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MEF2C Transcription Factor Controls Chondrocyte Hypertrophy and Bone Development

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SUMMARY

Chondrocyte hypertrophy is essential for endochondral bone development. Unexpectedly, we discovered that MEF2C, a transcription factor that regulates muscle and cardiovascular development, controls bone development by activating the gene program for chondrocyte hypertrophy. Genetic deletion of *Mef2c* or expression of a dominant-negative MEF2C mutant in endochondral cartilage impairs hypertrophy, cartilage angiogenesis, ossification, and longitudinal bone growth in mice. Conversely, a superactivating form of MEF2C causes precocious chondrocyte hypertrophy, ossification of growth plates, and dwarfism. Endochondral bone formation is exquisitely sensitive to the balance between MEF2C and the corepressor histone deacetylase 4 (HDAC4), such that bone deficiency of *Mef2c* mutant mice can be rescued by an *Hdac4* mutation, and ectopic ossification in *Hdac4* null mice can be diminished by a heterozygous *Mef2c* mutation. These findings reveal unexpected commonalities in the mechanisms governing muscle, cardiovascular, and bone development with respect to their regulation by MEF2 and class II HDACs.

INTRODUCTION

The vertebrate skeleton is comprised of endochondral and membranous bones, which form through different mechanisms (Olsen et al., 2000; Nakashima and de Crombrugghe, 2003; Kronenberg, 2003; Karsenty, 2003). Endochondral bones, which account for all the bones of the body, with the exception of the craniofacial bones and the clavicle, develop from a cartilaginous template. In contrast, membranous bones are derived from mesenchymal cells, which differentiate directly into osteoblasts without a cartilaginous intermediate. There are also permanent cartilaginous structures throughout the body, such as those located in ears, nose, throat, joints, and segments of the ribs. Why the cartilage in these regions fails to undergo ossification is unknown.

Endochondral bone formation begins with the aggregation of mesenchymal cells and their differentiation into chondrocytes. Chondrocyte hypertrophy, which initiates in the center of cartilaginous skeletal elements, drives longitudinal bone growth. This process is coupled to exit from the cell cycle and is marked by the expression of specific extracellular matrix molecules, such as $\alpha_1(X)$ collagen (Col10a1). Chondrocyte hypertrophy is followed by apoptosis; invasion of blood vessels, osteoclasts, and other mesenchymal cells from the perichondrium; and production of the mature bone matrix. The ultimate sizes and structures of endochondral bones depend on the coordinated regulation of chondrocyte proliferation, maturation, and hypertrophy in response to multiple extracellular signals. Indian hedgehog (Ihh) and Parathyroid hormone-related peptide (PTHrP) play critical roles in coordinating these processes (Karaplis et al., 1994; Lanske et al., 1996; Weir et al., 1996; Vortkamp et al., 1996; St-Jacques et al., 1999). Ihh produced by prehypertrophic chondrocytes induces the expression of PTHrP, which regulates the rate at which chondrocytes exit the cell cycle and undergo hypertrophy. Ihh also stimulates chondrocyte proliferation and controls the differentiation of mesenchymal cells into osteoblasts within the bone collar. VEGF expressed by hypertrophic chondrocytes is required for chondrocyte survival and cartilage angiogenesis (Zelzer et al., 2004).

Several transcription factors that control the patterning and maturation of the endochondral skeleton have been identified (reviewed in Karsenty, 2003; Zelzer and Olsen, 2003; Lefebvre and Smits, 2005). Runx2 and Runx3, members of the Runt family of transcription factors, are necessary for chondrocyte hypertrophy and osteoblast differentiation (Komori et al., 1997; Yoshida et al., 2004). Chondrocyte hypertrophy is arrested during the late stages of maturation in *Runx2* null mice, and it is completely blocked in mice lacking Runx2 and Runx3,

suggesting redundant roles for these factors in chondrocyte differentiation. The zinc finger transcription factor osterix acts downstream of Runx2 and is required for osteoblast differentiation and bone formation (Nakashima et al., 2002, Nishio et al., 2006). Mice lacking the HMG box transcription factor Sox9 display hypoplasia of endochondral skeletal elements, resembling the defects associated with campomelic dysplasia in humans (Bi et al., 2001). Sox5 and Sox6 have also been implicated in chondrocyte differentiation (Smits et al., 2001).

Recently, we reported that histone deacetylase 4 (HDAC4) acts as a negative regulator of chondrocyte hypertrophy (Vega et al., 2004). *Hdac4* null mice die during the perinatal period due to premature and ectopic chondrocyte hypertrophy and ossification of endochondral cartilage. HDAC4 and other class II HDACs establish corepressor complexes with DNA-binding transcription factors (Miska et al., 1999; Lu et al., 2000; reviewed in Verdin et al., 2003), including myocyte enhancer factor-2 (MEF2), a MADS (MCM1, Agamous, Deficiens, serum response factor) box factor implicated in muscle and cardiovascular development (reviewed in McKinsey et al., 2002; Black and Olson, 1998). There are four mammalian *Mef2* genes, *Mef2a*, *Mef2b*, *Mef2c*, and *Mef2d*, which are expressed in complex and overlapping patterns in embryonic and adult tissues (Edmondson et al., 1994). *Mef2c* null mice die by embryonic day 9.5 (E9.5) from abnormal cardiovascular development (Lin et al., 1997, 1998), whereas *Mef2a* null mice die perinatally from a spectrum of heart defects (Naya et al., 2002).

Here, we demonstrate the previously undescribed expression of *Mef2* genes in developing chondrocytes, and we show that MEF2C plays an unexpected role in endochondral bone development. Conditional deletion of *Mef2c* or expression of a dominant-negative mutant of MEF2C in developing cartilage impairs chondrocyte hypertrophy and subsequent growth plate vascularization and endochondral ossification. Conversely, a superactivating form of MEF2C promotes precocious chondrocyte hypertrophy and ossification of endochondral bones. Deletion of *Hdac4* restores normal bone formation in heterozygous *Mef2c* mutant mice, and deletion of a single *Mef2c* allele prevents excessive and ectopic bone formation in *Hdac4* mutant mice. We conclude that MEF2C acts as an essential, early regulator of bone development by orchestrating transcriptional and cell-cell signaling events involved in chondrocyte hypertrophy. These findings highlight the partnership of MEF2 and class II HDACs as a nodal point in the control of the seemingly unrelated processes of muscle, cardiovascular, and bone development.

