

# Genetic Inactivation of ERK1 and ERK2 in Chondrocytes Promotes Bone Growth and Enlarges the Spinal Canal

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**ABSTRACT:** Activating mutations in FGFR3 cause the most common forms of human dwarfism: achondroplasia and thanatophoric dysplasia. In mouse models of achondroplasia, recent studies have implicated the ERK MAPK pathway, a pathway activated by FGFR3, in creating reduced bone growth. Our recent studies have indicated that increased Fgfr3 and ERK MAPK signaling in chondrocytes also causes premature synchondrosis closure in the cranial base and vertebrae, accounting for the sometimes fatal stenosis of the foramen magnum and spinal canal in achondroplasia. Conversely, whether the decrease—or inactivation—of *ERK1* and *ERK2* promotes bone growth and delays synchondrosis closure remains to be investigated. In this study, we inactivated *ERK2* in the chondrocytes of *ERK1*-null mice using the *Col2a1-Cre* and *Col2a1-CreER* transgenes. We found that the genetic inactivation of *ERK1* and *ERK2* in chondrocytes enhances the growth of cartilaginous skeletal elements. We also found that the postnatal inactivation of *ERK1* and *ERK2* in chondrocytes delays synchondrosis closure and enlarges the spinal canal. These observations make ERK1 and ERK2 an attractive target for the treatment of achondroplasia and other FGFR3-related skeletal syndromes. © 2010 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. *J Orthop Res*

**Keywords:** ERK; chondrocytes; bone growth; synchondrosis; achondroplasia

The extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway consists of a series of kinases that play important roles in skeletal development. The ERK MAPK pathway is activated by various cytokines and growth factors, including family members of fibroblast growth factor (FGF). Activating mutations in FGF receptor 3 (FGFR3) cause the most common forms of human dwarfism—achondroplasia, thanatophoric dysplasia, and hypochondroplasia.<sup>1–6</sup> Mutations in the signaling molecules in the ERK MAPK pathway also cause a number of human skeletal disorders, including Noonan, Costello, and cardio-facio-cutaneous syndromes.<sup>7–9</sup>

The growth inhibitory roles of FGFR3 have been demonstrated in a number of mouse models and human skeletal syndromes. Mice that express a gain-of-function mutant of Fgfr3 show a dwarf phenotype similar to the human dwarfisms achondroplasia and thanatophoric dysplasia.<sup>10–13</sup> In contrast, *Fgfr3*-null mice show skeletal overgrowth similar to that seen in human camptodactyly, tall stature, and hearing loss (CATSHL) syndrome, which is caused by a loss-of-function mutation in FGFR3.<sup>14–16</sup> Several lines of evidence have indicated that the ERK MAPK pathway is a critical downstream effector of Fgfr3. Growth plate chondrocytes expressing a human achondroplasia mutant of Fgfr3, for example, show increased levels of phosphorylated ERK1 and ERK2 and their upstream kinase MEK1.<sup>17,18</sup> Furthermore, mice expressing a constitutively active mutant of MEK1 in chondrocytes show an achondroplasia-like dwarfism.<sup>17</sup> In addition, the overexpression of C-type natriuretic peptide (CNP) in chondrocytes causes

skeletal overgrowth and rescues the dwarf phenotype in a mouse model of achondroplasia.<sup>18</sup> One of the actions of CNP is to inhibit the ERK MAPK pathway.<sup>18,19</sup>

Recent work in our laboratory has indicated that Fgfr3 and the ERK MAPK pathway also regulate synchondrosis closure.<sup>20</sup> A synchondrosis consists of two opposed growth plates with a common zone of resting chondrocytes. We found premature synchondrosis closure in the vertebrae and the cranial base of human samples of homozygous achondroplasia and thanatophoric dysplasia. We also observed premature synchondrosis closure in the cranial base and vertebrae of mice expressing the Fgfr3 G374R achondroplasia mutant and mice expressing a constitutively active MEK1 mutant in chondrocytes. These observations strongly suggest that premature synchondrosis closure accounts for the foramen magnum and spinal canal stenosis in achondroplasia. A common and serious complication of achondroplasia,<sup>6,21</sup> foramen magnum and cervical spinal stenosis associated with compression of the brainstem and spinal cord can lead to sudden death during early childhood.<sup>22</sup> In addition, spinal canal stenosis can cause other neurologic complications such as radiculopathy, claudication, and paraparesis.

