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RESEARCH ARTICLE

c-Raf promotes angiogenesis during normal growth plate maturation

Eva S. Liu[1,2,3], Adalbert Raimann[2,3,4], Byongsoo Timothy Chae[2], Janaina S. Martins[2,3], Manuela Baccarini[5] and Marie B. Demay[2,3,*]

ABSTRACT

Extracellular phosphate plays a key role in growth plate maturation by inducing Erk1/2 (Mapk3/1) phosphorylation, leading to hypertrophic chondrocyte apoptosis. The Raf kinases induce Mek1/2 (Map2k1/2) and Erk1/2 phosphorylation; however, a role for Raf kinases in endochondral bone formation has not been identified. Ablation of both A-Raf (Araf) and B-Raf (Braf) in chondrocytes does not alter growth plate maturation. Because c-Raf (Raf1) phosphorylation is increased by extracellular phosphate and c-Raf is the predominant isoform expressed in hypertrophic chondrocytes, chondrocyte-specific *c-Raf* knockout mice (*c-Raf$^{f/f}$;ColII-Cre^{+}*) were generated to define a role for c-Raf in growth plate maturation. *In vivo* studies demonstrated that loss of c-Raf in chondrocytes leads to expansion of the hypertrophic layer of the growth plate, with decreased phospho-Erk1/2 immunoreactivity and impaired hypertrophic chondrocyte apoptosis. However, cultured hypertrophic chondrocytes from these mice did not exhibit impairment of phosphate-induced Erk1/2 phosphorylation. Studies performed to reconcile the discrepancy between the *in vitro* and *in vivo* hypertrophic chondrocyte phenotypes revealed normal chondrocyte differentiation in *c-Raf$^{f/f}$;ColII-Cre^{+}* mice and lack of compensatory increase in the expression of A-Raf and B-Raf. However, VEGF (Vegfa) immunoreactivity in the hypertrophic chondrocytes of *c-Raf$^{f/f}$;ColII-Cre^{+}* mice was significantly reduced, associated with increased ubiquitylation of VEGF protein. Thus, c-Raf plays an important role in growth plate maturation by regulating vascular invasion, which is crucial for replacement of terminally differentiated hypertrophic chondrocytes by bone.

KEY WORDS: c-Raf, Growth plate, Chondrocyte apoptosis, VEGF, Angiogenesis, Mouse

INTRODUCTION

During endochondral bone formation, mesenchymal cells condense to differentiate into proliferative chondrocytes. These cells then differentiate into pre-hypertrophic chondrocytes, which undergo terminal differentiation to become hypertrophic chondrocytes (Kronenberg, 2003). Hypertrophic chondrocytes secrete angiogenic factors that promote vascular invasion and undergo apoptosis, leading to replacement of cartilage with bone (Carlevaro et al., 2000; Maes et al., 2010). Vascular endothelial growth factor A (VEGF; VEGF-A or Vegfa) is an angiogenic factor secreted by hypertrophic chondrocytes that is crucial for growth plate maturation (Carlevaro et al., 2000; Maes et al., 2004). Blocking VEGF action in growing mice by administration of a VEGF decoy receptor leads to expansion of the hypertrophic chondrocyte layer of the growth plate, suggesting that VEGF is required for replacement of the hypertrophic chondrocytes by bone (Gerber et al., 1999).

Mek1/2-Erk1/2 (Mapk3/1-Map2k1/2) signaling is crucial for normal endochondral bone formation. Ablation of Erk1/2 in chondrocytes using ColII-Cre or Osx-Cre leads to widening of the hypertrophic chondrocyte layer of the growth plate (Matsushita et al., 2009; Chen et al., 2015b), establishing the crucial role of Erk1/2 signaling in growth plate maturation. Mice expressing a constitutively active *Mek1* transgene in chondrocytes exhibit narrower zones of hypertrophic chondrocytes (Murakami et al., 2004), consistent with observations that Mek1/2 signaling modulates Erk1/2 phosphorylation during endochondral bone formation. Treatment of mice with a Mek1/2 inhibitor prevents Erk1/2 phosphorylation in hypertrophic chondrocytes, impairing hypertrophic chondrocyte apoptosis and leading to an expansion of the hypertrophic chondrocyte layer of the growth plate (Miedlich et al., 2010). Supporting a key role for Erk1/2 phosphorylation in hypertrophic chondrocyte apoptosis, PTH/PTHrP signaling suppresses both Erk1/2 phosphorylation and hypertrophic chondrocyte apoptosis *in vivo* in mice and in cultures of primary hypertrophic chondrocytes (Liu et al., 2014).

Previous studies have demonstrated that induction of Erk1/2 phosphorylation by extracellular phosphate promotes hypertrophic chondrocyte apoptosis *in vivo* and *in vitro* (Miedlich et al., 2010). Correspondingly, inhibition of Mek1/2 in cultured hypertrophic chondrocytes impairs phosphate-induced Erk1/2 phosphorylation (Kimata et al., 2010; Miedlich et al., 2010). These experiments define a crucial role for phosphate-induced Erk1/2 phosphorylation in growth plate maturation and demonstrated that Mek1/2 mediates the effects of phosphate on Erk1/2 phosphorylation.

The Raf kinases A-Raf, B-Raf and c-Raf (Araf, Braf and Raf1, respectively – Mouse Genome Informatics) activate Mek1/2 (Wojnowski et al., 2000; Cseh et al., 2014); however, a role for these kinases in growth plate maturation has not been identified. A-Raf and B-Raf expression in the growth plate is reported to be limited to proliferative chondrocytes (Provot et al., 2008). Ablation of A-Raf and B-Raf in chondrocytes does not lead to abnormalities in chondrocyte differentiation or growth plate maturation in embryonic mice (Provot et al., 2008). Because c-Raf is the predominant isoform expressed in hypertrophic chondrocytes (Kaneko et al., 1994), and c-Raf phosphorylation is induced by extracellular phosphate (Kimata et al., 2010), mice with chondrocyte-specific c-Raf ablation were generated to identify a role for Raf signaling in growth plate maturation.