RESULTS

Expression of *Mef2* Genes in the Developing Skeleton

While analyzing the expression of *Mef2* genes during mouse embryogenesis, we found that *Mef2c* was expressed in the mesenchymal primordium of the endochondral cartilage at E12.5 (Figure 1Ac) and that both *Mef2c* and *Mef2d* were expressed in prehypertrophic and hypertrophic chondrocytes, as well as in the spongiosa of developing endochondral bones at later stages of bone development (Figure 1A). It is noteworthy that the level of *Mef2c* and *Mef2d* expression in hypertrophic chondrocytes was comparable to that in surrounding skeletal muscle (Figure 1A). *Mef2a* transcripts were detectable at lower levels, and *Mef2b* expression was not detectable in the developing skeleton (Figure 1A).

A Heterozygous *Mef2c* Mutation Impairs Bone Development

Mice homozygous for a *Mef2c* null mutation die at E9.5 from defects in cardiovascular development (Lin et al., 1997), precluding an analysis of potential functions of *Mef2c* at later developmental stages. In light of the expression of *Mef2c* in the developing endochondral skeleton, we analyzed *Mef2c*$^{+/-}$ mice for possible skeletal abnormalities and noticed a lack of ossification within the sternum at postnatal day (P) 1 (compare Figure 1Ba and Figure 1Bb). Typically, the sternum contains trabeculated bone within each sternebra, with growth plates at both ends. In contrast to wild-type mice in which the sternebrae and xiphoid process are ossified by P1, these structures remained cartilaginous in mutant mice (Figure 1B). Histological analysis and von Kossa staining, which detects calcium deposits within the mineralized cartilaginous matrix, showed only residual hypertrophic chondrocytes and almost a complete absence of trabeculated bone in the sternum of *Mef2c*$^{+/-}$ mutants at P1. We did not detect abnormalities in other endochondral skeletal elements of *Mef2c*$^{+/-}$ mutants, indicating that the sternum is most sensitive to levels of *Mef2c* expression at this stage.

Mice homozygous for a *Mef2d* null allele are viable and show no skeletal abnormalities (unpublished data). In light of the expression of *Mef2d* in the developing skeleton, we generated *Mef2c*$^{+/-}$; *Mef2d*$^{+/-}$ double heterozygous mutant mice to test for potential functional redundancy of these genes in skeletal development. Indeed, *Mef2c*$^{+/-}$; *Mef2d*$^{+/-}$ mutants displayed a deficiency in ossification of the sternum more severe than that of *Mef2c*$^{+/-}$ mutants (Figure 1B). Histological analysis revealed almost no hypertrophic chondrocytes in the sternum of *Mef2c*$^{+/-}$; *Mef2d*$^{+/-}$ mice. In situ hybridization showed α_1(II) collagen (*Col2a1*) expression, a marker of proliferating chondrocytes, throughout the sternum of *Mef2c*$^{+/-}$; *Mef2d*$^{+/-}$ mice at P1, whereas *Col2a1* expression was excluded from the hypertrophic and mineralized regions of the wild-type sternum (Figure 1C). In contrast, *Col10a1*, *Col1a1*, and *bone sialoprotein*, which are markers of hypertrophic chondrocytes, trabeculated bone, and differentiated osteoblasts, respectively, were not expressed in the mutants (Figure 1C). *Mef2c*$^{+/-}$; *Mef2d*$^{+/-}$ mutant mice died within a day after birth; thus, we were unable to analyze them for possible skeletal abnormalities later in postnatal development. These findings suggested that MEF2C played an essential role in bone development, and that MEF2D augmented this function of MEF2C.
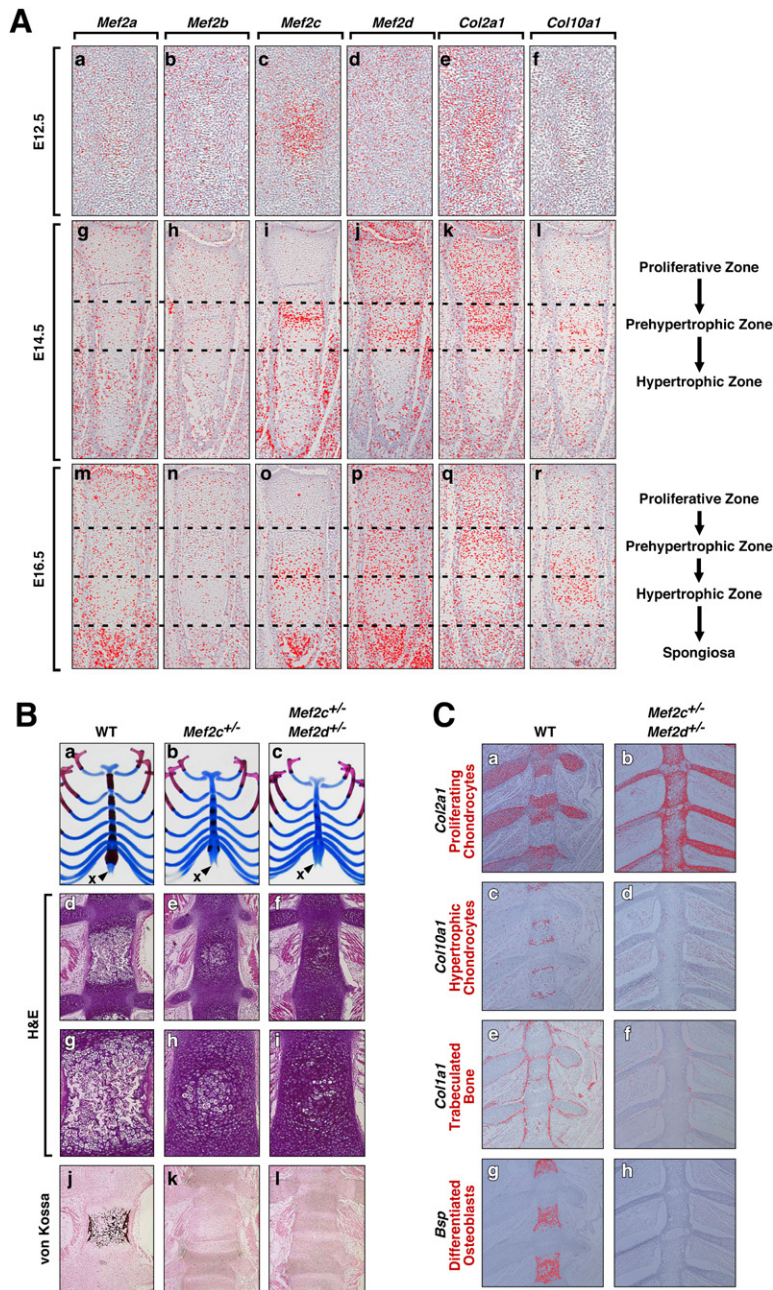
Figure 1. *Mef2* Expression in Developing Endochondral Bones and Skeletal Defects in Heterozygous *Mef2* Mutant Mice

