ORIGINAL ARTICLE

FGFR1 signaling in hypertrophic chondrocytes is attenuated by the Ras-GAP neurofibromin during endochondral bone formation

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Abstract

Aberrant fibroblast growth factor receptor 3 (FGFR3) signaling disrupts chondrocyte proliferation and growth plate size and architecture, leading to various chondrodysplasias or bone overgrowth. These observations suggest that the duration, intensity and cellular context of FGFR signaling during growth plate chondrocyte maturation require tight, regulated control for proper bone elongation. However, the machinery fine-tuning FGFR signaling in chondrocytes is incompletely defined. We report here that neurofibromin, a Ras-GAP encoded by Nf1, has an overlapping expression pattern with FGFR1 and FGFR3 in prehypertrophic chondrocytes, and with FGFR1 in hypertrophic chondrocytes during endochondral ossification. Based on previous evidence that neurofibromin inhibits Ras-ERK signaling in chondrocytes and phenotypic analogies between mice with constitutive FGFR1 activation and Nf1 deficiency in Col2a1-positive chondrocytes, we asked whether neurofibromin is required to control FGFR1-Ras-ERK signaling in maturing chondrocytes in vivo. Genetic Nf1 ablation in Fgfr1-deficient chondrocytes reactivated Ras-ERK1/2 signaling in hypertrophic chondrocytes and reversed the expansion of the hypertrophic zone observed in mice lacking Fgfr1 in Col2a1-positive chondrocytes. Histomorphometric and gene expression analyses suggested that neurofibromin, by inhibiting Rankl expression, attenuates pro-osteoclastogenic FGFR1 signaling in hypertrophic chondrocytes. We also provide evidence suggesting that neurofibromin in prehypertrophic chondrocytes, downstream of FGFRs and via an indirect mechanism, is required for normal extension and organization of proliferative columns. Collectively, this study indicates that FGFR signaling provides an important input into the Ras-Raf-MEK-ERK1/2 signaling axis in chondrocytes, and that this input is differentially regulated during chondrocyte maturation by a complex intracellular machinery, of which neurofibromin is a critical component.

Introduction

Endochondral bone formation is a complex, highly regulated and orderly multi-step process by which long bones elongate during development and heal following fracture. It relies on tight spatiotemporal interactions and crosstalk between growth factors and signaling from their receptors in target cells, including osteochondroprogenitors, chondrocytes, osteoblasts and osteoclasts. Proper signaling within the growth plate between chondrocytes and with neighboring cells is essential for the successful and timely transition of resting chondrocytes to proliferating and then hypertrophic chondrocytes (1,2). Failure or dysregulation of any of these steps during development typically leads to shortened stature or dwarfism, structural bone abnormalities (3), whereas failure during fracture healing in adults leads to fracture non-union (pseudarthrosis) (4).

Fibroblast growth factor (FGF) signaling during endochondral bone formation has been the subject of intense scientific inquiry. In the growth plate, the receptors for FGFs (mainly FGFR3 and FGFR1) were shown to be expressed in distinct zones: FGFR3 in...
proliferating chondrocyte columns and FGFR1 in hypertrophic chondrocytes (5–9). Constitutive FGFR3 activity in the growth plate, caused by activating Fgfr3 mutations, inhibits chondrocyte proliferation, leading to growth arrest and inhibition of maturation to hypertrophy (10–12). Moleculally, activating FGFR3 mutations cause overactive downstream ERK and STAT1 signaling in the developing growth plate, leading to several forms of dwarfism which vary in severity depending on the degree of FGFR3 constitutive activity and ERK activity (13–15). The function of FGFR1 in endochondral bone growth, on the other hand, remains unclear. Activating FGFR1 mutations have been associated with craniosynostososes, caused by defects in intramembranous ossification (20–22). In the growth plate, the non-overlapping expression patterns of FGFR1-FGFR3 suggest that these receptors have unique functions, mediated by differences in their ligand-binding specificity and/or downstream signaling. In addition, human cases indicate that FGFR1 signaling is crucially important for endochondral ossification. Activating mutations in FGFR1 indeed cause osteopetrosis, a rare syndrome characterized by rhizomelic dwarfism, craniosynostosis and non-ossifying bone lesions, fracture healing deficits, scoliosis, tibial bowing, low bone mineral density, hypertelorism and pectus excavatum (23–28).

Activation of ERK1/2 and p38 in response to FGF has been observed in multiple cell types, whereas the activities of the JNK kinases, the PI3K-AKT pathway and the PLC pathway vary depending on cell type (29). FGF treatment of RCS chondrocytes and PC12 adrenal cells, which leads to growth arrest, was shown to be accompanied by strong and sustained ERK1/2 activation, whereas epidermal growth factor (EGF) treatment, was not (30,31). These results suggest the existence of cell-specific and possibly differentiation stage-specific mechanisms to control the intensity or duration of FGFR-Grb2-Sos-Ras-RAF-MEK-ERK signaling.

One of the intracellular regulators of this pathway is neurofibromin, a Ras GTPase activating protein (Ras-GAP) that promotes the conversion of active Ras bound to GTP to the inactive form of Ras bound to GDP. Loss-of-function mutations in NF1, the gene encoding neurofibromin, causes chronic and unregulated Ras and ERK1/2 activity, which is at the origin of the multiple conditions associated with neurofibromatosis type-1 (NF1). Approximately 40% of individuals with NF1 display skeletal pathologies, including non-ossifying bone lesions, pseudarthrosis, shortened stature, idiopathic or dystrophic scoliosis, asymmetry of facial bones, osteosclerosis, tibial bowing, hypertelorism, pectus excavatum and low bone mineral density (32–36), which are reminiscent of the bone manifestations observed in individuals with osteoglycopenic dysplasia, although more focal and heterogeneous than the latter. Our previous studies have shown that NF1 deletion in Col2a1-positive murine chondrocytes leads to Ras-ERK1/2 chronic activation, to a reduction in the proliferation and hypertrophic zones of the growth plate and to dwarfism compared with wild-type (WT) littermates (37,38). In addition, these mice display increased number of osteoclasts at the osteochondral border, and NF1-deficient chondrocytes favored osteoclastogenesis ex vivo, suggesting that in chondrocytes, neurofibromin serves to limit osteoclastogenesis and growth plate catabolism during development.