[1]Division of Endocrinology, Diabetes, and Hypertension, Brigham and Women's Hospital, Boston, MA 02115, USA. [2]Endocrine Unit, Massachusetts General Hospital, Boston, MA 02114, USA. [3]Harvard Medical School, Boston, MA 02115, USA. [4]Department of Pediatrics and Adolescent Medicine, Medical University Vienna, 1090, Vienna, Austria. [5]Department of Microbiology, Immunobiology and Genetics, Center of Molecular Biology, Max F. Perutz Laboratories, University of Vienna, Doktor-Bohr-Gasse 9, Vienna 1030, Austria.

*Author for correspondence (demay@helix.mgh.harvard.edu)

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RESULTS

Chondrocyte-specific ablation of c-Raf leads to expansion of the hypertrophic chondrocyte layer of the growth plate and decreased hypertrophic chondrocyte apoptosis *in vivo*

Extracellular phosphate induces hypertrophic chondrocyte apoptosis through activation of the Mek1/2-Erk1/2 signaling pathway (Miedlich et al., 2010). Since extracellular phosphate induces c-Raf phosphorylation, which is known to activate Mek1/2-Erk1/2 signaling, and c-Raf is the predominant isoform in hypertrophic chondrocytes (Kaneko et al., 1994; Provot et al., 2008), investigations were undertaken to identify a role for c-Raf in growth plate maturation. Mice expressing Cre recombinase under the control of collagen type II (*Col2a1*) regulatory elements (ColII-Cre) were mated to mice with exon 3 floxed *c-Raf* alleles (*c-Raf*$^{f/f}$) (Jesenberger et al., 2001) to generate mice with chondrocyte-specific c-Raf ablation (*c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$). *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ mice were born at normal Mendelian frequency and did not exhibit evidence of growth retardation relative to their control *c-Raf*$^{f/f}$*;ColII-Cre*$^{-}$ littermates.

Analyses of the growth plate phenotype demonstrated that chondrocyte-specific ablation of c-Raf leads to an expansion of the growth plate [embryonic day (E) 18.5 and postnatal day (P) 2, 18 and 35 tibiae]. This is due to expansion of the hypertrophic chondrocyte layer, as observed with H&E staining and *in situ* hybridization for collagen type X (*ColX*; *Col10a1*) and osteopontin (*Op*; *Spp1*), which are markers of hypertrophic chondrocytes (Fig. 1). The number of hypertrophic chondrocytes per column was significantly greater in the growth plates of *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ mice than in those of control mice at all time points examined (E18.5, 11.5±0.7 versus 7.5±0.7; P2, 19.7±1.5 versus 15.0±1.0; P18, 11.3±1.2 versus 6.0±1.0; P35, 7.7±0.6 versus 3.7±0.6; $P<0.05$ for all time points). Expansion of the hypertrophic chondrocyte layer in the *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ mice was associated with a decrease in p-Erk1/2 immunoreactivity at E18.5, P2 and P18 (Fig. 1), but not P35 (Fig. 1).

Because inhibition of Erk1/2 phosphorylation impairs hypertrophic chondrocyte apoptosis (Miedlich et al., 2010), TUNEL assays were performed to determine if growth plate expansion in the *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ mice was associated with impaired hypertrophic chondrocyte apoptosis. Consistent with the decrease in phospho (p)-Erk1/2 immunoreactivity, a decrease in hypertrophic chondrocyte apoptosis was observed at E15.5, P2 and P18 in *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ mice (Fig. 2A,B; TUNEL-positive cells in control versus *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$: E15.5, 10.8±1.3 versus 1.3±1.0; P2, 3.3±1.5 versus 1.0±0.9; P18, 4.5±1.9 versus 1.6±0.7; $P<0.05$ for all time points). Normalization of hypertrophic chondrocyte apoptosis paralleled the restoration of p-Erk1/2 immunoreactivity in P35 *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ mice (Fig. 2A,B; TUNEL-positive cells in control versus *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$: 6.2±1.3 versus 5.7±2.2; $P=0.64$).

In vitro investigations have shown that extracellular phosphate activates the mitochondrial apoptotic pathway resulting in cleavage of caspase 9 (Miedlich et al., 2010). Consistent with impaired activation of the mitochondrial apoptotic pathway, cleaved caspase 9 immunoreactivity was attenuated in hypertrophic chondrocytes of *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ tibiae relative to that observed in control mice (Fig. 2C).

c-Raf is not required for activation of phosphate-induced Erk1/2 phosphorylation *in vitro*

Extracellular phosphate induces Erk1/2 phosphorylation in hypertrophic chondrocytes, leading to hypertrophic chondrocyte apoptosis (Miedlich et al., 2010; Liu et al., 2014). Based on the decrease in p-Erk1/2 immunoreactivity in the hypertrophic zone of the growth plates in *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ mice, studies were undertaken to address the role of c-Raf in phosphate-induced Erk1/2 phosphorylation *in vitro*. Hypertrophic chondrocytes from *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ and control mice were treated with sodium phosphate or sodium sulfate for 30 min prior to the isolation of cell lysates (Fig. 3). The dose-dependent induction of p-Erk1/2 observed with phosphate treatment of hypertrophic chondrocytes from *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ was indistinguishable from that of their control littermates, demonstrating that c-Raf is not essential for phosphate-induced Erk1/2 phosphorylation *in vitro*.

c-Raf is not essential for chondrocyte differentiation *in vitro*

Studies were undertaken to evaluate the discrepancy between the *in vitro* and *in vivo* effects of c-Raf ablation on Erk1/2 phosphorylation in hypertrophic chondrocytes. To determine whether expansion of the hypertrophic chondrocyte layer and decreased p-Erk1/2 in *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ mice are a result of impaired chondrocyte differentiation, P2 costal chondrocytes were cultured for 3 to 14 days to evaluate the program of chondrocyte differentiation. Chondrocytes isolated from *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ mice demonstrated a progressive decrease in *ColX* expression accompanied