(A) Detection of *Mef2* transcripts by in situ hybridization to sections of the developing radius at (Aa–Ad) E12.5, (Ag–Aj) E14.5, and (Am–Ap) E16.5. Expression of *Mef2c* is initially observed in the cartilage primordium at E12.5. *Mef2c* and *Mef2d* are expressed in prehypertrophic and hypertrophic chondrocytes at later stages. Expression of (Ae, Ak, and Aq) *Col2a1* and (Af, Al, and Ar) *Col10a1* are also described to show chondrocyte differentiation.

(B) (Ba–Bc) Anterior views of the sternum and ribs of mice stained for bone (red) and cartilage (blue) at P1. The xiphoid process (x) is indicated. Note the reduction in ossification of the sternum in (Bb) and the complete absence of ossification in (Bc). Hematoxylin and eosin (H&E)-stained sections are shown in (Bd)–(Bf), and a high-magnification view of the sternebrae is shown below in (Bg)–(Bi). Note the absence of hypertrophic chondrocytes and trabeculation in the mutants. (Bj–Bl) von Kossa-stained sections are shown at the bottom.

(C) Detection of markers of bone development by in situ hybridization to sections of sternum from wild-type and *Mef2c*^{+/−}; *Mef2d*^{+/−} mice at P1.

Generation of a Conditional *Mef2c* Allele

To further investigate the potential involvement of *Mef2c* in bone development, we generated a conditional *Mef2c* null allele, called *Mef2c*[loxP], that contained loxP sites in the introns flanking the second coding exon. This exon encodes the MADS- and MEF2-specific domains, which, in turn, mediate DNA binding, dimerization, and cofactor interactions. Deletion of the genomic region between the loxP sites inactivates the gene (Figure S1, see the Supplemental Data available with this article online). A similar genomic region was deleted in our original *Mef2c*[KO] null allele (Lin et al., 1997). Mice homozygous for the *Mef2c*[loxP] allele or trans-heterozygous for this allele and the *Mef2c*[KO]

allele showed no apparent abnormalities, indicating that the *Mef2c*[loxP] allele functioned normally.

Conditional Deletion of *Mef2c* Disrupts Bone Development

We used two mouse lines that express Cre recombinase in different skeletal elements and their precursors to conditionally delete *Mef2c* in vivo. We deleted *Mef2c* from the early precursors of all cell types of the developing bone by using the *Twist2-Cre* knockin (Yu et al., 2003), or we deleted the gene specifically in the cartilaginous template of endochondral bones by using *Col2-Cre* (Long et al., 2001). The expression of *Mef2c* was effectively reduced
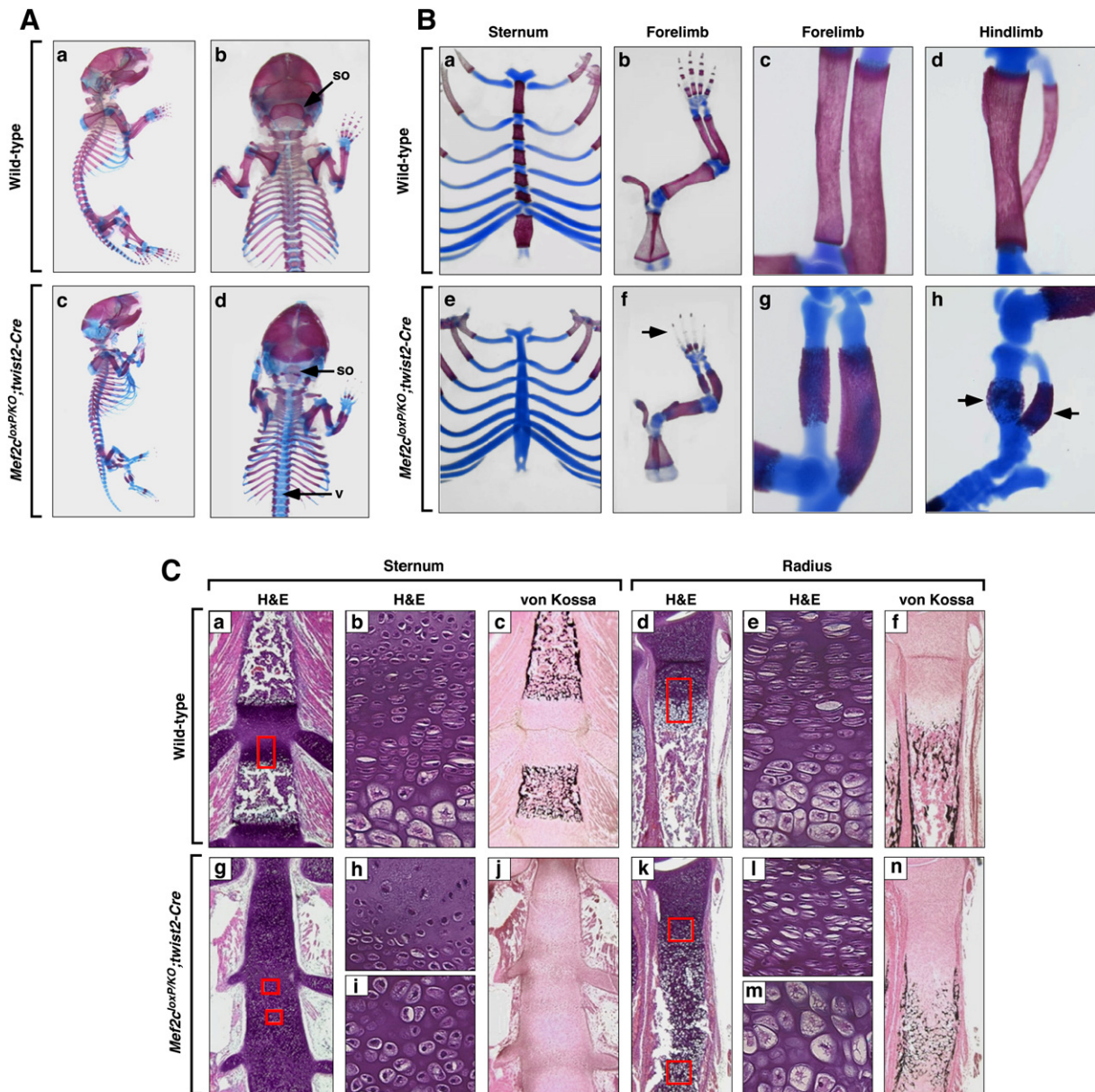
Figure 2. Failure in Endochondral Ossification after Conditional Deletion of *Mef2c* by Using *Twist2-Cre*