Genetic mouse models with Nf1 deficiency or Fgfr1 gain- or loss-of-function mutations support a negative association between FGFR1 signaling and neurofibromin activity. First, we found that the activation of ERK1/2 by FGF2 treatment is dampened to baseline activity within 30 min in WT chondrocytes, whereas it lasts more than 1 h in Nf1-deficient chondrocytes. These findings suggested that neurofibromin acts as a brake on FGFR signaling in chondrocytes (38). Second, the hypertrophic zone of Fgfr1Col2a1KO mice, in which Fgfr1 is ablated in Col2a1-positive chondrocytes, is longer than in WT littermates, in contrast to the shortened hypertrophic zones of the Nf1Col2a1KO mice, which lack Nf1 in the same Col2a1-positive cells (7,38). Third, Fgfr1Pro250Arg mice, harboring a constitutively active Fgfr1Pro250Arg allele at the endogenous Fgfr1 locus (39), are dwarved as are Nf1Col2a1KO mice (37–39). Lastly, at the molecular level, the growth plate expression of matrix metalloproteinase 9 (Mmp9) and osteopontin (Opn), two proteins contributing to growth plate catabolism and osteoclastogenesis at the osteochondral front, are downregulated in Fgfr1Col2a1KO mice while they are upregulated in Nf1Col2a1KO mice (7,38). Therefore, constitutive activation of FGFR1 in Fgfr1Pro250Arg mice leads to similar phenotypes to the ones observed in Nf1Col2a1KO mice lacking Nf1 in chondrocytes, whereas loss of FGFR1 in Fgfr1Col2a1KO mice triggers phenotypes that are mostly opposite to the ones observed in Nf1Col2a1KO mice. Collectively, these observations led us to hypothesize that neurofibromin acts as a brake on FGFR1 signaling in the developing growth plate. In this study, we generated double mutant mice lacking both Nf1 and Fgfr1 in Col2a1-positive chondrocytes to ask whether loss of activity of neurofibromin and thus activation of Ras-ERK1/2 signaling reverses the aberrant hypertrophic zone length and decreased osteoclastogenesis caused by lack of FGFR1 signaling in Fgfr1Col2a1KO mice (Fig. 1B). Our results support a model whereby neurofibromin controls: (i) the cartilage catabolism-promoting function of FGFR1 signaling in hypertrophic chondrocytes and (ii) the organization and extension of proliferative chondrocyte columns, via regulation of FGFR3 (and possibly FGFR1) signaling in prehypertrophic chondrocytes.

**Results**

**Fgfr1, Fgfr3 and Nf1 expression is localized to distinct zones in the growth plate**

A first requirement for neurofibromin to control FGFR1 signaling is for the two proteins to be expressed in the same chondrocyte population. To address this question, we performed in situ hybridization (ISH) on serial paraffin sections on postnatal (P) WT long bone growth plates, using Indian hedgehog (Ihh) as a marker of the prehypertrophic zone. At both P0 and P7, Fgfr1 and Nf1 transcripts were both detected in the hypertrophic zone, with minimal detectable expression in the proliferation zone, where Fgfr3 was localized primarily. Both Fgfr1 and Nf1 transcripts had weaker expression detectable in the prehypertrophic zone (Fig. 1A). Thus Fgfr1 expression overlapped with Nf1 expression in the prehypertrophic, hypertrophic and perichondrium areas, while Fgfr3 expression only overlapped with Nf1 expression in prehypertrophic chondrocytes at both P0 and P7. Although co-expression does not prove functional interaction, these results suggest that neurofibromin may regulate FGFR1 signaling rather than FGFR3 signaling in hypertrophic growth plate chondrocytes.

**Nf1 ablation in Fgfr1-deficient chondrocytes reverses the alterations in growth plate maturation/hypertrophy observed in Fgfr1Col2a1KO mice**

The overlap in Fgfr1 and Nf1 expression in postmitotic prehypertrophic and hypertrophic chondrocytes (Fig. 1A and (5,9,38,40)), the largely opposite growth plate phenotypes between Fgfr1Col2a1KO and Nf1Col2a1KO mice (7,38,37) and the fact that FGF activation and Nf1 mutation/ablation both trigger Ras-Raf-MEK-ERK activation
in chondrocytes (38), led us to the hypothesis that neurofibromin inhibits, among the many receptor tyrosine kinases (RTKs) expressed in the growth plate, the signaling of FGFR1 in mature chondrocytes (Fig. 1B). This hypothesis is not functionally testable in vitro due to the dynamic nature of growth plate development and challenges in generating mature chondrocytes in vitro, and can be best addressed genetically and in vivo, by either overexpressing neurofibromin in chondrocytes to rescue the phenotypes of cells with activation of FGFR1, or by reducing neurofibromin activity in cells lacking FGFR1 to artificially reactivate Ras-ERK1/2 signaling in these cells. In the latter option, we reasoned that lack of neurofibromin, leading to Ras-ERK1/2 pathway activation (38) (Fig. 1C, bottom left and right), in FGfr1-deficient chondrocytes (in which Ras-ERK1/2 signaling is reduced, Fig. 1C, top right), should reverse the growth plate phenotypes of FGfr1Col2cKO mice. We thus generated double mutant mice lacking both Nf1 and Fgf1 in Col2a1-expressing chondrocytes (DbfCol2a1KO mice) and compared their skeletal phenotypes to single knockout, Fgf1Col2a1KO and Nf1Col2a1KO littermates.

