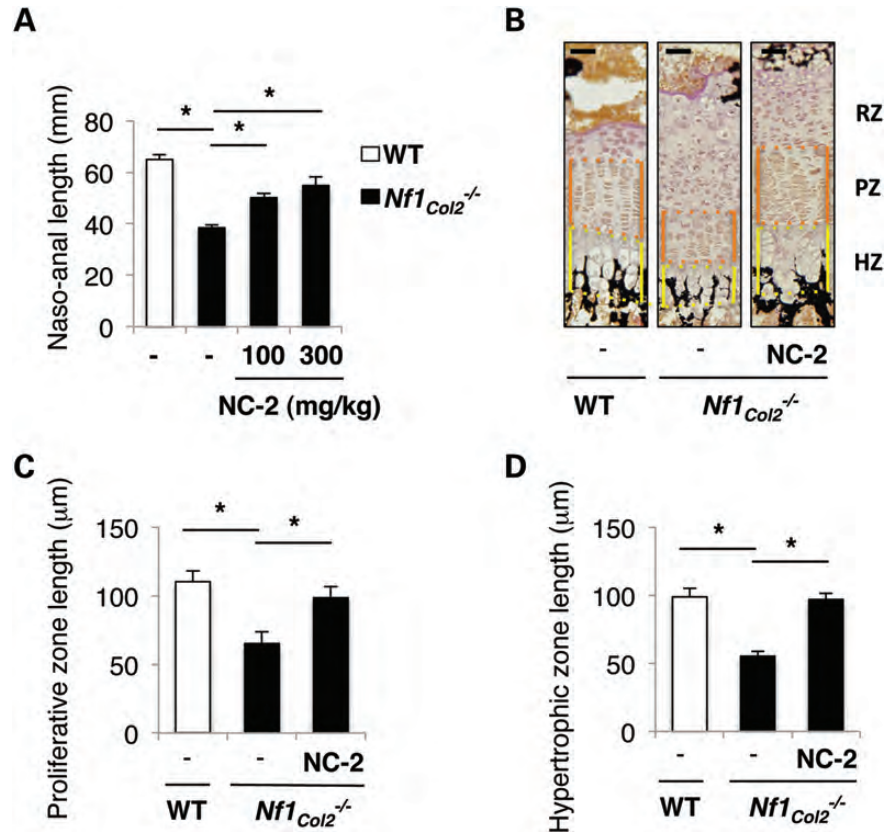


Figure 6. CNP treatment corrects the growth retardation of *Nf1*^{-/-} Col2^{-/-} mice. **(A)** Increased naso-anal length (mm) in *Nf1*^{-/-} Col2^{-/-} mice treated with two concentrations of NC-2 (**P* < 0.01, *n* = 7). **(B)** Representative images of undecalcified femur sections stained by Von Kossa/Van Gieson, from 18-day-old WT and *Nf1*^{-/-} Col2^{-/-} mice treated with or without NC-2 (300 mg/kg). Scale bar; 50 μm. NC-2 treatment corrects the shortened proliferating and hypertrophic zone lengths typical of *Nf1*^{-/-} Col2^{-/-} mice (**P* < 0.01, *n* = 7). **(C and D)** Increased proliferating (C) and hypertrophic (D) femoral zone lengths in WT and *Nf1*^{-/-} Col2^{-/-} mice following NC-2 treatment for 18 days (**P* < 0.01, *n* = 7).

that neurofibromin is an important regulator of *Ihh* expression in prehypertrophic chondrocytes. Whether neurofibromin regulates *Ihh* expression downstream of FGFR3 or FGFR1, or both, in prehypertrophic chondrocytes, and the nature of the signaling pathway between Ras and *Ihh* transcriptional activation remains to be determined.