While increased ERK MAPK signaling has been implicated in reduced bone growth and premature synchondrosis closure, whether or not the specific inactivation of *ERK1* and *ERK2* in chondrocytes indeed accelerates bone growth and delays synchondrosis closure has remained to be investigated. In this study, we examined the effects of the genetic inactivation of *ERK1* and *ERK2* on bone growth and synchondrosis closure. We show here that the prenatal inactivation of *ERK1* and *ERK2* in chondrocytes results in increased growth of the long bones and vertebral bodies. We further show that the postnatal inactivation of *ERK1* and *ERK2* in chondrocytes delays synchondrosis closure in the

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vertebrae and enlarges the spinal canal. These observations clearly demonstrate the potential of targeting ERK1 and ERK2 for promoting bone growth and preventing premature synchondrosis closure in human dwarfisms such as achondroplasia.

## MATERIALS AND METHODS

### Mice

The institutional animal care and use committee approved all animal procedures. *ERK1*-null mice, mice with the floxed *ERK2* allele, and *Col2a1-Cre* transgenic mice were described previously.<sup>23–25</sup> The generation of *ERK1*<sup>-/-</sup>; *ERK2*<sup>lox/lox</sup>; *Col2a1-Cre* (*ERK1/2/Col2a1Cre*) mice and their initial characterization were reported elsewhere.<sup>26</sup> For postnatal analysis, we used the *Col2a1-CreER* transgene to circumvent the perinatal lethality of *ERK1/2/Col2a1Cre* mice.<sup>27</sup> One milligram tamoxifen was injected subcutaneously into *ERK1*<sup>-/-</sup>; *ERK2*<sup>lox/lox</sup>; *Col2a1-CreER* (*ERK1/2/Col2a1CreER*) and control *ERK1*<sup>-/-</sup>; *ERK2*<sup>lox/lox</sup> mice at postnatal days 4 and 6 (P4, P6), and the animals were sacrificed at P8. Alternatively, 1 mg tamoxifen was injected subcutaneously at P7 and P9, and animals were sacrificed at P14.

### Skeletal Preparations and Histological Examination

Skeletal preparations were stained with alcian blue and alizarin red following the standard protocol. Isolated bones were laid next to a ruler and photographed using a dissection microscope, a digital camera, and Leica Application software. Scion Image software (Scion Corporation, Frederick, MD) was used to measure bone lengths. For histological analysis, tissues were fixed in 10% formalin, demineralized in 0.5 M EDTA, and embedded in paraffin. Seven-micrometer thick sections were stained with hematoxylin, eosin, and alcian blue. Photographs were taken using Leica Application software, and the dimensions of the vertebral body and foramen were measured using Scion Image software. For consistency, only the 1st and 2nd lumbar vertebrae were used for the measurement. For immunostaining of CD31, sections were incubated with anti-CD31 (Abbotec, San Diego, CA) antibody followed by horseradish-peroxidase/Fab polymer conjugate secondary antibody (Invitrogen, Carlsbad, CA, Picture Kit). Non-immune rabbit IgG was used as a control. Color was developed using 3,3'-diaminobenzidine (DAB). Osteoclasts were identified by TRAP staining.<sup>26</sup> TUNEL assay was performed using ApopTag Plus Fluorescein *In Situ* Apoptosis Detection Kit (Millipore, Ballerica, MA).