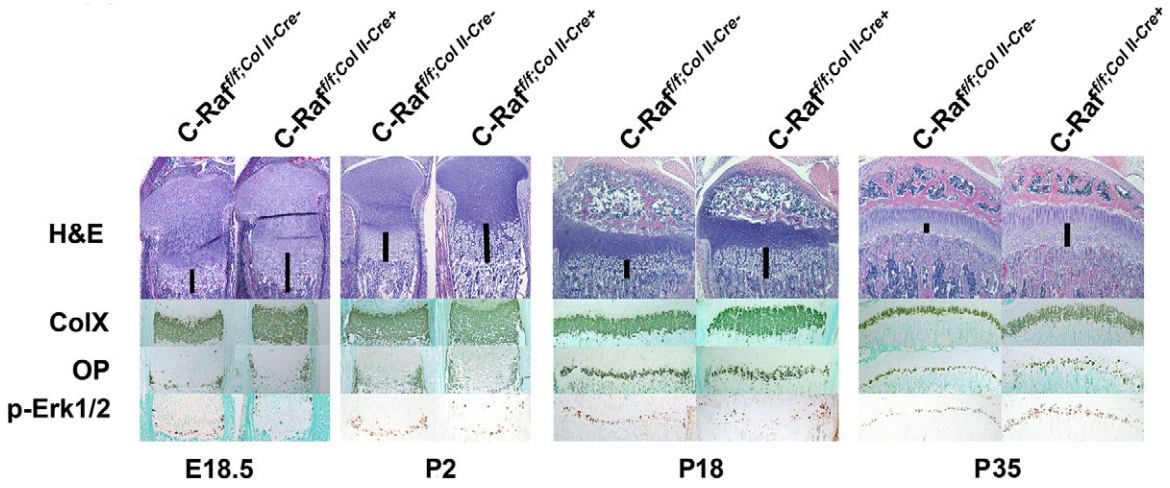

Fig. 1. c-Raf is required for normal growth plate maturation. H&E staining, *in situ* hybridization for collagen type X (*ColX*) and osteopontin (*Op*), and immunohistochemistry for p-Erk1/2 were performed on tibiae from E18.5, P2, P18 and P35 *c-Raf*$^{f/f}$*;ColII-Cre*$^{-}$ (control) and *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ mice. Data are representative of three mice per genotype. Bars indicate the length of the hypertrophic chondrocyte layer.

Development (2016) 143, 348-355 doi:10.1242/dev.127142

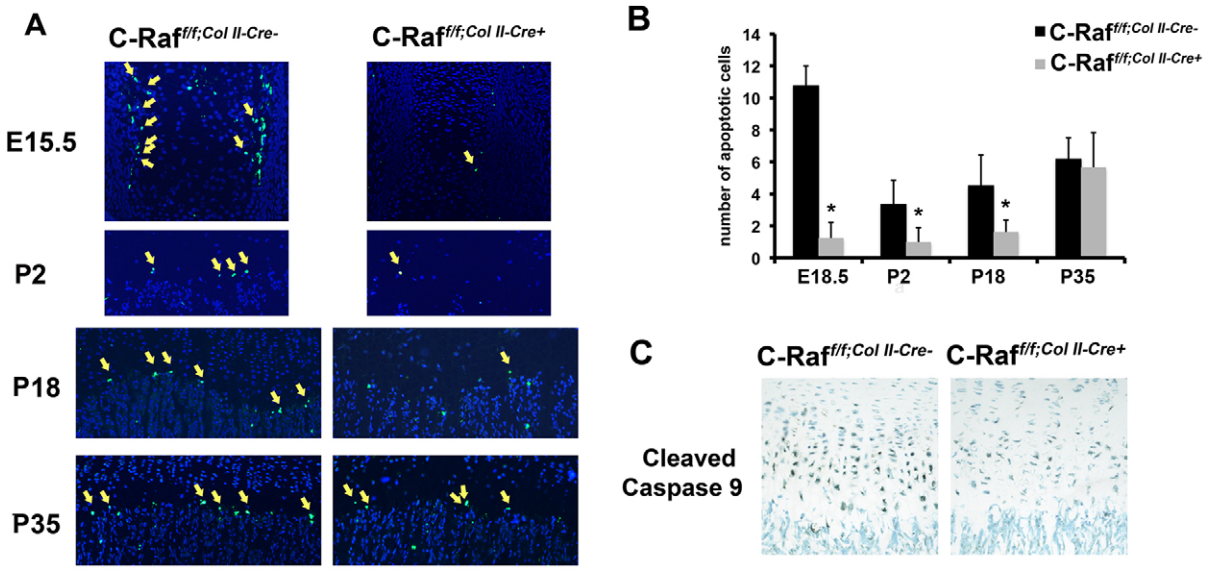

Fig. 2. Ablation of c-Raf in chondrocytes impairs hypertrophic chondrocyte apoptosis *in vivo*. (A) TUNEL labeling (green) was performed on the radii from E15.5 and the tibiae from P2, P18 and P35 *c-Raf$^{f/f}$;ColII-Cre^{-}* (control) and *c-Raf$^{f/f}$;ColII-Cre^{+}* mice. Arrows indicate TUNEL-positive cells. Blue, DAPI-stained nuclei. (B) TUNEL-positive cells were quantitated in the distal two rows of the hypertrophic chondrocyte layer. Data are representative of three mice per genotype. *P<0.05, versus control mice. Error bars indicate s.d. (C) Immunohistochemistry for cleaved caspase 9 performed on tibiae from P18 *c-Raf$^{f/f}$;ColII-Cre^{-}* and *c-Raf$^{f/f}$;ColII-Cre^{+}* mice. Data are representative of three mice per genotype.

by increasing expression of *Op*, a marker of terminally differentiated hypertrophic chondrocytes, from day 3 to day 14 in culture, similar to that observed in the chondrocyte cultures of control mice (Fig. 4A).