The distinct expression patterns of FGFR1 and FGFR3 in the growth plate and the overlapping localization of neurofibromin and FGFR1 in hypertrophic chondrocytes, as well as in the perichondrium (45,46,56,59,90), suggest that neurofibromin controls FGFR1 signaling in hypertrophic chondrocytes. This hypothesis is supported by the strikingly similar phenotypes shared by *Nf1*^{-/-} Col2^{-/-} mice and mice overexpressing a chimeric FGFR containing the extracellular domain of FGFR3_{ach} replaced with the intracellular domain of FGFR1, under the control of the *Col2a1*-cre promoter (59). Conversely, mice with *Col2a1*-cre-driven chondrocyte-specific inactivation of *Fgfr1* exhibit phenotypes opposite to *Nf1*^{-/-} Col2^{-/-} mice, including expansion of their hypertrophic type X collagen-positive zone, reduced *Opn* and *Mmp9* expression and decreased number of TRAP-positive osteoclasts at the chondro-osseous junction at embryonic stages (56). Despite this collection of concordant indirect evidence, genetic crosses between *Fgfr*- and *Nf1*-mutant mice will be required

to further dissect the intricate regulatory mechanisms linking neurofibromin and FGFR signaling in chondrocytes.

The role of neurofibromin in growth plate development appears to be particularly important during post-natal growth, as *Nf1*^{-/-} Col2^{-/-} mice are dwarf postnatally but born with a size similar to WT littermates. This suggests that neurofibromin plays a non-redundant and necessary role during the embryonic to post-natal transition period, but does not exclude that its activity is required during embryonic growth plate development and osteogenesis, as supported by the shortening of the hypertrophic zone, the reduced osteogenesis and increased cortical bone porosity that are measurable at birth already. The alterations in gene expression and the length reduction of the hypertrophic zone in *Nf1*^{-/-} Col2^{-/-} embryos are indicative of altered chondrocyte maturation in the absence of *Nf1*. The similar *in situ* staining pattern for *Ihh* expression between WT and *Nf1*^{-/-} Col2^{-/-} pups (37) suggests that the reduced expression of *Ihh* observed in *Nf1*^{-/-} chondrocyte cultures and in the growth plates of *Nf1*^{-/-} Col2^{-/-} mice is due to suppression of *Ihh* gene expression/protein synthesis rather than a reduction in the number of cells expressing *Ihh*. On the other hand, increased osteoclastogenesis in transgenic mice expressing the *Fgfr3* (G369C/+) activating mutation has been reported, and the phenotype was attributed to a direct effect of

FGFR3 activation in osteoclasts (91). Our gene expression and chondrocyte/monocyte coculture data point to chondrocyte-derived RANKL, MMP9 and 13, and possibly OPN as alternative or additional cause(s) of increased osteoclastogenesis and premature cartilage removal in this mouse model and possibly in patients with chondrodysplasia (66,92–95). In addition, the increase in *Mmp9* and *Mmp13* expression in *Nf1*^{-/-} chondrocytes, and shortening of the hypertrophic zone in *Nf1*^{-/-}*Col2* embryos, are in agreement with previous reports indicating that FGF2 stimulates *Mmp9* and *Mmp13* expression in chondrocytes (70), and with the elongated hypertrophic zone observed in mice lacking MMP9 and 13 (69). It is also noteworthy that the MMP9 immunoreactivity pattern was distinct from the distribution of osteo/chondroclasts at the osteochondral border, indicating that increased MMP9 activity from both mature osteoclasts and hypertrophic chondrocytes may contribute to the promotion of growth plate catabolism in *Nf1*^{-/-}*Col2* mice. These data together thus indicate that neurofibromin activity in hypertrophic chondrocytes is necessary during embryonic development to limit cartilage matrix degradation at the ossification front. It is noteworthy that *Rankl* and *Opn* appear both to be direct targets of neurofibromin signaling in chondrocytes, as their expression is reduced upon short-term ERK inhibition. This observation suggests that because of unrestrained ERK1/2 activity, both genes are ectopically expressed by *Nf1*^{-/-} hypertrophic chondrocytes *in vivo*, hence explaining the increase in genes commonly considered as hypertrophic chondrocyte markers, in the setting of reduced expression of *Ihh* and *Col10a1*.