### Real-Time PCR

Epiphyseal cartilage of the tibia was dissected out from *ERK1/2/Col2a1CreER* mice and control mice at P8 and stored in *RNAlater* (Applied Biosystems, Carlsbad, CA). Tissues were homogenized using Powergen 500 (Fisher, Waltham, MA). RNA was extracted using RNeasy Mini Kit (Qiagen, Germantown, MD) and reverse transcribed by High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed on the Applied Biosystems 7500 real-time PCR detection system using TaqMan probe sets (*Erk2*; Mm00442479\_m1, *Gapdh*; 4352932E, Applied Biosystems). The comparative cycle threshold (Ct) method was used to compare gene expression levels.

### Statistical Analysis of Data

Statistical analysis was performed using Analyse-it (Analyse-it Software, Leeds, UK). A one-way ANOVA analysis with a

post hoc pairwise Scheffe test was used to compare three genotypes at E18.5. The Mann–Whitney test was used to compare *ERK1/2/Col2a1CreER* and control mice at P8 and P14.

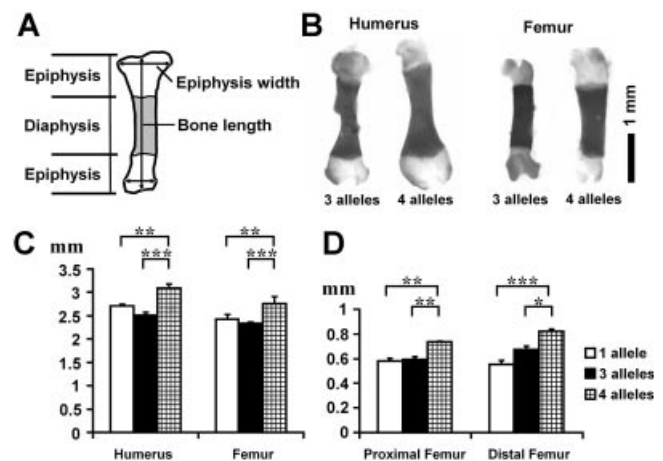
## RESULTS

### ERK1 and ERK2 Inactivation Enhances the Bone Growth of Proximal Long Bones

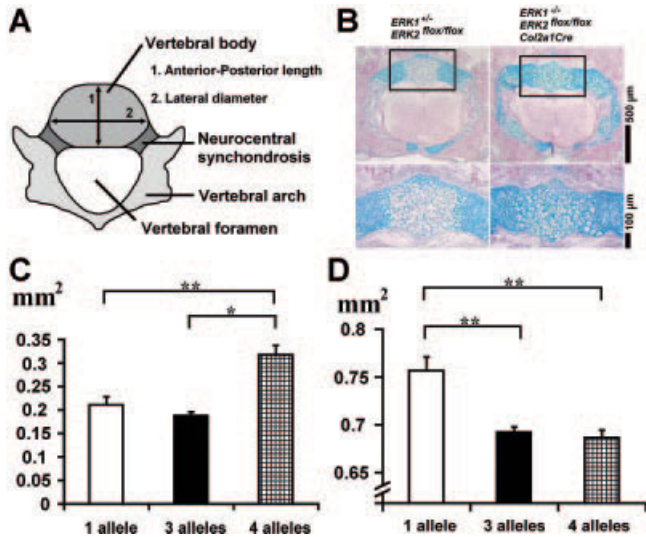
The length of each long bone was measured using skeletal preparations of E18.5 embryos (Fig. 1A). While the radius, ulna, and tibia did not show statistically significant differences among genotypes, the humerus and femur were significantly longer in *ERK1/2/Col2a1-Cre* embryos in comparison to the embryos in which only one allele (*ERK1*<sup>-/+</sup>; *ERK2*<sup>lox/lox</sup>) or three alleles (*ERK1*<sup>-/+</sup>; *ERK2*<sup>lox/lox</sup>; *Col2a1-Cre*) of *ERK1/2* were inactivated (Fig. 1B,C). The width of each epiphysis was also measured at its widest point. *ERK1/2/Col2a1Cre* embryos had significantly wider epiphyses in the proximal and distal humerus and femur (Fig. 1D). *ERK1/2/Col2a1Cre* embryos also showed wider epiphyses in the distal tibia (data not shown). These observations indicate that the inactivation of *ERK1* and *ERK2* in chondrocytes causes increased bone growth that is more pronounced in the proximal long bones.