To determine whether incomplete ablation of c-Raf or compensatory increases in A-Raf or B-Raf were responsible for the *in vitro* phenotype, mRNA expression of the three Raf isoforms was evaluated. RT-qPCR (Fig. 4A) of RNA isolated from control and *c-Raf$^{f/f}$;ColII-Cre^{+}* primary chondrocytes on days 3, 7 and 14 in culture revealed a 94.0±2.7% reduction in exon 3-containing *c-Raf* transcripts in the *c-Raf$^{f/f}$;ColII-Cre^{+}* mice, demonstrating efficient c-Raf ablation (Fig. 4A). RT-qPCR did not demonstrate a compensatory upregulation in the expression of *A-Raf* or *B-Raf* mRNA in the hypertrophic chondrocytes of *c-Raf$^{f/f}$;ColII-Cre^{+}* mice (Fig. 4A). To exclude the possibility that c-Raf ablation in cells other than chondrocytes results in the phenotype observed, immunohistochemical analyses on P18 tibiae were performed. These data confirmed that c-Raf is expressed in hypertrophic chondrocytes of control mice and that the *ColII-Cre* transgene ablates c-Raf protein in hypertrophic chondrocytes, but not in cortical osteocytes or trabecular osteoblasts of *c-Raf$^{f/f}$;ColII-Cre^{+}* mice (Fig. 4B).

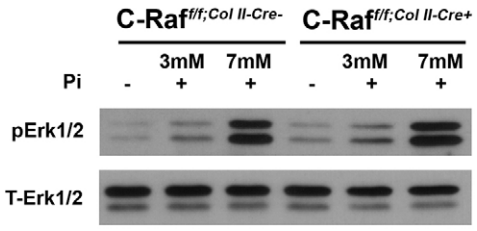

Fig. 3. c-Raf is not necessary for phosphate-induced Erk1/2 phosphorylation *in vitro*. Hypertrophic chondrocytes were incubated with 3 mM or 7 mM sodium sulfate (–) or sodium phosphate (+) for 30 min prior to western analysis of whole-cell extracts for phospho (p) and total (T) Erk1/2. Data are representative of three independent chondrocyte preparations per genotype.

The observation that phosphate-induced Erk1/2 phosphorylation is not impaired in hypertrophic chondrocytes of *c-Raf$^{f/f}$;ColII-Cre^{+}* mice *in vitro* suggested that the *in vivo* phenotype could be secondary to impaired paracrine signaling, which directly or indirectly modulates chondrocyte Erk1/2 phosphorylation. Thus, the expression of signaling molecules and transcription factors that regulate chondrocyte hypertrophy and apoptosis in the developing and maturing growth plate was examined in intact neonatal femurs. Fgf2 and Fgf18, secreted by perichondrial cells, promote initiation of chondrocyte hypertrophy and hypertrophic chondrocyte apoptosis, respectively (Sahni et al., 2001; Liu et al., 2002), whereas Wnt5a, Hdac4 and Nkx3.2 inhibit chondrocyte hypertrophy (Vega et al., 2004; Provot et al., 2006; Park et al., 2007; Bradley and Drissi, 2010). However, RT-qPCR failed to detect a difference in expression of the transcripts encoding these proteins using RNA isolated from whole femurs or from the distal femoral metaphysis of P1 *c-Raf$^{f/f}$;ColII-Cre^{+}* mice relative to control mice (data not shown).

Chondrocyte-specific ablation of c-Raf impairs vascular invasion at the growth plate

During endochondral bone development, hypertrophic chondrocytes secrete angiogenic factors that promote vascular invasion, leading to apoptosis of hypertrophic chondrocytes (Gerber et al., 1999). VEGF, produced by hypertrophic chondrocytes, is an important inducer of vascular invasion during endochondral bone formation (Carlevaro et al., 2000). It is expressed as multiple alternatively spliced isoforms, of which VEGF164 and VEGF188 have been shown to be crucial for normal metaphyseal vascularization (Maes et al., 2004).

To determine whether c-Raf ablation alters VEGF expression in hypertrophic chondrocytes, VEGF mRNA and protein levels were examined in hypertrophic chondrocytes from *c-Raf$^{f/f}$;ColII-Cre^{+}* and control mice. Although mRNA levels of *Vegf164* and *Vegf188* were not altered in the chondrocytes from *c-Raf$^{f/f}$;ColII-Cre^{+}* mice relative to those from control mice (Fig. 5A), western analyses demonstrated a decrease in VEGF protein levels in chondrocytes cultured from *c-Raf$^{f/f}$;ColII-Cre^{+}* mice relative to those of control littermates