(A) Alizarin red and Alcian blue staining of P1 wild-type and *Mef2c*^loxP/KO^*; Twist2-Cre* whole skeletons in (Aa and Ac) lateral and (Ab and Ad) dorsal views. Arrows in (Ab) and (Ad) indicate the supraoccipital bone (so) and vertebral bodies (v), which fail to ossify in *Mef2c*^loxP/KO^*; Twist2-Cre* mice.

(B) Alizarin red and Alcian blue staining of P1 wild-type and *Mef2c*^loxP/KO^*; Twist2-Cre* bones of the sternum, forelimb, and hindlimb, as indicated. Many phalangeal bones of the forelimb fail to ossify in the mutant (arrow in [Bf]). Arrows indicate the tibia and fibula in (Bh), in which ossification is severely disrupted. Bones of *Mef2c*^loxP/KO^*; Twist2-Cre* mice contain excessive cartilage, and bone collars are disorganized.

(C) Histologic sections of P1 wild-type and *Mef2c*^loxP/KO^*; Twist2-Cre* sternum and radius stained with H&E or by von Kossa's method. The sternum of *Mef2c*^loxP/KO^*; Twist2-Cre* mice does not contain hypertrophic chondrocytes, while the radius is filled with cartilage that has undergone limited hypertrophic differentiation. (Cb), (Ce), (Ch), (Ci), (Cl), and (Cm) show enlargements of the boxed regions in (Ca), (Cd), (Cg), and (Ck), respectively.

as a result of Cre expression (Figure S1D and data not shown).

Mef2c^loxP/KO^*; Twist2-Cre* mice were readily identifiable at birth by their shortened limbs (Figures 2A and 2B). A fraction of these conditional *Mef2c* mutants also displayed difficulty breathing, evidenced by gasping and accumulation of air in their intestines, and none survived beyond the first week of postnatal life. Analysis of the skeletons of *Mef2c*^loxP/KO^*; Twist2-Cre* mice revealed severe defects in ossification of nearly all endochondral bones (Figures 2A and 2B). This phenotype was especially pronounced in the sternum (compare Figure 2Ba and Figure 2Be). Similarly, the vertebral bodies failed to ossify, as did the supraoccipital bone, which defines the dorsal margin of the

foramen magnum (arrows in Figure 2Ad). There was also obvious foreshortening of the ossified regions of the bones of the fore and hindlimbs and lack of ossification in many phalangeal bones of the digits (Figure 2B). Truncation of the tibia and fibula was particularly severe (compare Figure 2Bd and Figure 2Bh). Ossification of the bone collar also appeared disorganized in these mutant mice (Figure 2B).

Histological sections of the sternum and radius revealed that the defects in endochondral ossification in the mutants resulted from a failure in chondrocyte hypertrophy (Figure 2C). von Kossa staining showed that the sternum and radius of $Mef2c^{loxP/KO}$; Twist2-Cre mice were completely devoid of trabeculated bone (Figure 2C). However, weak von Kossa staining was seen at the midpoint of the mutant radius and in the surrounding bone collar of the mutants, indicative of a few chondrocytes in the late stages of hypertrophic differentiation. Bone collar formation is secondary to chondrocyte hypertrophy; therefore, it did not occur in the mutant sternum, where chondrocyte hypertrophy failed to take place (Figure 2C). In the mutant radius, a disorganized bone collar was present only near the midpoint of the bone, where residual hypertrophic chondrocytes were present (Figure 2C).

Deletion of *Mef2c* with *Col2-Cre*

To distinguish whether the skeletal defects in $Mef2c^{loxP/KO}$; Twist2-Cre mice reflected a role for MEF2C in chondrocytes or osteoblasts, we specifically deleted Mef2c in proliferating chondrocytes by using a Cre transgene controlled by the Col2a1 promoter, called Col2-Cre. The resulting mutants were identifiable at birth by shortened limbs, an absence of ossification of the sternum (Figure 3A), and a failure in chondrocyte hypertrophy (Figure 3B), similar to the defects seen in $Mef2c^{loxP/KO}$; Twist2-Cre mice.

$Mef2c^{loxP/KO}$; Col2-Cre mice survived to adulthood and remained distinguishable from wild-type littermates by their waddling gait due to their shortened limbs. Bone and cartilage staining of skeletons of 3-month-old mutant mice highlighted the truncation of all long bones of the limbs (Figure 3C). The structures of the distal ribs, radii, and sternabrae were also distorted by disorganized cartilaginous remnants of the growth plate and aberrant ossification (Figure 3D), demonstrating the requirement for MEF2C in timely chondrocyte maturation.