It is at this point still unclear if the shortening of the hypertrophic zone observed in *Nf1*^{-/-}*Col2* mice is predominantly caused by altered differentiation of *Nf1*^{-/-} chondrocytes, their increased sensitivity to apoptosis, or by premature or enhanced growth plate cartilage resorption. Since the mineralized cartilage matrix serves as a template for bone formation and since osteogenesis is coupled to chondrogenesis (6,96,97), it is also possible that the increased growth plate catabolism observed in *Nf1*^{-/-}*Col2* mice contributes, along with the low level of chondrocyte-derived IHH, to the reduced osteogenesis and increased cortical bone porosity measured in these mice (37). The fact that the type II collagen-cre transgene inactivates the floxed *Nf1* gene not only in chondrocytes, but also in embryonic osteochondroprogenitors (37,56) giving rise to bone marrow osteoblasts in this particular mouse model, however, did not allow us to tease apart the relative contribution of *Nf1* in type II collagen-expressing cells to growth plate development versus bone formation. The signaling mechanism whereby neurofibromin controls *Rankl*, *Mmp9* and *Mmp13* expression also remains to be identified. Regardless of the respective contribution of *Nf1* in osteoblasts and chondrocytes, these data identify *Nf1* as a necessary component for normal growth plate homeostasis during development, regulating chondrocyte proliferation, differentiation, apoptosis and growth plate catabolism (Fig. 7).

Mutations in *NF1* in patients with neurofibromatosis type I cause various bone abnormalities, of which two of the more severe are tibial pseudoarthrosis (non-union following fracture) and dystrophic scoliosis. NF1 patients are haploinsufficient for *NF1* and, as observed in *Nf1*^{+/-} mice, do not present with a significant reduction in size, although they are on average

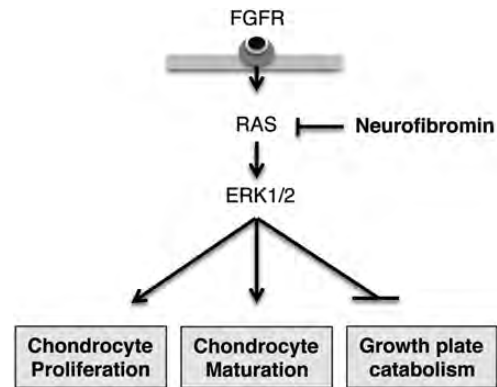


Figure 7. Model summarizing the role of neurofibromin during growth plate development. Neurofibromin, expressed in prehypertrophic and hypertrophic chondrocytes, restrains Ras-ERK1/2-dependent FGFR signaling, thereby promoting chondrocyte proliferation and maturation, and inhibiting growth plate catabolism to maintain proper growth plate homeostasis.

slightly shorter (see (98) for review). The clinical presentation of the NF1 focal dysplasias and results from genetic mouse models support a model whereby somatic *NF1* loss of heterozygosity in a subset of bone marrow osteochondroprogenitors causes the focal and dystrophic skeletal maladies in NF1 (37,99–101). The severity of the bone growth phenotype in *Nf1*^{-/-}*Col2* mice contrasts, however, with the nearly normal body size of NF1 patients and suggests that *NF1* loss-of-function in chondroprogenitors or chondrocytes during early development is not the cause of the moderate size reduction seen in NF1 patients. Our results suggest, however, that loss of neurofibromin function in growth plate osteochondroprogenitors might contribute to the severe delay of fracture healing observed in some of these patients, as bone repair requires the timely transition through endochondral bone formation steps, where developmental genes are reactivated in adults. If this is verified, the ability of the NPR-B agonist NC-2 to blunt ERK chronic activation typical of *Nf1*^{-/-} chondrocytes and to reverse the chondrocyte phenotypes of *Nf1*^{-/-} mice might translate to beneficial outcomes in the treatment of NF1 pseudoarthrosis. Lastly, although the majority of known cases of chondrodysplasias is caused by mutations in *FGFR1* or *FGFR3*, this study raises the possibility that some of the ACH cases with no mutation detected in these two receptors could stem from somatic inactivating mutations of *NF1*.