Development (2016) 143, 348-355 doi:10.1242/dev.127142

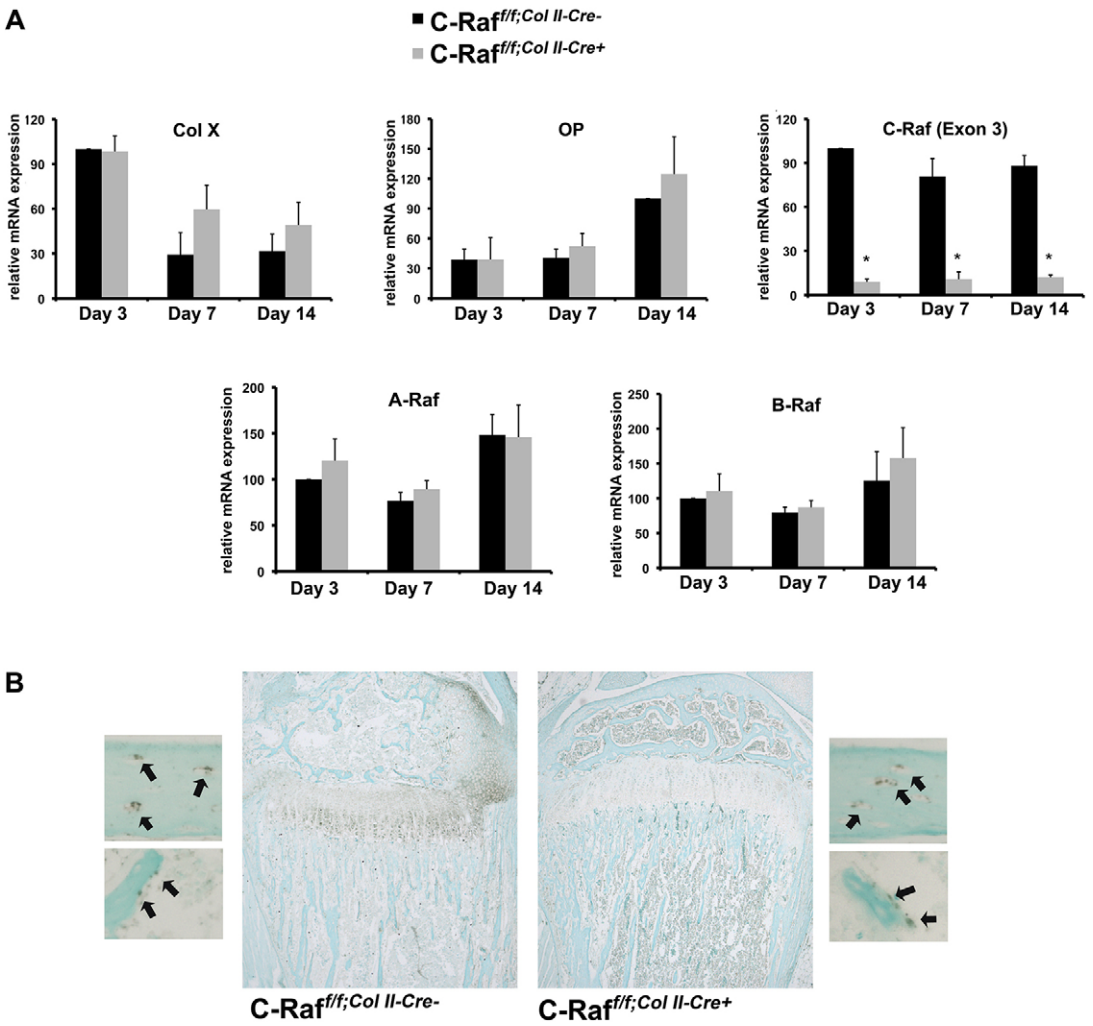

Fig. 4. c-Raf ablation does not alter chondrocyte differentiation *in vitro*. (A) RT-qPCR was performed to analyze mRNA expression of markers of chondrocyte differentiation *ColX* and *Op* and Raf isoforms in chondrocytes isolated from *c-Raf*$^{f/f}$*;ColII-Cre*$^{-}$ (control) and *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ mice. RNA expression was normalized to that of actin in the same sample. Data are reported as normalized to mRNA expression in the control mice (14 days in culture for *Op* and 3 days in culture for the others). Data represent mean±s.d. of RNA isolated from three independent chondrocyte preparations per genotype subjected to RT-qPCR. *P<0.05, versus control mice. (B) Immunohistochemistry for c-Raf performed on tibiae from P18 *c-Raf*$^{f/f}$*;ColII-Cre*$^{-}$ and *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ mice. Side panels show magnified images of cortical osteocytes (top) and trabecular osteoblasts (bottom). Arrows indicate c-Raf-positive cells. Data are representative of three mice per genotype.

(Fig. 5B). Immunohistochemistry demonstrated a decrease in VEGF immunoreactivity in the hypertrophic chondrocytes of P18 *c-Raf*$^{f/f}$*; ColII-Cre*$^{+}$ mice (Fig. 5C). Since VEGF is required for vascular invasion at the metaphysis, vascular invasion was evaluated by immunohistochemistry for CD31 (Pecam1), an endothelial cell marker. Chondrocyte-specific c-Raf ablation led to a delay in vascular invasion at E15.5, as evidenced by a decrease in CD31 immunoreactivity in the hypertrophic chondrocyte zone of *c-Raf*$^{f/f}$*; ColII-Cre*$^{+}$ radii, and this persisted postnatally (Fig. 5D). These results suggest that the expansion of the hypertrophic chondrocyte layer in mice with chondrocyte-specific c-Raf ablation is secondary to impaired vascular invasion. Consistent with the normalization of hypertrophic chondrocyte apoptosis seen in *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ tibiae at P35, an increase in VEGF and CD31 immunoreactivity was observed in the growth plates of these mice (Fig. 5C,D).

In the setting of normal mRNA levels, a decrease in VEGF protein levels in *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ mice could result from alteration in mRNA translation or protein stability. Several proteins have been implicated in stabilizing VEGF protein levels. Connective tissue growth factor (Ctgf) has both positive and negative effects on angiogenesis. Notably, VEGF protein levels are decreased in the hypertrophic chondrocytes of embryonic *Ctgf* null mice despite normal mRNA levels (Ivkovic et al., 2003). αB-crystalin (Cryab), a chaperone in the unfolded protein response pathway, prevents degradation of VEGF (Ivkovic et al., 2003; Ruan et al., 2011). Neither *Ctgf* nor *Cryab* mRNA levels were altered in *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ hypertrophic chondrocytes (Fig. 6A).

The initiation of VEGF translation is enhanced by angiotensin II in an Akt-dependent manner (Feliers et al., 2005). Consistent with this, treatment of mice with an angiotensin receptor blocker leads to expansion of the hypertrophic chondrocyte zone of the growth plate (Chen et al., 2015a). However, c-Raf ablation did not alter p-Akt levels in cultured chondrocytes (Fig. 6B).

To determine whether the absence of c-Raf enhances the ubiquitylation of VEGF, polyubiquitylated proteins were isolated from control and *c-Raf*$^{f/f}$*;ColII-Cre*$^{+}$ primary hypertrophic chondrocytes and subjected to VEGF western analysis. These studies demonstrated an increase in ubiquitylated VEGF in *c-Raf*$^{f/f}$*;*