Chondrocyte Deletion of *Mef2c* and *Mef2d*

Our earlier finding that deletion of one Mef2d allele exacerbated the bone defects in $Mef2c^{+/-}$ mice suggested that these two Mef2 genes acted cooperatively to control chondrocyte hypertrophy. Because $Mef2c^{+/-}$; $Mef2d^{+/-}$ mice were not viable, it was necessary to generate a conditional Mef2d allele in order to examine possible redundant functions of MEF2C and MEF2D in the developing skeleton. As shown in Figures 3Ai–3Al, homozygous deletion of both genes by using Col2-Cre resulted in a skeletal phenotype more severe than that of $Mef2c^{loxP/KO}$; Col2-Cre mice, in which nearly all mineralized endochondral skeletal elements were missing.

Requirement of MEF2C for Activation of the Gene Program for Chondrocyte Hypertrophy and Vascularization

To precisely define the molecular defects resulting from the absence of MEF2C expression in the endochondral skeleton, we analyzed numerous markers of skeletal development in the radius (Figure 4) and sternum (Figure S2) of $Mef2c^{loxP/KO}$; Twist2-Cre mice at E14.5 and P1. At both stages, Col2a1, a marker of proliferating chondrocytes, failed to be downregulated in mutant chondrocytes (Figures 4A and 4B). Col10a1, which is expressed in a specific zone of hypertrophic chondrocytes of normal bones, was not expressed in the radius of $Mef2c^{loxP/KO}$; Twist2-Cre mutant mice at E14.5, and at P1 we detected only diffuse Col10a1 expression in a few residual hypertrophic chondrocytes scattered throughout the length of the radius (Figures 4A and 4B), suggesting a severe developmental delay in the timing and extent of chondrocyte hypertrophy. There was no detectable expression of Col1a1, a marker of ossification, in the radius or sternum of the mutants at these stages. Similarly, osteocalcin, a marker of osteoblasts, was not detected within the endochondral skeleton of mutant mice, consistent with the failure in endochondral ossification.

Runx2, which is normally expressed at high levels in prehypertrophic and hypertrophic chondrocytes and osteoblasts, failed to be upregulated in endochondral skeletal elements of $Mef2c^{loxP/KO}$; Twist2-Cre mutant mice at P1 (Figure 4B), although Runx2 expression was detected in the bone collar of the mutant. Ihh is expressed in prehypertrophic chondrocytes and induces PTHrP expression in adjacent chondrocytes, resulting in a delay in chondrocyte hypertrophy (Vortkamp et al., 1996; St-Jacques et al., 1999). In mutant mice, Ihh was expressed diffusely in prehypertrophic chondrocytes, but it did not show the normally localized expression to the prehypertrophic zone (Figure 4B). The diffuse expression of Ihh likely reflects, at least in part, the failure in chondrocyte hypertrophy, which typically results in the organization of chondrocytes into a distinct prehypertrophic cell layer. The PTHrP receptor (Ppr) was expressed in prehypertrophic chondrocytes of wild-type and mutant skeletal elements. VEGF is normally upregulated in hypertrophic chondrocytes of the growth plate, where it stimulates cartilage vascularization (Zelzer et al., 2004). VEGF failed to be upregulated in endochondral cartilage of $Mef2c^{loxP/KO}$; Twist2-Cre mice, and there was a complete absence of vascularization, as assayed by staining for endomucin (Figure 4B). In contrast, the perichondrium and surrounding tissue were normally vascularized in the mutant. Thus, MEF2C is required specifically in endochondral cartilage for VEGF expression and blood vessel invasion.

Blockade to Chondrocyte Hypertrophy by Dominant-Negative MEF2C

The residual chondrocyte hypertrophy and ossification seen in Mef2c; Mef2d mutant mice could reflect partial functional redundancy with Mef2a or incomplete deletion of these conditional alleles. As an independent means of
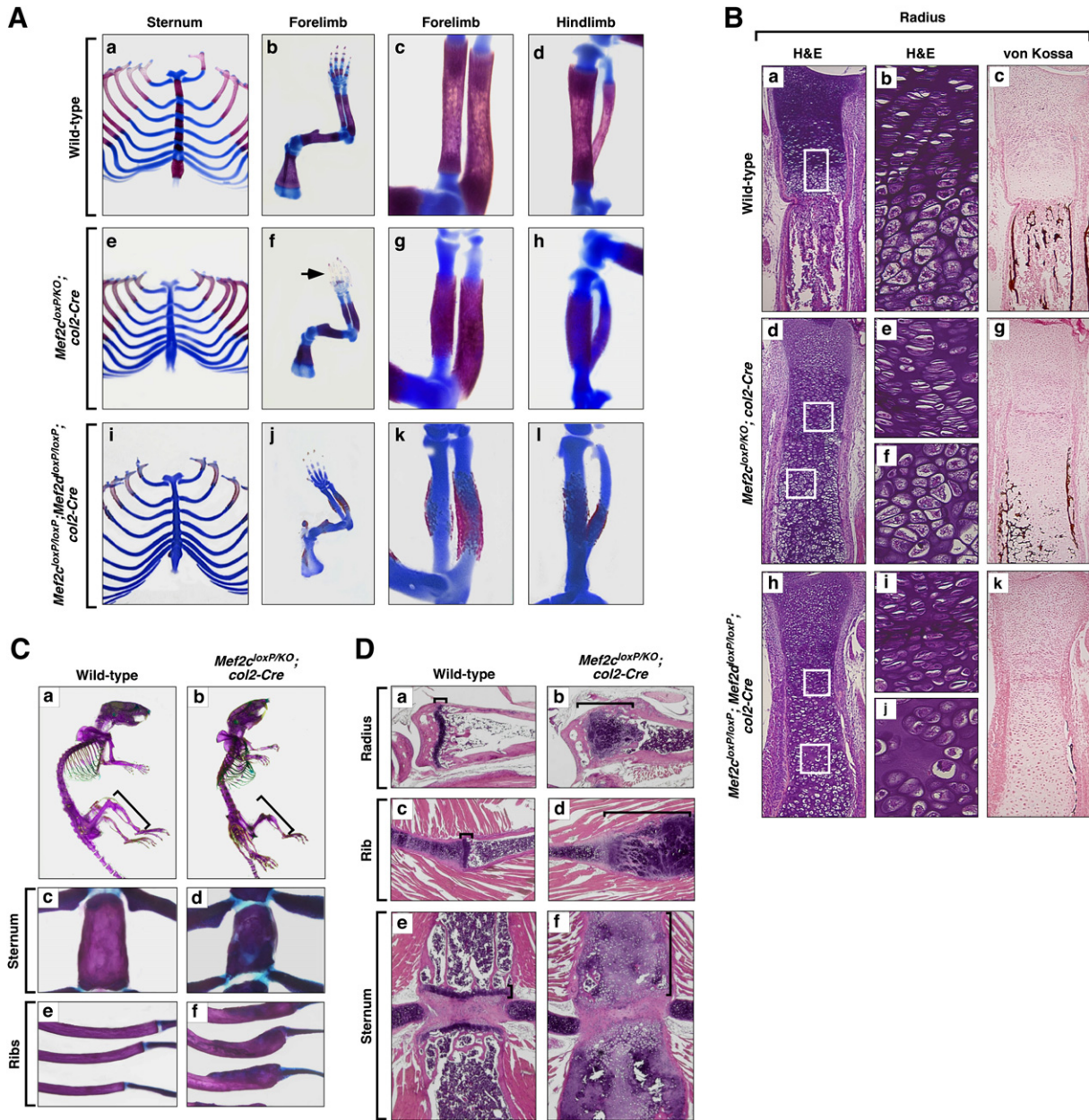
Figure 3. Failure in Endochondral Ossification after Conditional Deletion of *Mef2c* and *Mef2d* by Using *Col2-Cre*