### ERK1 and ERK2 Inactivation Promotes the Growth of Cartilaginous Vertebrae

We also examined the dimensions of the vertebrae using histological sections of the lumbar vertebrae at E18.5 (Fig. 2A). Hematoxylin, eosin, and alcian blue staining showed a delay in ossification in *ERK1/2/Col2a1Cre* embryos compared with littermate control embryos (Fig. 2B). While the vertebral body of control embryos was mostly ossified, the vertebral body of *ERK1/2/*



**Figure 1.** Skeletal preparation and long bone measurements at E18.5. The inactivation of all four alleles of *ERK1* and *ERK2* in chondrocytes promotes bone growth. (A) Scheme of long bone measurement. (B) Skeletal preparation of the femur and humerus of *ERK1*<sup>-/+</sup>; *ERK2*<sup>lox/lox</sup>; *Col2a1-Cre* (three alleles) and *ERK1*<sup>-/-</sup>; *ERK2*<sup>lox/lox</sup>; *Col2a1-Cre* (four alleles) embryos at E18.5. (C) Length of humerus and femur at E18.5. (D) Epiphysis width of proximal and distal femur at E18.5. Values are the mean  $\pm$  SD. One allele: *ERK1*<sup>-/+</sup>; *ERK2*<sup>lox/lox</sup> ( $n = 10$ ), three alleles: *ERK1*<sup>-/+</sup>; *ERK2*<sup>lox/lox</sup>; *Col2a1-Cre* ( $n = 10$ ), four alleles: *ERK1*<sup>-/-</sup>; *ERK2*<sup>lox/lox</sup>; *Col2a1-Cre* ( $n = 5$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

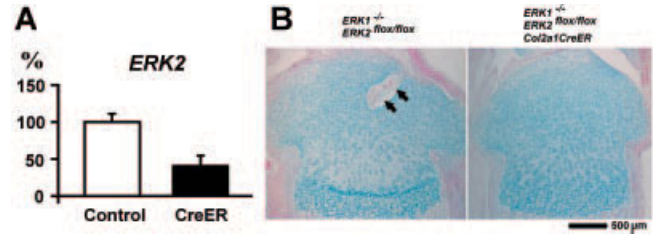


**Figure 2.** Histology and measurement of lumbar vertebrae at E18.5. (A) Scheme of vertebral measurement. (B) Cross-section of lumbar vertebrae stained with hematoxylin, eosin, and alcian blue showing a delay in the ossification of vertebral body and arch in *ERK1*<sup>-/-</sup>; *ERK2*<sup>flox/flox</sup>; *Col2a1-Cre* embryos. The vertebral body (boxed area in the upper panel) is magnified in the lower panel. (C) Cross-sectional area of vertebral body at E18.5. (D) Cross-sectional area of vertebral foramen at E18.5. Values are the mean ± SD. One allele: *ERK1*<sup>-/+</sup>; *ERK2*<sup>flox/flox</sup> (*n* = 10), three alleles: *ERK1*<sup>-/+</sup>; *ERK2*<sup>flox/flox</sup>; *Col2a1-Cre* (*n* = 10), four alleles: *ERK1*<sup>-/-</sup>; *ERK2*<sup>flox/flox</sup>; *Col2a1-Cre* (*n* = 5). \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

*Col2a1Cre* embryos remained entirely cartilaginous. *ERK1/2/Col2a1Cre* embryos also showed the persistent presence of hypertrophic chondrocytes both in the vertebral body and arch. The cross-sectional area of the vertebral body was significantly larger in *ERK1/2/Col2a1Cre* embryos compared with control embryos, indicating increased growth (Fig. 2C). The vertebral body of *ERK1/2/Col2a1Cre* embryos was larger both in the anterior–posterior and lateral directions (data not shown). In contrast to the measurements of the vertebral body, the cross-sectional area of the vertebral foramen was significantly smaller in *ERK1/2/Col2a1Cre* embryos compared with control *ERK1*<sup>-/+</sup>; *ERK2*<sup>flox/flox</sup> embryos (Fig. 2D).