Development (2016) 143, 348-355 doi:10.1242/dev.127142

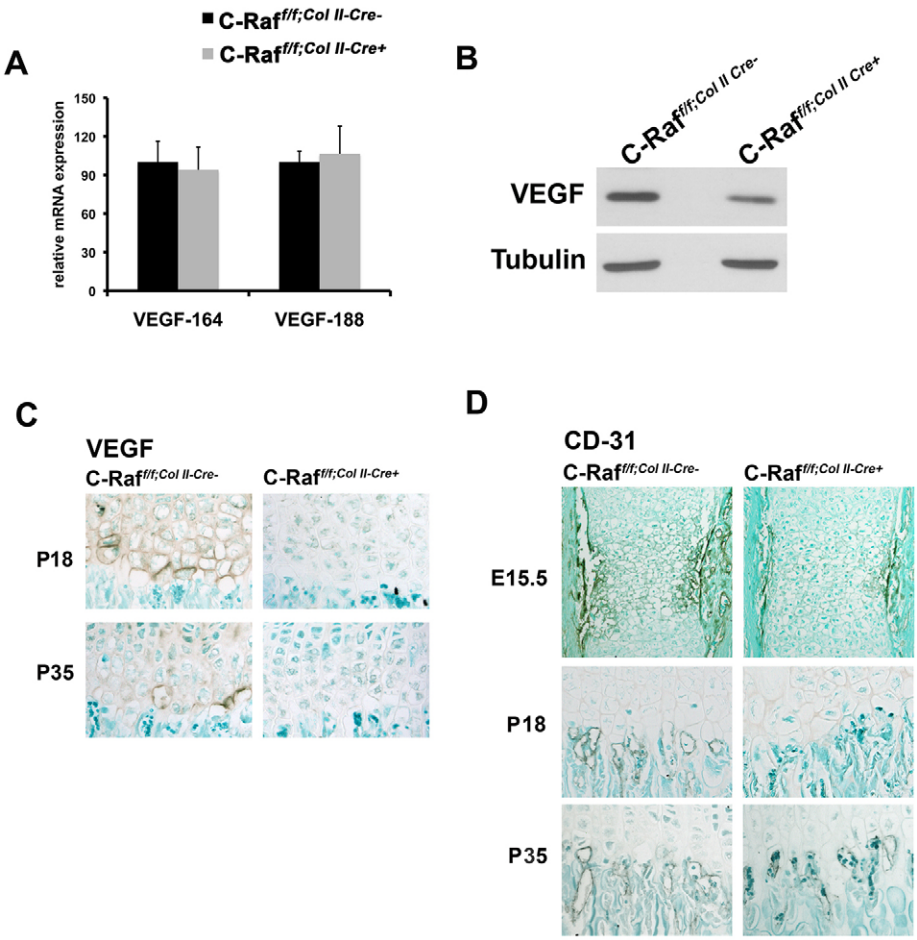

Fig. 5. c-Raf regulates VEGF protein expression. (A) Expression of *Vegf164* and *Vegf188* mRNA in P1 femurs was analyzed by RT-qPCR and normalized to that of actin in the same sample. Data are normalized to mRNA expression in *c-Raf$^{f/f}$;ColII-Cre^{-}* (control) mice. Data represent mean±s.d. of RNA isolated from three P1 femurs per genotype subjected to RT-qPCR. (B) Whole-cell extracts from hypertrophic chondrocytes isolated from *c-Raf$^{f/f}$;ColII-Cre^{-}* and *c-Raf$^{f/f}$;ColII-Cre^{+}* mice were evaluated by western analysis for VEGF protein levels with β-tubulin as a loading control. Data are representative of three independent chondrocyte preparations per genotype. (C) VEGF immunohistochemistry on tibiae from P18 and P35 *c-Raf$^{f/f}$;ColII-Cre^{-}* and *c-Raf$^{f/f}$;ColII-Cre^{+}* mice. (D) CD31 immunohistochemistry performed on E15.5 radius and P18 and P35 tibiae from *c-Raf$^{f/f}$;ColII-Cre^{-}* and *c-Raf$^{f/f}$;ColII-Cre^{+}* mice. Data are representative of three mice for each genotype.

ColII-Cre^{+} hypertrophic chondrocytes (Fig. 6C). Since ubiquitylation targets proteins for degradation by proteasomes (Wilkinson, 2000), control and *c-Raf$^{f/f}$;ColII-Cre^{+}* hypertrophic chondrocytes were treated with the proteasomal inhibitor MG132 for 24 h. Inhibition of proteasomal degradation normalized VEGF protein levels in *c-Raf$^{f/f}$;ColII-Cre^{+}* hypertrophic chondrocytes (Fig. 6D).

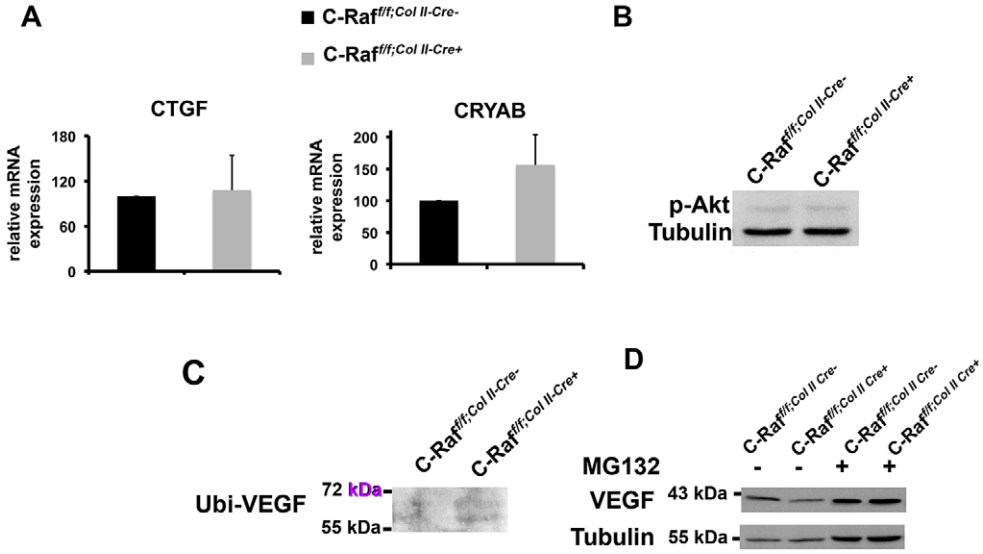

Fig. 6. c-Raf modulates post-translational modification of VEGF. (A) Expression of *Ctgf* and *Cryab* mRNA in primary chondrocytes was analyzed by RT-qPCR and normalized to that of actin in the same sample. Data are normalized to mRNA expression in *c-Raf$^{f/f}$;ColII-Cre^{-}* (control) mice. Data represent mean±s.d. of RNA isolated from three independent chondrocyte preparations per genotype subjected to RT-qPCR. (B,C) Western analysis for phospho-Akt (p-Akt) with β-tubulin as a loading control (B) and ubiquitylated VEGF (ubi-VEGF) (C) from hypertrophic chondrocytes isolated from *c-Raf$^{f/f}$;ColII-Cre^{-}* and *c-Raf$^{f/f}$;ColII-Cre^{+}* mice. Data are representative of three chondrocyte preparations per genotype. (D) Western analysis for VEGF from hypertrophic chondrocytes isolated from *c-Raf$^{f/f}$;ColII-Cre^{-}* and *c-Raf$^{f/f}$;ColII-Cre^{+}* mice treated with MG132. Data are representative of three chondrocyte preparations per genotype.