MATERIAL AND METHODS

Animals and drugs

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Vanderbilt University Medical Center. WT and *Nf1*^{-/-}*Col2* were generated by crossing *Nf1*^{flx/flx} mice and *Nf1*^{flx/+} mice; *α1(II) collagen-Cre* mice (55,102). *Nf1*^{flx/flx} mice and *Nf1*^{flx/flx} mice; *α1(II) collagen-Cre* mice were used as WT and cKO, respectively. NC-2 (100 or 300 mg/kg) or phosphate buffer saline vehicle were injected daily and subcutaneously from birth for 18 days. NC-2 is composed of the human CNP22 sequence,

preceded by six amino acids from the propeptide, fused to the C-terminus of the Fc domain of human immunoglobulin G1 by using an intervening glycine-rich linker (GGGS)₂. The compound binds specifically to the NPR-B receptor and stimulates intracellular production of cGMP with an EC₅₀ of 60 nM (data not shown).

Cell culture

Primary chondrocytes were extracted from the rib cages of P0 (newborn) mice. The cartilaginous part of the rib was dissected and the soft tissue was removed, then digested by collagenase D (3 mg/ml, Roche, Nutley, New Jersey, USA) and trypsin/ethylenediamine-tetraacetic acid (EDTA) (0.625 × 10⁻³%, GIBCO, Grand Island, NY, USA) in serum-free Dulbecco's Modified Eagle Medium (DMEM) medium (Invitrogen, Grand Island, NY, USA) for 1 h. Digestion medium was then discarded and samples were further digested overnight at 37°C (103). The extracted cells were filtered through 40-µm nylon mesh (BD Bioscience, Bedford, MA, USA), collected by centrifugation and used as primary chondrocytes. Primary chondrocyte cell cultures were maintained in DMEM+/+, DMEM supplemented with 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin (Cellgro, Manassas, VA, USA) in a humidified, 5% CO₂ incubator at 37°C. After reaching confluence, primary chondrocytes were lifted using 0.25% trypsin (Invitrogen, Grand Island, NY, USA), and replated into experimental wells. To induce differentiation, cells were treated 50 µg/ml ascorbic acid (Sigma, St Louis, MO, USA) and 5 mM β-glycerophosphate (Sigma, St Louis, MO, USA). For micromass culture, 15 µl of concentrated (2 × 10⁷ cells/ml) cells were plated on 6 well (49). After 4 h of incubation, 2 ml of DMEM+/+ was slowly added. FGF2 (10 ng/ml, R&D systems, Minneapolis, MN, USA) and EGF (100 ng/ml, R&D systems, Minneapolis, MN, USA) were used to stimulate primary chondrocytes. Osteoclastogenesis assays were performed with spleen-derived osteoclast precursors cocultured with rib-derived primary chondrocytes. Osteoclast precursor cells were separated from the single-cell suspension of whole spleens by a ficoll gradient (LSM, MP Biomedicals, Solon, OH, USA) and plated (5 × 10⁵ cells/ml) on confluent primary chondrocytes in DMEM+/+ (104). Osteoclastogenesis was induced following treatment with 10⁻⁸ M of 1,25(OH)₂ vitamin D₃ (Sigma, St Louis, MO, USA) (105).

RT-PCR, genomic PCR and qPCR

Total RNA was extracted using TRIzol (Invitrogen, Grand Island, NY, USA), and cDNAs were synthesized following DNase I treatment using the high-capacity cDNA reverse-transcription kit (Applied Biosystems, USA). Quantitative PCR (qPCR) were performed by using TaqMan gene expression assays or SYBR green qPCR. The probe and primer sets for *Rankl* (Mm00441908_m1), *Opg* (Mm00441906_m1), *Ihh* (Mm00439613_m1), *Runx2* (Mm00501578_m1), *Tnsap* (Mm00475834_m1), *Fgfr1* (Mm00438930_m1), *Fgfr3* (Mm00433294_m1) and the normalizer *Hprt* (Mm00446968_m1) were obtained from Applied Biosystems (Foster City, CA, USA). The primers were: *Mmp9* (forward; GCCCTACAGCGCCCCCTACT,