(A) Alizarin red and Alcian blue staining of P1 wild-type, *Mef2c^{loxP/KO}; Col2-Cre*, and *Mef2c^{loxP/loxP}; Mef2d^{loxP/loxP}; Col2-Cre* bones of the sternum, forelimb, and hindlimb, as indicated. Bones of the *Mef2c^{loxP/KO}; Col2-Cre* mice contain large amounts of cartilage, and bone collars are disorganized. Many phalangeal bones of the paw fail to ossify in the mutant (arrow in [Af]). In *Mef2c; Mef2d* double mutants, there is almost a complete absence of bone formation.

(B) Histologic sections of P1 wild-type, *Mef2c^{loxP/KO}; Col2-Cre* and *Mef2c^{loxP/loxP}; Mef2d^{loxP/loxP}; Col2-Cre* radius stained with H&E or by von Kossa's method. The radius of the mutant is filled with cartilage that has undergone only limited hypertrophic differentiation and no mineralization. (Bb), (Be), (Bf), (Bi), and (Bj) show enlargements of the boxed regions of the adjacent panels (Ba, Bd, and Bh). Note the more severe defects in *Mef2c; Mef2d* double mutants than in *Mef2c* mutants (compare [Bg] and [Bk]).

(C) Alizarin red and Alcian blue staining of 3-month-old wild-type and *Mef2c^{loxP/KO}; Col2-Cre* bones of the (Ca and Cb) whole skeleton, (Cc and Cd) sternum, and (Ce and Cf) ribs. The limbs of the *Mef2c^{loxP/KO}; Col2-Cre* skeleton are shortened. Brackets in (Ca) and (Cb) correspond to the length of the wild-type tibia. The sternum and ribs of the *Mef2c^{loxP/KO}; Col2-Cre* skeleton contain large cartilaginous growth plate remnants that distort the bone architecture.

(D) Histologic sections of 3-month-old wild-type and *Mef2c^{loxP/KO}; Col2-Cre* (Da and Db) radius, (Dc and Dd) ribs, and (De and Df) sternum stained with H&E. The radius and ribs of the *Mef2c^{loxP/KO}; Col2-Cre* skeleton contain large cartilaginous growth plate remnants, and the sternum is filled with partially ossified cartilage. Brackets indicate the position and length of the remaining growth plate cartilages.
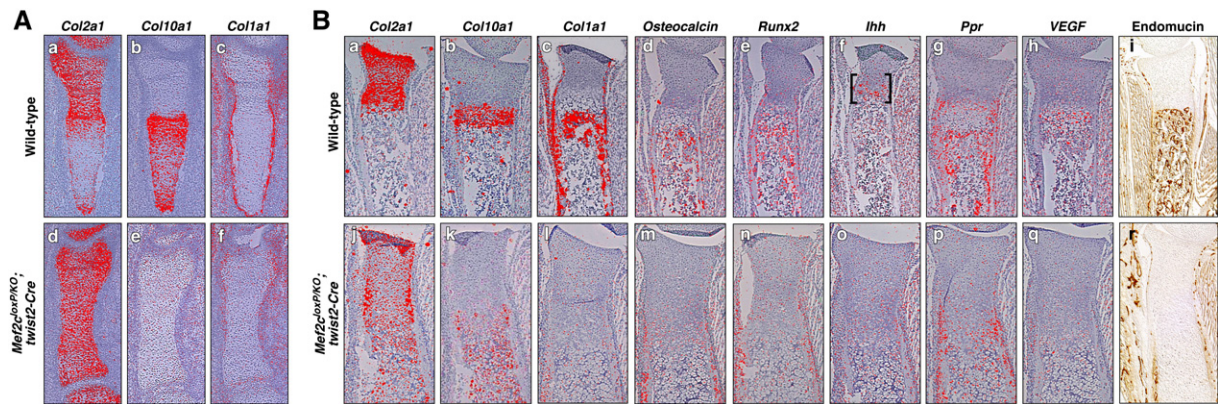
Figure 4. Detection of Markers of Bone Development in Wild-Type and *Mef2c^loxP/KO^; Twist2-Cre* Mice

(A and B) Transcripts for the indicated markers of bone development were detected by in situ hybridization to sections of the radius of wild-type (top row) and *Mef2c^loxP/KO^; Twist2-Cre* (bottom row) mice at (A) E14.5 and (B) P1. Endomucin expression was detected by immunostaining. Brackets in (Bf) show the region of prehypertrophic chondrocytes.