Development (2016) 143, 348-355 doi:10.1242/dev.127142

DISCUSSION

Raf kinases activate Mek1/2, leading to phosphorylation of Erk1/2 (Cseh et al., 2014). Because Erk1/2 phosphorylation is required for hypertrophic chondrocyte apoptosis, the current studies sought to identify a role for Raf signaling in growth plate maturation. Ablation of both A-Raf and B-Raf in chondrocytes does not result in alterations in chondrocyte differentiation (Provot et al., 2008). However, c-Raf is the predominant isoform expressed in hypertrophic chondrocytes (Kaneko et al., 1994; Provot et al., 2008). Global ablation of c-Raf in mice results in severe defects in embryonic development and growth, associated with a delay in the appearance of primary ossification centers (Wojnowski et al., 1998). However, these studies were unable to establish whether the absence of c-Raf in chondrocytes per se was responsible for the skeletal phenotype observed. Therefore, studies were undertaken to ablate c-Raf specifically in chondrocytes.

c-Raf$^{f/f}$;ColII-Cre^{+} mice exhibit expansion of the hypertrophic layer, accompanied by a decrease in p-Erk1/2 immunoreactivity and impaired hypertrophic chondrocyte apoptosis, demonstrating that c-Raf is necessary for normal maturation of the hypertrophic zone *in vivo*. Although chondrocyte-specific ablation of c-Raf impairs Erk1/2 phosphorylation in hypertrophic chondrocytes *in vivo*, *in vitro* experiments demonstrated that chondrocytes from *c-Raf$^{f/f}$; ColII-Cre^{+}* mice do not exhibit alterations in phosphate-induced Erk1/2 phosphorylation. These results reveal that c-Raf is not an essential mediator of phosphate in the signaling cascade that leads to Erk1/2 phosphorylation and hypertrophic chondrocyte apoptosis. Prior *in vivo* studies have shown that A-Raf and B-Raf expression is limited to proliferative chondrocytes (Provot et al., 2008). However, our *in vitro* data demonstrate that A-Raf expression in hypertrophic chondrocytes is 7.6±3.3% of that of c-Raf, while B-Raf expression is 42.7±14.5% of that of c-Raf in control mice, suggesting that A-Raf and/or B-Raf might functionally compensate for the loss of c-Raf in hypertrophic chondrocytes. Investigations in *B-Raf* and *c-Raf* knockout mice demonstrate increasing developmental abnormalities with progressive decreases in Raf gene dosage (Wojnowski et al., 2000), suggesting that B-Raf might mediate the effects of phosphate on Erk1/2 phosphorylation during growth plate maturation in the absence of c-Raf.

Hypertrophic chondrocyte apoptosis is first seen at the time of vascular invasion in E14.5 humeri, suggesting that vascular invasion is required for hypertrophic chondrocyte apoptosis (Zalutskaya et al., 2009). Consistent with this hypothesis, the hypertrophic chondrocytes of avascular cultured metatarsals do not undergo apoptosis under normal culture conditions, implicating circulating factors, including phosphate, in the induction of hypertrophic chondrocyte apoptosis *in vivo* (Zalutskaya et al., 2009). Impaired vascular invasion in the growth plates of *c-Raf$^{f/f}$; ColII-Cre^{+}* mice would be expected to attenuate the exposure of hypertrophic chondrocytes to extracellular phosphate, resulting in a decrease in Erk1/2 phosphorylation and in hypertrophic chondrocyte apoptosis. Therefore, studies were performed to address the hypothesis that the expansion of the hypertrophic chondrocyte layer in mice with chondrocyte-specific c-Raf ablation was due to impaired vascular invasion.

VEGF, secreted by hypertrophic chondrocytes, is sequestered in the extracellular matrix (Houck et al., 1992). Metalloproteinase 9 (Mmp9) produced by nearby osteoclasts releases matrix-bound VEGF, which promotes vascular invasion and the replacement of terminally differentiated hypertrophic chondrocytes by the primary spongiosa of bone (Engsig et al., 2000; Ortega et al., 2010). Mice deficient in Mmp9 exhibit a phenotype similar to that of mice lacking VEGF, characterized by expansion of the hypertrophic layer of the growth plate accompanied by delayed hypertrophic chondrocyte apoptosis and impaired angiogenesis (Vu et al., 1998; Gerber et al., 1999; Haigh et al., 2000). Similar to what is observed in *Mmp9* knockout mice (Ortega et al., 2010), the *c-Raf$^{f/f}$; ColII-Cre^{+}* mice do not exhibit altered chondrocyte *Vegf* mRNA expression, but rather have decreased bioavailability of VEGF protein, which leads to expansion of the hypertrophic layer. The phenotypes of these two mouse lines are also similar in that the expansion of the hypertrophic chondrocyte layer of the growth plates resolves with aging (Vu et al., 1998). In the *c-Raf$^{f/f}$;ColII-Cre^{+}* mice, the resolution of the phenotype was accompanied by normalization of hypertrophic chondrocyte apoptosis, increased p-Erk1/2 immunoreactivity in the hypertrophic chondrocyte layer and recovery of metaphyseal vascular invasion, as evidenced by the increase in CD31 immunoreactivity observed by P35.

Several factors have been reported to modulate VEGF protein levels. Ctgf promotes growth plate maturation by regulating VEGF protein levels in hypertrophic chondrocytes (Ivkovic et al., 2003), while Cryab inhibits the proteolytic degradation of VEGF (Ruan et al., 2011). However, the expression of these factors was not altered in *c-Raf$^{f/f}$;ColII-Cre^{+}* chondrocytes. Akt signaling has also been shown to increase *Vegf* mRNA stability (Feliers et al., 2005); however, p-Akt levels were not impaired in *c-Raf$^{f/f}$;ColII-Cre^{+}* hypertrophic chondrocytes. During post-translational processing of proteins, ubiquitylation targets proteins for degradation by proteasomes (Wilkinson, 2000). *c-Raf$^{f/f}$;ColII-Cre^{+}* hypertrophic chondrocytes have increased levels of ubiquitylated VEGF and proteasome inhibition restores VEGF protein levels in these chondrocytes. Thus, c-Raf plays an important role in growth plate maturation and vascular invasion by attenuating ubiquitin-dependent VEGF degradation.