Gross examination over the period of rapid growth before weaning indicated that both body length (Fig. 2A) and body weight (Supplementary Material, Fig. S1) were reduced in both Nf1Col2a1KO and DbfCol2a1KO mice compared with WT littermates and Fgf1Col2a1KO mice. Similarly, both genotypes had significantly shorter bones than WT and Fgf1Col2a1KO mice at P18 (Fig. 2B). Fgf1Col2a1KO mice also had reduced stature (by P4), body weight (by P9) and tibial length (P18) compared with WT littermates, despite the increased size of their hypertrophic zone (Fig. 2D) (7). Body length, body weight and tibia length were not significantly different between Nf1Col2a1KO and DbfCol2a1KO mice (Fig. 2A and B).

To assess changes in growth plate structure between WT, single mutant Fgf1Col2a1KO and Nf1Col2a1KO and DbfCol2a1KO mice, growth plate histomorphometry was performed, analyzing the FGFR3-rich proliferation and FGFR1-rich hypertrophic zones longitudinally at P0, 7 and 14 days of age. The proliferation zones of all four groups of mice were not significantly different until P14, at which time the proliferation zones of Nf1Col2a1KO and DbfCol2a1KO were significantly shorter than that of WT and Fgf1Col2a1KO mice, although they were not statistically different from each other (Fig. 2C). In contrast, the hypertrophic zone across all three postnatal time points was elongated by inactivation of Fgf1r1 in Col2a1-expressing chondrocytes (Fig. 2D and E), in agreement with previous studies (7). Ablation of Nf1 in double mutant DblCol2a1KO mice reduced the size of this zone compared with WT and Fgf1Col2a1KO littermates at P0, 7 and 14, to values similar to the ones observed in Nf1Col2a1KO mice (the hypertrophic zones of Nf1Col2a1KO and DblCol2a1KO were not statistically different from each other). In addition, lack of Nf1 in Fgf1-deficient hypertrophic chondrocytes led to disturbances in the orderly stacking of the proliferative chondrocyte columns observed in WT and Fgf1Col2a1KO mice (Fig. 2E). Increased phospho-ERK1/2 immunostaining in the growth plate prehypertrophic and hypertrophic regions in DblCol2a1KO mice compared with Fgf1Col2a1KO mice (Fig. 1C, bottom
Figure 2. Nf1 ablation in Fgfr1Col2cKO mice reverses their hypertrophic zone phenotype. (A) P0-P18 body length of WT, Fgfr1Col2cKO, Nf1Col2cKO and DblCol2cKO mice. Body length was not significantly different between Nf1Col2cKO and DblCol2cKO mice, but both genotypes had a body length significantly less than WT and Fgfr1Col2cKO mice (*P < 0.05 versus WT unless indicated, n = 4 per group, repeated measure two-way ANOVA). (B) P18 tibial length of WT, Fgfr1Col2cKO, Nf1Col2cKO and DblCol2cKO mice. Tibial length was not significantly different between Nf1Col2cKO and DblCol2cKO mice, but it was significantly less in DblCol2cKO than in WT and Fgfr1Col2cKO mice (*P < 0.05 versus WT unless indicated, n = 7 per group, one-way ANOVA). (C and D) Length quantification of proximal tibial proliferation (C) and hypertrophic (D) zones of P0, 7 and 14 WT, Fgfr1Col2cKO, Nf1Col2cKO and DblCol2cKO mice (*P < 0.05 versus WT unless indicated, n = 4 per group, two-way ANOVA). The length of the proliferation zones in Nf1Col2cKO and DblCol2cKO mice were significantly shorter than WT but not significantly different from each other at P14. The same was true of the hypertrophic zone, but at P0, 7 and 14. (E) H&E staining of proximal tibial growth plates of WT, Fgfr1Col2cKO, Nf1Col2cKO and DblCol2cKO mice at P0, 7 and 14 and high magnification of the proliferation zone (PZ) at P14. Arrows indicate disorganized proliferative columns. Scale bar: 100 μm.
right) confirmed reactivation of the Ras-ERK1/2 pathway upon Nf1 ablation in these mice. In addition, in situ hybridization and quantitative RT-PCR (qPCR) verified efficient Fgfr1 deletion in the growth plate of Fgfr1Col2cKO mice and that Fgfr1 expression was not affected with regard to level of expression or growth plate localization upon Nf1 ablation (see Fig. 5A and C). Thus, Nf1 ablation in Fgfr1-deficient chondrocytes reverses the increase in growth plate hypertrophic zone observed in Fgfr1Col2cKO mice, and has a deleterious effect on the stacking of the proliferative chondrocyte columns, which was not affected in mice lacking Fgfr1 only.

Lack of Nf1 in Fgfr1-deficient chondrocytes reverses the reduction in osteochondral osteoclast number observed in Fgfr1Col2cKO mice

Osteoclast number at the chondro-osseous junction (the bone marrow zone immediately adjacent to the growth plate) was shown to be reduced in Fgfr1Col2cKO mice, and Mmp9 and Opn expression were lower in Fgfr1 null chondrocytes compared with WT chondrocytes (7). In contrast, Fgfr1Col2cKO mice display an increase in osteoclast number at the osteochondral border (37,38,41), and Nf1 null chondrocytes have elevated expression of Rankl, Opm, Mmp9 and Mmp13, all of which are important for extracellular bone matrix catabolism (38). These observations suggested that FGFR1 and neurofibromin were both involved, in an antagonistic fashion, in the mechanism by which mature chondrocytes stimulate growth plate catabolism. To determine whether the reduced osteoclast density at the osteochondral border in Fgfr1Col2cKO mice could be rescued by concurrent deletion of Nf1 in growth plate chondrocytes, we quantified osteoclast number in TRAP-stained thin bone sections from WT, Nf1Col2cKO, Fgfr1Col2cKO and DblCol2cKO mice. When Fgfr1 was deleted in Col2a1-expressing chondrocytes of newborn and P14 mice, osteoclast number at the chondro-osseous junction was reduced (Fig. 3A–C), in line with previous studies in Fgfr1Col2cKO embryos (7). Upon Nf1 deletion, a clear increase in the number of TRAP-positive multinucleated osteoclasts was observed in DblCol2cKO growth plates versus WT mice. Osteoclast number was not significantly different between DblCol2cKO and Nf1Col2cKO growth plates at P0 and P14 (Fig. 3A–C). Consistently, we found that Rankl expression was elevated in Nf1Col2cKO and DblCol2cKO growth plates, yet decreased in Fgfr1Col2cKO growth plates taken from 5-day-old pups, compared with WT animals (Fig. 3D). Opg