inactivating the multiple MEF2 factors expressed in developing endochondral cartilage, we generated transgenic mice that expressed a fusion protein of the MEF2C DNA-binding domain and the Engrailed repressor in proliferating chondrocytes under control of the *Col2a1* promoter. This MEF2C-Engrailed fusion protein inactivates all MEF2 proteins by preventing DNA binding and creating transcriptionally inactive heterodimers with wild-type MEF2 proteins. Ossification in nearly every region of the endochondral skeleton was blocked in MEF2C-Engrailed transgenic mice, while formation of cartilage-independent membranous bones was unaffected (Figure 5). All endochondral bones of these transgenic mice were also hypoplastic, resulting in shortening of the limbs, spine, and pelvis and deformation of the rib cage. In the most strongly affected animals, the endochondral bones at the base of the skull, the hyoid bone, the bones of the limbs, the sternum, and the pelvis remained entirely cartilaginous (Figures 5A, 5B, and 5D and data not shown). Milder defects were observed in transgenic mice carrying fewer copies of the transgene (data not shown). Histologic sections of the sternum and forelimb demonstrated that chondrocyte hypertrophy in these transgenic mice was completely absent (Figure 5C).

Excessive Ossification in Response to MEF2C-VP16

To determine if MEF2 was sufficient to induce chondrocyte hypertrophy and bone formation, we generated transgenic mice that expressed a superactive MEF2C-VP16 fusion protein under control of the *Col2a1* promoter. In contrast to the MEF2C-Engrailed transgenic mice, expression of MEF2C-VP16 in endochondral cartilage of transgenic mice resulted in premature endochondral ossification at E18.5, with consequent foreshortening of the limbs (Figure 5). This phenotype of excessive ossification was especially apparent in the sternum and vertebrae (Figure 5B). Growth plates separating the sternebrae were prematurely ossified, resulting in complete ossification along the length of the sternum (Figure 5Bh). In con-

trast to normal vertebrae at E18.5, which contain a cartilaginous growth plate surrounding the vertebral body (Figure 5Ba), the entire vertebra was ossified, due to premature fusion of the growth plates between the vertebral body and the lateral vertebral processes (Figure 5Bg). Growth plate cartilages of the limbs were also inappropriately ossified in MEF2C-VP16 transgenic mice, resulting in distortion of the forelimb (Figure 5Bi). Inappropriate ossification in MEF2C-VP16 transgenic mice was also prominent in the base of the skull, where premature fusion of the endochondral growth plates resulted in deformation of the cranial vault (Figures 5Ac and 5Dc). In contrast, formation of cartilage-independent membranous bones was unaffected. Histologic examination of the sternum and forearm confirmed that growth plate maturation was accelerated, particularly in the sternum, where nearly all chondrocytes in the growth plate were consumed by premature differentiation (Figure 5Cf).

Activation of the *Col10a1* Promoter by MEF2C

The bone abnormalities resulting from *Mef2c* deletion and MEF2C-Engrailed expression suggested that MEF2 was required for activation of a hypertrophic gene program in chondrocytes. Analysis of the promoter region of the *Col10a1* gene, the prototypical marker of chondrocyte hypertrophy (Gebhard et al., 2004), revealed several evolutionarily conserved sequences resembling MEF2-binding sites (Figure 6A), and gel mobility shift assays confirmed that MEF2 bound with varying affinities to a subset of these sites (Figure 6C). Transfection assays also showed that MEF2 could activate the *Col10a1* promoter, and mutation of the MEF2 sites eliminated MEF2 responsiveness (Figure 6B). As previously reported (Gebhard et al., 2004), the 5′ flanking region of the *Col10a1* gene was able to direct the expression of a lacZ reporter in hypertrophic chondrocytes of developing endochondral bones of transgenic mice, in a pattern that recapitulated the endogenous expression pattern of the *Col10a1* gene (Figures 6D and 6E). Mutation of the MEF2 DNA-binding sites in the
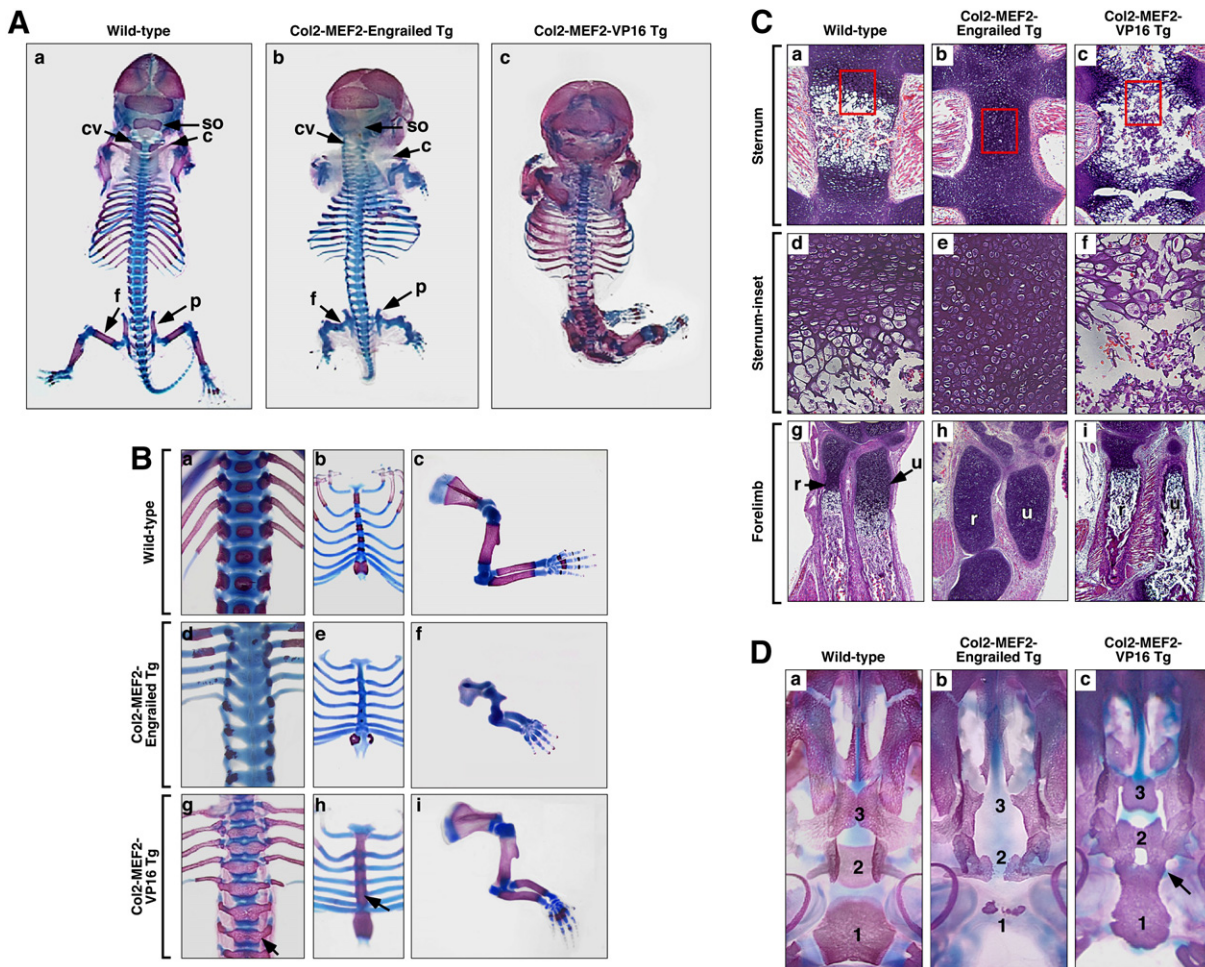
Figure 5. Regulation of Bone Development by Dominant-Negative and Activating Forms of MEF2C