**Postnatal ERK1 and ERK2 Inactivation Delays Synchronosis Closure and Enlarges the Spinal Canal**

To examine the roles of ERK1/2 in postnatal skeletal development, we inactivated *ERK2* using the *Col2a1-CreER* transgene. Following tamoxifen injection at P4 and P6, *ERK2* expression in the tibial epiphysis was inhibited about 60% in *ERK1/2/Col2a1CreER* mice at P8 (Fig. 3A). Histological analysis indicated that the overall architecture of the epiphysis was not significantly affected (Fig. 3B). However, *ERK1/2/Col2a1CreER* mice consistently showed a delay in vascular invasion in the developing secondary ossification centers at P8 (Fig. 3B). We further examined VEGF expression in the epiphyses by real time PCR, but we did not observe differences between *ERK1/2/Col2a1CreER* and control mice (data not shown). There were no obvious differ-

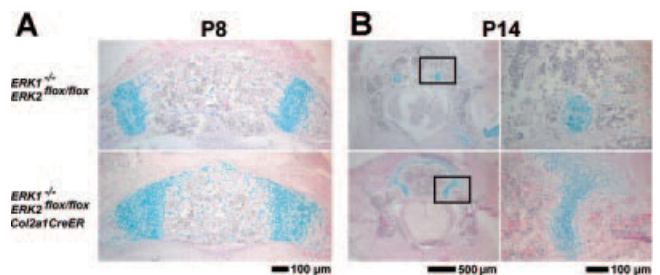


**Figure 3.** *ERK2* expression and histology of tibial epiphysis of *ERK1*<sup>-/-</sup>; *ERK2*<sup>flox/flox</sup> and *ERK1*<sup>-/-</sup>; *ERK2*<sup>flox/flox</sup>; *Col2a1-CreER* mice at P8 following tamoxifen injection at P4 and P6. (A) Real-time PCR analysis of *ERK2* expression in the tibial epiphysis. Control: *ERK1*<sup>-/+</sup>; *ERK2*<sup>flox/flox</sup> (*n* = 5), CreER: *ERK1*<sup>-/+</sup>; *ERK2*<sup>flox/flox</sup>; *Col2a1-CreER* (*n* = 6). Values are the mean ± SD. (B) Hematoxylin, eosin, and alcian blue staining of the proximal tibia at P8. Arrows indicate vascular invasion in the developing secondary ossification center.

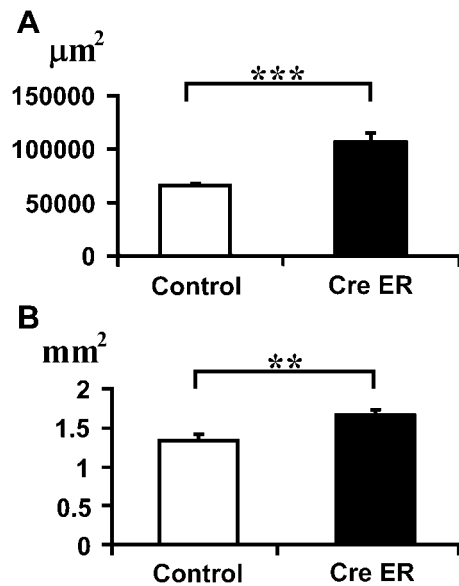
ences in the dimensions of long bones at P8 and P14 (data not shown).