Figure 3. Nf1 ablation in Fgfr1Col2cKO mice reverses their decrease in osteoclastogenesis at the osteochondral junction. TRAP staining in distal femurs from P0 pups (A) and quantification of the number of TRAP-positive (red) osteoclasts per osteochondral border surface (BS) in P0 (B) and P14 (C) distal femur sections showed that the number of osteoclasts at the chondro-osseous junction in Fgfr1Col2cKO mice is reduced compared with WT littermates, and that Nf1 deletion in DblCol2cKO mice leads to higher number of osteoclasts compared with Fgfr1Col2cKO mice. Osteoclast number in Nf1Col2cKO and DblCol2cKO was not significantly different from each other. The osteochondral border surface is indicated by a dotted line (*P<0.05 versus WT unless indicated, n=4 per group, one-way ANOVA, scale bar: 100 μm). (D) Rankl expression is increased in P5 long bone growth plate cartilage from Nf1Col2cKO and DblCol2cKO mice and decreased in Fgfr1Col2cKO P5 growth plate cartilage when compared with WT (*P<0.05 versus WT unless indicated, n=4 per group, one-way ANOVA, qPCR).
expression was unchanged in all four genotypes (Supplementary Material, Fig. S2). These findings suggest that neurofibromin attenuates FGFFR1 signaling in hypertrophic growth plate chondrocytes to limit their osteoclastogenic-promoting function.

**NF1 ablation in Col10a1-positive hypertrophic chondrocytes reduces hypertrophic zone width but does not impair bone elongation**

The results above suggest a predominant role of neurofibromin, downstream of FGFFR1, in hypertrophic chondrocytes, however, neurofibromin is also expressed in prehypertrophic chondrocytes (Fig. 1) and osteoblasts (37,38,40). In an effort to determine the function of neurofibromin more specifically in hypertrophic growth plate chondrocytes, we generated Nf1Col10cKO mice lacking NF1 in Col10a1-positive cells, using the Col10-Cre driver transgenic mice (42). We first used these mice bred with Rosa26:LacZ reporter mice (43) to examine the activity of the Col10-Cre transgene specifically during the active phase of postnatal bone elongation at P0, P7 and P14, since prior studies only reported activity embryonically and shortly after birth (P1, P3) or 5 weeks after birth (42,44,45). We found, at all ages analyzed, that the Col10-Cre transgene is active in hypertrophic chondrocytes, as demonstrated by positive β-galactosidase staining in this area of the growth plate, but not in proliferative columns. β-galactosidase activity was also observed in osteoblast-like cells in the primary spongiosa and embedded osteocyte-like cells (Fig. 4A, data not shown). Nf1flox/flox mice were then bred to Col10-Cre mice to generate, through two rounds of breeding, Nf1Col10cKO mice and WT littermates. Unexpectedly, Nf1Col10cKO mice had a normal stature and physical appearance at all time points analyzed (from P0 to P18) compared with WT littermates (Fig. 4B). Histologically, the proliferation zone length of Nf1Col10cKO growth plates was not significantly shorter than WT at P0 or P18 (Fig. 4C). The hypertrophic zone of Nf1Col10cKO growth plates was, however, significantly shorter than WT at P0 and P18 (Fig. 4C). The hypertrophic zone of Nf1Col10cKO growth plates was, however, significantly shorter than WT at P0 and P18 (Fig. 4C). We also found that the number of osteoclasts at the chondro-osseous border was significantly higher in Nf1Col10cKO compared with WT mice (Fig. 4E and F). The growth plates of Nf1Col10cKO mice appeared normal with orderly proliferative chondrocyte columns as opposed to the disorganized columns seen in Nf1Col2cKO growth plates, in which NF1 is ablated in prehypertrophic and hypertrophic cells (Fig. 4G). Collectively, these results suggest that NF1 loss-of-function in prehypertrophic chondrocytes, where NF1 expression overlaps with both Fgfr3 and Fgfr1, is responsible for the size and growth plate columnar defects observed in Nf1Col2cKO mice, whereas NF1 in hypertrophic chondrocytes primarily regulates osteoclastogenesis in the primary spongiosa.

**Fgfr3 expression is not altered upon Fgfr1 ablation in Col2a1-positive chondrocytes**

Our analyses suggest that lack of NF1 in prehypertrophic chondrocytes causes alterations in the size and organization of NF1 and Fgfr1-negative, but Fgfr3-positive, proliferative chondrocyte columns. Ectopic Fgfr3 upregulation was previously observed in Fgfr1-deficient osteoblasts (7), and it has been shown that chondrocytic FGF and ERK1/2 signaling in general are inhibitory to chondrocyte proliferation/differentiation (although these effects were not attributed to an individual or specific FGFR receptor) (11,46). These observations prompted us to question whether NF1 or Fgfr1 deficiency altered growth plate Fgfr3 expression level or tissue localization, thus potentially causing some of the observed changes in proliferation and hypertrophic zones. Hence, we assessed Fgfr3 expression by in situ hybridization and qPCR analyses in the growth plates of Nf1Col2cKO and Dbl−/− mice. We found that Fgfr3 expression was not mislocalized when NF1 was deleted alone or in combination with Fgfr1, and remained primarily in the proliferation zone of the growth plate with some expression in the prehypertrophic zone at P0 (Fig. 5A). Furthermore, quantitative assessment by qPCR showed no significant differences in Fgfr3 expression in Fgfr1Col10cKO, Nf1Col10cKO and Dbl−/− growth plates versus WT growth plates 5 days after birth (Fig. 5B and C).