MATERIALS AND METHODS

Animal studies

Animal studies were approved by the Massachusetts General Hospital Institutional Animal Care Committee. All mice were on a C57BL/6J background, maintained in a virus- and parasite-free barrier facility and exposed to a 12 h light/dark cycle. Both male and female mice were studied. P18 mice were weaned onto house chow. Mice with chondrocyte-specific ablation of c-Raf were generated by mating mice expressing Cre recombinase under the control of the collagen type II (*Col2a1*; here referred to as *ColII*) promoter with mice bearing exon 3 floxed *c-Raf* alleles (Jesenberger et al., 2001; Mikula et al., 2001). The phenotype of mice homozygous for chondrocyte-specific c-Raf ablation [homozygous for the floxed *c-Raf* allele and heterozygous for the *ColII-Cre* transgene (*c-Raf$^{f/f}$; ColII-Cre^{+}*)] was compared with that of Cre-negative littermates homozygous for the *c-Raf* floxed allele (*c-Raf$^{f/f}$;ColII-Cre^{-}*) and with mice wild type for c-Raf expressing the *ColII-Cre* transgene.

Histology

Bones were fixed in 4% paraformaldehyde in PBS (pH 7.4), demineralized in 20% EDTA (pH 8.0) and processed for paraffin sectioning. Morphology was assessed by Hematoxylin and Eosin (H&E) staining of 5 µm paraffin sections. p-Erk1/2 and CD31 immunohistochemistry was performed as previously described (Miedlich et al., 2010; Liu et al., 2014). For cleaved caspase 9, c-Raf and VEGF immunohistochemistry, sections were blocked with TNB [0.1 M Tris pH 7.5, 0.15 M NaCl, 0.5% blocking reagent (Perkin Elmer)]. Antigen retrieval using sodium citrate buffer (pH 6.0) at 95°C, trypsin, and proteinase K was performed for cleaved caspase 9, c-Raf, and VEGF antibodies, respectively. Sections were incubated with rabbit anti-cleaved caspase 9 (1:100; AbCam, ab32539), anti-c-Raf (1:300; AbCam, ab32025) or anti-VEGF (1:600; AbCam, ab46154) overnight at 4°C. Signals were detected using secondary antibody [biotinylated goat

Development (2016) 143, 348-355 doi:10.1242/dev.127142

anti-rabbit antibody (1:200 or 1:1000, Vector Laboratories, BA-1000) or goat anti-rabbit IgG HRP antibody (1:2000, Santa Cruz, sc-2004)], followed by biotinyl tyramide amplification (PerkinElmer). *In situ* hybridization was performed on paraffin sections using digoxigenin-labeled probes as previously reported (Zalutskaya et al., 2009). The TUNEL assay for apoptotic cells was performed using an *In Situ* Cell Death Detection Kit (Roche Diagnostics) (Sabbagh et al., 2005).

Cell culture

Primary chondrocytes were isolated from the rib cages of P2 pups by sequential collagenase II digestions and plated onto gelatin-coated 6-well plates at a density of $3\times10^5/cm^2$ (Lefebvre et al., 1994; Sabbagh et al., 2005). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 25 µg/ml ascorbic acid at 37°C, 5% CO_2 for up to 2 weeks. To evaluate phosphate induction of Erk1/2 phosphorylation, chondrocytes were serum restricted (0.5% FBS) overnight prior to exposure to sodium sulfate or sodium phosphate. To determine the effect of proteasome inhibition on chondrocytes, serum-restricted cells were treated with 10 µM MG132 (Sigma) for 24 h.

Real-time PCR

Whole femurs, or the distal femoral metaphysis containing the growth plate and perichondrium, were isolated from adjacent tissue using a dissecting microscope. Total RNA from tissue or cultured cells was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed with SuperScript II (Roche). Quantitative real-time PCR (RT-qPCR) was performed using the QuantiTect SYBR Green RT-PCR Kit (Qiagen) on an Opticon DNA Engine (MJ Research). Primer sequences are listed in Table S1. Gene expression was normalized to that of actin (*Actb*) for each sample, using the methods of Livak and Schmittgen (2001).

Western analysis

Whole-cell lysates of primary chondrocytes were prepared as previously described (Miedlich et al., 2010). Protein concentration was calculated using the BCA protein assay (Pierce) and 7 µg protein was subjected to western analysis. Membranes were blocked with 5% non-fat dried milk prior to incubation with rabbit antibodies against p-Erk1/2 (1:1000; Cell Signaling, #9101), Erk1/2 (1:2000; Cell Signaling, #9102), β-tubulin (1:1000; Cell Signaling, #5346) and VEGF (1:1000; AbCam, ab46154). Following incubation with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG HRP antibody; 1:2000; Santa Cruz, sc-2004), signals were detected using ECL Plus (Amersham Biosciences).

Isolation of ubiquitylated protein

Ubiquitylated proteins were isolated from cultured hypertrophic chondrocyte protein lysates (0.15 mg per sample) using a ubiquitin affinity resin (Pierce Ubiquitin Enrichment Kit, Thermo Fisher Scientific). Ubiquitylated proteins were subjected to western analysis as described above.

Statistical analysis

Student's *t*-test was used to analyze significance between two groups. $P<0.05$ was considered significant.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Project design: E.S.L., A.R. and M.B.D. M.B. provided *c-Raf*$^{fl/fl}$ mice. Mouse colony management: E.S.L., A.R. and B.T.C. Molecular biology experiments and histological analyses: E.S.L., A.R., J.S.M. and B.T.C. Cell culture experiments: A.R., E.S.L. and B.T.C.; Manuscript preparation: E.S.L. and M.B.D.

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Supplementary information

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