Histological analysis at P8 and P14 of the vertebrae of *ERK1/2/Col2a1CreER* mice showed a significant delay in the closure of neurocentral synchondroses, which connect the ossification centers in the vertebral body and arch (Fig. 4A,B). The cross-sectional area of the neurocentral synchondrosis was significantly greater in *ERK1/2/Col2a1CreER* mice at P8, indicating a delay in cartilage resorption (*p* < 0.001) (Fig. 5A). At P14, the neurocentral synchondroses were closed in five out of eight control *ERK1*<sup>-/-</sup>; *ERK2*<sup>flox/flox</sup> mice, while the synchondroses were open in all seven *ERK1/2/Col2a1CreER* mice. Furthermore, the cross-sectional area of the vertebral foramen was significantly greater in *ERK1/2/Col2a1CreER* mice both at P8 (*p* < 0.01) and P14 (*p* < 0.01), indicating that postnatal ERK1/2 inactivation in chondrocytes enlarges the spinal canal (Fig. 5B and data not shown).

Since *ERK1/2/Col2a1-CreER* mice showed a delay in cartilage resorption, we examined the number of osteoclasts by TRAP staining and examined chondrocyte apoptosis by TUNEL assay. We did not observe obvious differences in the number of osteoclasts and in chondrocyte apoptosis between genotypes (data not shown). We also examined vascular invasion by



**Figure 4.** Hematoxylin, eosin, and alcian blue staining of the lumbar vertebrae, showing a delay in synchondrosis closure in *ERK1*<sup>-/-</sup>; *ERK2*<sup>flox/flox</sup>; *Col2a1-CreER* mice (lower panels) compared with *ERK1*<sup>-/+</sup>; *ERK2*<sup>flox/flox</sup> mice (upper panels). (A) Vertebral body and neurocentral synchondroses at P8 following tamoxifen injection at P4 and P6. (B) Lumbar vertebrae at P14 following tamoxifen injection at P7 and P9. Neurocentral synchondroses (boxed areas in the left panels) are magnified in the right panels.

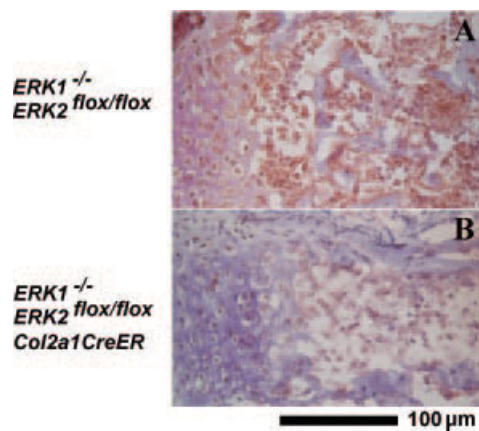


**Figure 5.** Cross-sectional area of neurocentral synchondrosis (A) and vertebral foramen (B) of lumbar vertebrae at P8. Values are the mean ± SD. Control: *ERK1*<sup>-/-</sup>; *ERK2*<sup>flox/flox</sup> (n = 5), CreER: *ERK1*<sup>-/-</sup>; *ERK2*<sup>flox/flox</sup>; *Col2a1-CreER* (n = 7). \*\**p* < 0.01, \*\*\**p* < 0.001.

immunohistochemistry for endothelial marker CD31. We observed decreased staining for CD31 in endothelial cells surrounding the neurocentral synchondroses of *ERK1/2/Col2a1-CreER* mice, suggesting reduced vascular invasion (Fig. 6). This is consistent with the delayed vascularization of the developing secondary ossification center in the long bones. Reduced vascular invasion may at least in part account for the delayed closure of neurocentral synchondroses.

## DISCUSSION

Our histological analyses and skeletal measurements of *ERK1/2/Col2a1Cre* embryos clearly indicated that *ERK1/2* is a negative regulator of endochondral bone growth. The inactivation of all four alleles of *ERK1* and



**Figure 6.** Reduced immunostaining for PECAM-1/CD31 adjacent to the neurocentral synchondrosis of *ERK1*<sup>-/-</sup>; *ERK2*<sup>flox/flox</sup>; *Col2a1-CreER* mice at P8. (A) *ERK1*<sup>-/-</sup>; *ERK2*<sup>flox/flox</sup>, (B) *ERK1*<sup>-/-</sup>; *ERK2*<sup>flox/flox</sup>; *Col2a1-CreER*.