We also sought to confirm whether expression levels and localization of Fgfr1 were altered by NF1 deletion in Nf1Col2cKO growth plates, and the efficiency of Fgfr1 deletion with the Col2-Cre driver. We found that Fgfr1 was normally localized in Nf1Col2cKO growth plates and that Fgfr1 expression was, as expected, reduced in Dbl−/− growth plates (Fig. 5A, left column). Quantitatively, Fgfr1 expression was efficiently lowered using the Col2-Cre driver as assessed by qPCR on P5 growth plates and was not reduced in Nf1Col2cKO growth plates (Fig. 5C). Together, these results confirm efficient Fgfr1 recombination in Fgfr1Col2cKO and Dbl−/− mutant mice, and indicate that Fgfr3 expression is not upregulated in response to Fgfr1 ablation in these two models.

**Pan-FGFR inhibition with BGJ-398 enhances bone growth in Nf1Col2cKO mice**

The difference in bone size between Nf1Col2cKO and Nf1Col10cKO mice and the overlap of expression between NF1, Fgfr1 and Fgfr3 in the prehypertrophic zone suggested that neurofibromin in prehypertrophic chondrocytes may control either Fgfr3 or Fgfr1 signaling and the mechanism by which these cells inhibit chondrocyte proliferation and column stacking. To address this question and because of the difficulties in generating triple conditional knockout mice lacking Fgfr1, Fgfr3 and NF1 with proper littermate controls, we chose to administer the pan-FGFR inhibitor BGJ-398 (47) or vehicle subcutaneously and daily to WT and Nf1Col2cKO newborns. We assessed body weight, body length, tibial length and growth plate parameters at the termination of the experiment 18 days later. Pan-FGFR inhibition by this dose of BGJ-398 reduced ERK1/2 activation in both prehypertrophic and hypertrophic chondrocytes in Nf1Col2cKO mice when compared with vehicle-treated animals (Supplementary Material, Fig. S3A), confirming that the drug was active and acting, at least in part, directly on growth plate chondrocytes. BGJ-398 significantly improved tibial length and the lengths of the proliferation and hypertrophic zones in the growth plates of Nf1Col2cKO mice (Fig. 6A–C), whereas it did not impact bone growth in WT mice. Body length and weight in Nf1Col2cKO mice treated by BGJ-398 were improved compared with vehicle control, but the differences did not reach statistical significance for these two parameters (Fig. 6D and Supplementary Material, Fig. S3B). Noticeably, BGJ-398 treatment improved the columnar organization of the proliferation zone (Fig. 6E). Since the proliferation zone in Fgfr1Col10cKO mice is normal in terms of width and organization, and since NF1 is not detectable in this zone, this effect of pan-FGFR inhibition on the width and organization of the proliferation zone in Nf1Col2cKO mice suggests that neurofibromin in prehypertrophic chondrocytes, and downstream of Fgfr3 mainly, is required for normal chondrocyte proliferation and column stacking.

**Discussion**

The respective role of FGFRs and FGFR3s in the growth plate, the intracellular machinery mediating their activity, and in
particular the potential contribution of Ras-GAP proteins to these activities remain incompletely characterized, in part because of the dynamic and complex nature of the process of endochondral bone formation and the overlap in expression and signaling pathways downstream of FGFRs. This study provides the first comprehensive experimental and genetic evidence indicating that the Ras-GAP activity of neurofibromin is required in prehypertrophic chondrocytes for normal growth plate elongation and proliferative column organization, and is required in hypertrophic chondrocytes, downstream of FGFR1, to attenuate the osteoclastogenic properties of these cells in contact with the bone marrow environment, and thus the coupling between chondrogenesis and bone modeling during development.

A number of signaling proteins are phosphorylated in response to chondrocyte FGF stimulation, including Shc, PLCγ, STAT1, Gab1 and FRS2α (48–50). These signaling events lead to

Figure 4. Nf1 ablation in hypertrophic chondrocytes does not affect bone size but increases osteochondral osteoclastogenesis. (A) β-galactosidase staining (blue) in P0, 7 and 14 proximal, Col10-Cre;Rosa26lacZ tibiae shows Col10-Cre transgene activity in hypertrophic chondrocytes and primary spongiosa osteoblasts, but not in prehypertrophic chondrocytes. Sections were counterstained with nuclear fast red. Black dotted lines delineate prehypertrophic and hypertrophic zones. Scale bar: 50 μm. (B) The body length of Nf1Col10cKO versus WT mice is not significantly different at P0, P9 or 18 (*P < 0.05 versus WT, n = 5 per group, repeated measure two-way ANOVA). (C and D) Length quantification of proximal tibial proliferation (C) and hypertrophic (D) zones of P0 and 18 WT and Nf1Col10cKO mice (*P < 0.05 versus WT, n = 5 per group, unpaired t-test). The length of the proliferation and hypertrophic zones between WT and Nf1Col10cKO mice were not significantly different at P0 or P18. (E and F) TRAP quantification of the number of TRAP-positive (red) osteoclasts per osteochondral border surface in P0 (E) and P18 (F) proximal tibial sections showed that the number of osteoclasts at the chondro-osseous junction in Nf1Col10cKO mice was significantly greater than WT (P < 0.05 versus WT, n = 5 per group, unpaired t-test, scale bar: 100 μm). (G) H&E staining of proximal tibial growth plates of WT (left column) and Nf1Col10cKO (middle column) littermates at P0 and P18. Nf1Col2cKO proximal tibial growth plates (right column) are shown for comparison, with arrows highlighting disorganized proliferative columns and black dotted lines delineating the hypertrophic zones. Scale bar: 100 μm.
the activation of intracellular signaling pathways that control cell shape, proliferation, differentiation, migration and survival. In particular, the docking proteins FRS2α and FRS2β are major mediators of the Ras/MAPK and PI3K/AKT signaling pathways that fine-tune the signal initiated by FGFR stimulation by negative feedback mechanisms. This study identifies the Ras-GAP neurofibromin as one critical intracellular components controlling FGFR signaling, specifically in postmitotic growth plate chondrocytes, and further supports the notion that the intensity or duration of FGFR signals during endochondral bone formation must be tightly controlled for harmonious development of the growth plate.