reverse; AGACACGCCCCCTTGCTGAACA), *Opn* (forward; CTCCTTGCGCCACAGAATG, reverse; TGGGCAACAGG GATGACA), *Col10a1* (forward; GGCAGCAGCATTACGACC CAAGAT, reverse; GAATAACAGACACCACCCCCTCAC), *Nf1* (forward; GTATTGAATTGAAGCACCTTTGTTTG, reverse; CTGCCCAAGGCTCCCCCAG). The primers were *Npr-b* (forward; TGTACCATGACCCCGACCTT, reverse; CCC GTTGCTCTGATGAAGT), *Gapdh* (forward; ACCACAGTC-CATGCCATCAC, reverse; TCCACCACCCTGTTGCTGTA). Specificity of amplification was verified by the presence of a single peak on the dissociation curve. Specific amplification conditions are available upon request. For genotyping, genomic DNA was isolated from tail tips by sodium hydroxide digestion, and PCR was performed using primers P1, P2 and P4, as described by Zhu *et al.* (55), resulting in a 280 bp *Cre*-mediated recombination band and a 350 bp non-recombined band. The *α1(II) collagen-Cre* transgene was detected using the forward primer: GAGTTGATA GCTGGCTGGTGGCAGATG and reverse: TCC TCCTGCTCCTAGGGCCTCCTGCAT to generate a 700 bp band.

Histology

Paraformaldehyde-fixed samples were decalcified in 0.5 M EDTA (pH 8.0) overnight or up to 1 week, depending on the age of the mice, and then dehydrated in graded series of ethanol, cleared in xylenes and embedded in paraffin. Five micrometers sagittal sections were cut and stained with H&E. Immunohistochemistry was performed according to standard protocols using an antibody against Neurofibromin (sc-67, Santa Cruz, Santa Cruz, CA, USA), Phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204, E10 mouse mAb #9106, Cell Signaling, Boston, MA, USA), MMP9 (ab38898, Abcam, Cambridge, MA, USA) or a non-immune IgG antibody, followed by horseradish peroxidase detection of the secondary antibody. *In vivo* proliferation assays were performed following i.p. injection with BrdU (0.1 mg/g) 2 h prior to sacrifice. Embryos were then harvested and processed for embedding and sectioning. BrdU was detected with a BrdU staining kit (Invitrogen, Grand Island, NY, USA) following the manufacturer's instructions. TRAP staining was performed by using the leukocyte acid phosphatase staining kit (Sigma, St Louis, MO, USA). Histomorphometric measurements were performed using the Bioquant Analysis System (BIOQUANT image analysis corporation, Nashville, TN, USA). *Fgfr1* *in situ* hybridization was performed on 5 µm sagittal paraffin sections. Sections were stored at 4°C until hybridization. The *Fgfr1* probe used is a 706 bp fragment of the 3' *Fgfr1* UTR (sequence available upon request). Anti-sense [³⁵S]-uridine triphosphate (Perkin Elmer, Waltham, MA, USA) probes were synthesized for hybridization.

Western blotting

Lysate from primary chondrocytes were prepared in RIPA buffer containing protease and phosphatase inhibitors (Roche, Nutley, New Jersey, USA). Proteins were separated by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membranes were then immunoblotted with indicated primary

antibodies, followed by incubation with a horseradish peroxidase-coupled anti-rabbit IgG antibody (sc-2004, Santa Cruz, Santa Cruz, CA, USA). Protein bands were visualized via chemiluminescence. Primary antibodies used for immunoblotting were ERK1/2 (#9102), phospho Erk1/2 (#9101), p70S6K (#9202), phospho p70S6K (#9205) and Caspase-9 antibodies (#9504, Cell Signaling, Boston, MA, USA).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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