*ERK2* resulted in longer and wider epiphyses of the long bones and larger vertebral bodies. These findings are consistent with our previous observation that increased MEK1 signaling in chondrocytes inhibits endochondral bone growth.<sup>17</sup> While the proximal long bones—the humerus and femur—were significantly longer in *ERK1/2/Col2a1Cre* embryos, the phenotype was less obvious in the distal bones. Similar overgrowth of the proximal long bones was also described in *Fgfr3*-deficient mice.<sup>14</sup> Therefore, it is interesting to speculate that chondrocytes in the proximal long bones are more sensitive to *Fgfr3* and its downstream *ERK1/2* signaling compared with chondrocytes in the distal bones. The observed difference may also be relevant to human achondroplasia, which is characterized by rhizomelic dwarfism, a disproportionate dwarfing of the proximal bones.<sup>6</sup> In contrast to our findings with *ERK1/2/Col2a1Cre* embryos, we did not observe obvious differences in the postnatal growth of long bones in *ERK1/2/Col2a1CreER* mice. It is possible that our 60% inhibition of *ERK2* in addition to *ERK1* is not sufficient to promote growth in these mice.

The increased bone growth of *ERK1/2/Col2a1Cre* embryos may be due to the increase in chondrocyte hypertrophy. We have recently found that *ERK1/2/Col2a1Cre* embryos show a remarkable expansion of the zone of hypertrophic chondrocytes in the long bones, while chondrocyte proliferation is strongly inhibited.<sup>26</sup> It has been shown that chondrocyte hypertrophy is a major determinant of longitudinal bone growth.<sup>28,29</sup> Chondrocyte hypertrophy is also likely to account for the growth in the width in addition to periosteal apposition. Since *ERK1/2/Col2a1Cre* embryos do not show an obvious periosteal phenotype, periosteal apposition may not be significantly affected in these embryos. The increase in the size of the vertebral body is also associated with the predominant presence of hypertrophic chondrocytes in our current study. Furthermore, these observations are consistent with our previous study showing that increased MEK1 signaling inhibits chondrocyte hypertrophy and bone growth.<sup>17</sup>

Since spinal canal stenosis is a common and serious complication of achondroplasia, the effects of *ERK1/2* inactivation on spinal canal development are of particular interest. While *ERK1/2/Col2a1Cre* embryos showed increased dimensions of the vertebral body in both the anterior–posterior and lateral directions, these embryos also showed a narrower spinal canal at E18.5. The increased growth of the cartilaginous vertebral body toward the spinal canal may account for the narrower spinal canal in *ERK1/2/Col2a1Cre* embryos. In contrast to *ERK1/2* inactivation in the prenatal period, the postnatal inactivation of *ERK1* and *ERK2* resulted in a delay in the closure of the neurocentral synchondroses and an increase in the size of the vertebral foramen, indicating that *ERK1/2* regulates the timing of synchondrosis closure and the size of the spinal canal. We have previously found premature synchondrosis closure in mouse models of achondroplasia and in human



samples of homozygous achondroplasia and thanatophoric dysplasia.<sup>20</sup> Our observations in *ERK1/2/Col2a1CreER* mice indicate that *ERK1* and *ERK2* would be a promising therapeutic target for spinal canal stenosis caused by activating mutations in *FGFR3*. Our results in *ERK1/2/Col2a1Cre* embryos also suggest that *ERK1/2* inactivation before the ossification of the vertebral body could result in a narrower spinal canal. The timing of *ERK1* and *ERK2* inactivation would be critical for the successful enlargement of the spinal canal in the future treatment of achondroplasia.

In summary, we have shown that genetic *ERK1* and *ERK2* inactivation in chondrocytes enhances the growth of cartilaginous skeletal elements. We have also shown that the postnatal inactivation of *ERK1* and *ERK2* in chondrocytes delays synchondrosis closure and enlarges the spinal canal. The efficacy of ERK inactivation for the treatment of *FGFR3*-related skeletal syndromes should be further tested by inactivating *ERK1* and *ERK2* in mouse models with activating *Fgfr3* mutations.

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