Dwarfishness in humans is primarily attributed to FGFR3-activating mutations and the resulting lack of extension of the growth plate proliferating zone (13–16), where FGFR3 is highly expressed. FGFR3 activation affects chondrocyte proliferation in a cell-autonomous fashion but also indirectly via repression of IHH signaling (51). Global knockout of Fgfr3 in prehypertrophic and hypertrophic chondrocytes, but not detectable in proliferating chondrocytes, these results suggest that neurofibromin is required in prehypertrophic chondrocytes to indirectly control the extension and organization of proliferative chondrocyte columns. This conclusion is further supported by the beneficial effect of pan-FGFR inhibition on bone size, proliferation zone width and columnar organization in growing Nf1Col2cKO mice (although other systemic or indirect effects of the drug cannot be excluded). These results, along with the fact that Fgfr1Col2cKO mice have no proliferation zone size phenotype and that Fgfr1-null prehypertrophic chondrocytes still are positive for phospho-ERK (Fig. 1C), suggest that in prehypertrophic chondrocytes, neurofibromin controls FGFR3 signaling to regulate, in an indirect, paracrine fashion, chondrocyte proliferation zone

Figure 5. Fgfr3 expression is not altered upon Fgfr1 ablation in Col2a1-positive chondrocytes. (A) In situ hybridization (red signal) in P0 distal femora shows that Fgfr1 expression (left column) remains localized to hypertrophic chondrocytes in Nf1 null chondrocytes and has a less intense signal in DblCol2cKO growth plates (bottom left). Fgfr3 expression (right column) remains restricted to the proliferation and prehypertrophic zones in Nf1Col2cKO (upper right) and DblCol2cKO (lower right) growth plates. Hoechst stained nuclei appear in blue. White boxes denote hypertrophic zones. Scale bar: 100 μm. (B) Fgfr3 expression is not significantly different in FS long bone growth plate cartilage between WT, Fgfr1Col2cKO, DblCol2cKO and Nf1Col2cKO mice (one-way ANOVA, n = 4 per group, qPCR). (C) Fgfr1 expression is reduced in FS long bone growth plate cartilage from Fgfr1Col2cKO and DblCol2cKO mice when compared with WT, while there is no significant difference in Fgfr1 expression between Nf1Col2cKO and WT mice (*P < 0.05 versus WT unless indicated, n = 4 per group, one-way ANOVA, qPCR).
length and their typical columnar organization. This does not rule out a role of FGFR1 signaling in prehypertrophic chondrocytes, based on the severe dwarfishness of individuals with osteoglophonic dysplasia and mice with FGFR1-activating mutations.

Concurrent ablation of \(Nf1\) and \(Fgfr1\) in the same \(Col2a1\)-expressing cell population and at the same time during development reversed the increase in hypertrophic zone width observed in \(Fgfr1^{Col2cKO}\) mice. These findings suggest that reactivation of the Ras-Raf-MEK-ERK1/2 pathway by \(Nf1\) deletion in \(Fgfr1\)-deficient hypertrophic chondrocytes functionally compensated for the reduced FGFR1 signaling in these cells in vivo. We have shown in a previous study that neurofibromin controls \(Rankl\) expression and osteoclastogenesis in mature chondrocytes, and thus that increased growth plate catabolism likely contributes to the reduction in hypertrophic width observed in \(Nf1^{Col2cKO}\) mice (38). The observation that chondrocytic ablation of \(Nf1\) reverses the reduction in osteoclast number observed in \(Fgfr1^{Col2cKO}\) mice, and the fact that the hypertrophic zone width in \(Nf1^{Col2cKO}\) mice is shorter (while being longer in \(Fgfr1^{Col2cKO}\) mice) compared with WT mice, suggests that neurofibromin, in hypertrophic chondrocytes, inhibits the pro-osteoclastogenic signals from FGFR1 in the chondrocytic lineage. It remains to be determined if activation of the transcription factor ATF4, resulting from the lack of \(Nf1\), contributes to the observed increase in \(Rankl\) expression, as was previously shown in mature osteoblasts (53). Though RANKL and OPG are obvious candidates to mediate changes in osteoclastogenesis at the osteochondral border, and though we indeed observed significant changes in \(Rankl\) expression in vivo,
we cannot rule out the possibility that other pro- or anti-osteoclastogenic molecules are differentially regulated in the presence/absence of neurofibromin or FGFR1. It must also be emphasized that the relatively modest increase in Rankl expression in the growth plates of Nf1<sup>Col2cKO</sup> mice is to be expected based on the nature of the cells expressing this cytokine in the growth plate, i.e. hypertrophic chondrocytes. This area of the growth plate indeed represents a small volume of the growth plate, is under constant catabolism, and contains cells that are hypertrophic (hence less cell number per volume) and rapidly eliminated. These characteristics are even more pronounced in the shortened growth plates of Nf1<sup>Col2cKO</sup> mice, hence the increase in Rankl expression in this transient area cannot be of high amplitude.

The width of the hypertrophic zone depends on multiple factors, including growth plate maturation, catabolism but also chondrocyte apoptosis. However, no difference in chondrocyte apoptosis has been detected between WT mice and mice lacking FGFR1 (Fgfri<sup>Col2cKO</sup> (7) or B-Raf (54) or ERK1/2 (55) in Col2a1-expressing chondrocytes. On the other hand, we have shown in previous studies that Nf2-null chondrocytes have enhanced sensitivity to phosphate-mediated apoptosis in vitro, although we failed to detect increased in vivo apoptosis in the growth plate of Nf1<sup>Col2cKO</sup> mice (38). Chondrocyte forced expression of Spry1, which leads to ERK activation, as well as an FGFR3-activating mutation, did result in premature chondrocyte apoptosis which leads to ERK activation, as well as an FGFR3-activating mutation, did result in premature chondrocyte apoptosis in vivo (56,57), although constitutive activation of MEK1 in these cells did not affect apoptosis (11). Therefore, Fgfr1 loss-of-function does not alter the rate of chondrocyte apoptosis, but ERK constitutive activation promotes it, via mechanism(s) that remains to be characterized.

Recalcitrant bone healing (pseudarthrosis) affects about 5% of children with NF1. In this condition, tibial bowing is followed by pseudarthrosis, particularly if treatment can be applied locally (47). Histology

**Histology**

Fixed tissues were decalcified for up to 1 week (depending on age) in 20% EDTA at 4°C, followed by dehydration by graded ethanol series, cleared in xylenes (or Histo-Clear for β-galactosidase staining, National Diagnostics, Atlanta, GA, USA) and embedded in paraffin. Serial, 5-micron sagittal sections were cut, Histo-Clear deparaffinized and rehydrated by graded ethanol series. Sections were then stained with hematoxylin and eosin using standard protocols. For osteoclast analyses, sections were TRAP-stained using standard protocols. For β-galactosidase staining, tissues were stained prior to fixation using standard protocols. Briefly, tissues were washed with phosphate buffered 0.02% NP-40, 0.01% deoxycholate and 2 mM MgCl₂, followed by overnight incubation in β-galactosidase staining solution (phosphate buffered 0.02% NP-40, 0.01% deoxycholate, 2 mM MgCl₂, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-Gal) at room temperature. Tissues were then fixed, decalcified, processed and sectioned as described earlier. Sections were counterstained with nuclear fast red.

**Materials and Methods**

**Animals and drugs**

The Vanderbilt University Institutional Animal Care and Use Committee (IACUC) approved all animal procedures. To generate Db1<sup>Col2cKO</sup> mice, we employed a two-armed breeding scheme (Supplementary Material, Fig. S4). In arm one, we bred Col2a1-Cre;Fgfri<sup>1lox/lox,Nf1<sup>lox/lox/+</sup></sup> males with Fgfri<sup>1lox/lox,Nf1<sup>lox/lox/+</sup></sup> females to generate WT (cre-negative), Fgfri<sup>1lox/2lox</sup>;Col2a1-Cre;Fgfri<sup>1lox/lox,Nf1<sup>lox/lox/+</sup>,Nf2<sup>−/+</sup></sup> and Db1<sup>Col2cKO</sup>;Col2a1-Cre;Fgfri<sup>1lox/lox,Nf1<sup>lox/lox</sup>,Nf2<sup>lox/lox</sup></sup> mice (59-61). In arm two, we bred Col2a1-Cre;Fgfri<sup>1lox/lox,Nf1<sup>lox/lox/+</sup></sup> males with Fgfri<sup>1lox/lox,Nf1<sup>lox/lox</sup> females to generate WT (cre-negative), Nf1<sup>Col2cKO</sup>;Col2a1-Cre;Fgfri<sup>1lox/lox,Nf1<sup>lox/lox</sup>,Nf2<sup>lox/lox</sup></sup> and Db1<sup>Col2cKO</sup>;Col2a1-Cre;Fgfri<sup>1lox/lox,Nf1<sup>lox/lox</sup>,Nf2<sup>lox/lox</sup></sup> mice. Arm two used Col2a1-Cre;Fgfri<sup>1lox/lox,Nf1<sup>lox/lox</sup>/+</sup> males rather than Col2a1-Cre;Fgfri<sup>1lox/lox,+</sup>,Nf1<sup>lox/lox</sup>/+</sup> males because Col2a1-Cre;Nf1<sup>lox/lox</sup> mice are infertile. Progenies were obtained at a Mendelian ratio (~12.5% Fgfri<sup>1lox/2lox</sup>, ~6.25% Nf1<sup>Col2cKO</sup> and ~12.5% or ~6.25% Db1<sup>Col2cKO</sup> (depending on breeding arm), indicating the absence of embryonic lethality.

Nf1<sup>Col2cKO</sup> and WT littermate mice were obtained by breeding Col10-Cre;Nf1<sup>lox/lox</sup> males with Nf1<sup>lox/lox</sup> females (42,60). Col10-Cre; Rosa26lacZ mice were obtained by breeding homozygous Col10-Cre mice with homozygous ROSA26 reporter mice (43). WT and Nf1<sup>Col2cKO</sup> littermate mice for drug studies were obtained by breeding Col2-Cre;Nf1<sup>lox/lox</sup> males with Nf1<sup>lox/lox</sup> females (59,60). Fgfri<sup>(B6.129S4-Fgfri<sup>tm5.15Torfr</sup>/J)</sup> and Nf1<sup>(STOCK Nf1<sup>tm1Parfr</sup>)</sup> mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Col2-Cre mice were originally obtained from Dr Gerard Karsenty (Columbia University). Col10-Cre;Rosa26;LacZ mice were a gracious gift from Dr Douglas Mortlock (Vanderbilt University, Nashville, TN, USA). Col10-Cre mice were originally generated by Dr K. von der Mark (University of Erlangen-Nuremberg, Germany).

Tissues were harvested at the indicated times and fixed at 4°C for 24 h in freshly prepared 4% paraformaldehyde (Sigma, St Louis, MO, USA). Body and tibia lengths were assessed by caliper and postmortem Faxitron X-Ray (Tucson, AZ, USA), respectively. Body length was measured while animals were in the prone position and taken from the tip of the nose to the anus with a digital caliper. BGI-398 (Selleckchem, Houston, TX, USA) or vehicle (PEG300/D5W, 2:1, v/v) was injected subcutaneously daily from birth and for 18 days. BGI-398 is most potent at FGFRs 1-3 with in vitro IC50s of 0.9–1.4 nM but has off target, sub-optimal activity at other non-FGFRs at 1IC50 concentrations of 180 nM or greater (47).
BIOQUANT software to employ a direct measurement technique. Specifically, the border of a zone and the border which constitutes the interface between adjacent zones were identified manually by blinded, properly trained laboratory members. The border between resting chondrocytes and the proliferation zone was defined by the appearance of a proliferative column of four or more chondrocytes of flattened morphology. The interface between the proliferation zone and hypertrophic zones was defined by the appearance of cells of larger size (hypertrophy) at the distal end of proliferative columns. The termination of the hypertrophic zone was defined as the interface between hypertrophic chondrocytes and the primary spongiosa. Along each defined border, BIOQUANT software selected a set of systematically random points with a fixed interval set by the laboratory member. For the neighborhood around each of these points, BIOQUANT computed a vector orthogonal to the interface. Each vector was extended across each respective zone until it intersected the opposite border; the length of each vector was recorded. The width of the zone was then reported as the mean of all such vectors. The width measured by this technique does not attempt to compensate for oblique sectioning, however, all sections included for measurement were orthogonally sectioned and at a similar position within each bone. The osteochondral border surface used for osteoclast measurements was manually defined by blinded, properly trained laboratory members as the interface between the hypertrophic chondrocyte and primary spongiosa compartments, across the entire bone element. Once the interface between these two zones was defined, BIOQUANT computed the length of the interface using standard 2-dimensional distance computations to generate a surface measurement. TRAP-positive osteoclasts in contact with this osteochondral border were counted for analyses.

**In situ hybridization (ISH)**

Tissues were fixed, decalciﬁed, processed and sectioned as described earlier. Sections were stored at 4°C until use. ISH was performed by standard protocols. Fgfr1, Fgfr3, Ihh ISH was performed using probes to the respective 3' UTRs (Fgfr1 and Fgfr3 sequences available upon request, Ihh as previously published) (62). Nf1 ISH was performed using a probe to the 5' translated region of the mRNA (40). Sense and anti-sense [35S]-uridine triphosphate (Perkin Elmer, Waltham, MA, USA) probes were synthesized for hybridization as described previously (62,63). Sections were stained with Hoechst 33258 to identify nuclei. Images were processed using Adobe Photoshop (San Jose, CA, USA).

**Immunohistochemistry**

Tissues were fixed, decalciﬁed, processed and sectioned as described earlier. Antigens were retrieved on graded ethanol rehydrated sections using DeCal Retrieval Solution per manufacturer’s instructions (Biogenex, Fremont, CA, USA). Immunostaining was performed using standard protocols with anti-phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204, E10 mouse mAb #9106, Cell Signaling, Boston, MA, USA) antibodies or non-immune IgG1 antibodies, followed by ImmPACT NovaRED (Vector Laboratories, Burlingame, CA, USA) horseradish peroxidase detection of the secondary antibody. Immunostained sections were counterstained with hematoxylin.

**Genomic PCR, RT–PCR and qPCR**

For genotyping, genomic DNA was isolated from tail biopsies by sodium hydroxide digestion and PCR was performed using appropriate primers. The Nf1 floxed allele was detected with primers P1, P3 and P4, as previously described (60). The Fgfr1 floxed allele was detected with primers intr5.53 and intr5.53 to generate a 750 bp band for the floxed allele and a 564 bp band for the WT allele, as previously described (61). The Col2-Cre transgene was detected using the forward primer: GAGTTGATAAGCTGCGT GGTCGCAAGATG and reverse: TCCTCCTGTCTCTAGGGCCCTCCTG CAT to generate a 700 bp band (59). The Col10-Cre transgene was detected using the P1 and P5 primers to generate a 305 bp band, as previously described (42). Total RNA was extracted from snap-frozen P5 (to avoid the presence of the secondary ossification center) murine growth plates (1 distal femoral epiphysis and 1 proximal tibial epiphysis per sample) using TRIzol (Life Technologies, Grand Island, NY, USA), and cDNAs were synthesized from 1 microgram RNAs following DNase I treatment using the high-capacity cDNA reverse-transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR (qPCR) were performed using TaqMan gene expression assays. The probe and primer sets for Rankl (Mm00441908_m1), Opg (Mm00425462_m1), Fgfr1 (Mm00438924_m1), Fgfr3 (Mm00433294_m1) and the normalizer Hprt (Mm0046968_m1) were obtained from LifeTechnologies. Normalized fold-expression values for Rankl, Opg, Fgfr1 and Fgfr3 were calculated using relative starting quantity values obtained using standard curve qPCR and normalized by relative starting quantity values obtained from standard curve Hprt qPCR.

**Statistical analysis**

All experimental values were analyzed using GraphPad PRISM (v6.0, La Jolla, CA, USA). When experimental conditions involved two groups, an unpaired t-test was used to determine differences between groups with significance determined by obtaining a P-value of <0.05. When experimental conditions involved three or more groups, one-way ANOVA was used to determine differences between groups. If differences were detected by ANOVA (P<0.05), post hoc significance was calculated by Holm-Sidak’s method correcting for multiple comparisons with significance determined by obtaining an adjusted P-value of <0.05. For analyses tracking body length and weight, repeated measure two-way ANOVA was used; post hoc calculated significance, if any, is graphed only for the terminal time point (P18). Graphs of growth plate zone lengths were graphed as grouped analyses for space saving purposes but were analyzed by one-way ANOVA at each time point specified. Data are presented as mean ± SEM.

**Supplementary Material**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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