(A) Alizarin red and Alcian blue staining of (Aa) wild-type, (Ab) Col2-MEF2C-Engrailed transgenic, and (Ac) Col2-MEF2C-VP16 transgenic E18.5 embryos depicting a dorsal view of whole skeletons. Arrows indicate the supraoccipital bone (so), the first cervical vertebrae (cv), the clavicle (c), the femur (f), and the pelvis (p).

(B) Alizarin red and Alcian blue staining of (Ba–Bc) wild-type, (Bd–Bf) Col2-MEF2C-Engrailed transgenic, and (Bg–Bi) Col2-MEF2C-VP16 transgenic E18.5 embryos depicting (Ba, Bd, and Bg) spine, (Bb, Be, and Bh) sternum, and (Bc, Bf, and Bi) forelimb. Elements of the endochondral skeleton remain cartilaginous in Col2-MEF2C-Engrailed transgenic embryos, and endochondral skeletal elements of Col2-MEF2C-VP16 transgenic embryos are prematurely ossified (arrows in [Bg] and [Bh]).

(C) Sections of (Ca, Cd, and Cg) wild-type, (Cb, Ce, and Ch) Col2-MEF2C-Engrailed transgenic, and (Cc, Cf, and Ci) Col2-MEF2C-VP16 transgenic E18.5 embryos stained with H&E depicting the (Ca–Cf) sternum or (Cg–Ci) forelimb of the indicated embryos. The radius (r) and ulna (u) are indicated in (Cg)–(Ci). (Cd)–(Cf) are enlargements of the boxed regions of (Ca)–(Cc), respectively. Chondrocytes in the sternum and radius of Col2-MEF2C-Engrailed transgenic embryos fail to initiate hypertrophic differentiation; conversely, in Col2-MEF2C-VP16 transgenic embryos, chondrocyte hypertrophic differentiation is accelerated and has consumed nearly all growth plate chondrocytes.

(D) Alizarin red and Alcian blue staining of (Da) wild-type, (Db) Col2-MEF2C-Engrailed transgenic, and (Dc) Col2-MEF2C-VP16 transgenic E18.5 embryos depicting the ventral skull. The endochondral basioccipital (1), basisphenoid (2), and presphenoid (3) bones are indicated. These bones fail to ossify in Col2-MEF2C-Engrailed transgenic embryos, and they prematurely fuse in Col2-MEF2C-VP16 transgenic embryos (arrow in [Dc]).

promoter diminished expression in hypertrophic chondrocytes in vivo. These results identify MEF2 as a direct activator of chondrocyte gene expression and a critical control point in regulating chondrocyte hypertrophy in vivo.

Genetic Antagonism between *Mef2c* and *Hdac4*

The decreased ossification observed in *Mef2c* mutant mice contrasts with the precocious and ectopic ossification of endochondral cartilage in mice lacking HDAC4,

a MEF2 corepressor (Vega et al., 2004). Thus, we wondered whether the repressive effects of HDAC4 on bone development might be mediated, at least in part, by MEF2. Consistent with this notion, expression of a constitutively nuclear HDAC4 blocked activation of the *Col10a1* promoter by MEF2C (Figure 6B).

To test for potential genetic interactions between *Mef2c* and *Hdac4* in vivo, we investigated whether deletion of one *Mef2c* allele might diminish the excessive and ectopic

ossification of endochondral cartilage seen in *Hdac4* null mice (Vega et al., 2004). Indeed, deletion of one *Mef2c* allele in *Hdac4*$^{-/-}$ mice normalized ossification of the chondrocostal cartilage and sternum (Figure 6F). Conversely, deletion of one copy of the *Hdac4* gene in the *Mef2c*$^{+/-}$ background partially restored ossification of the sternum and increased ossification in the xiphoid process, and deletion of both copies of *Hdac4* in the presence of a heterozygous *Mef2c* allele resulted in a nearly normal pattern of ossification in the sternum (Figure 6F). Similar rescue of normal ossification was seen in endochondral bones of the skull and the hyoid bone (data not shown). These findings demonstrate that ossification of endochondral cartilage depends on the balance between transcriptional activation by MEF2C and repression by HDAC4.

DISCUSSION

The results of this study reveal the following insights into the molecular basis of bone development and the role of MEF2C in this process. (1) MEF2C is a necessary early regulator of chondrocyte hypertrophy and subsequent growth plate maturation. The extent of chondrocyte hypertrophy and bone formation in vivo directly reflects the level of MEF2C transcriptional activity in chondrocytes, as shown by the perturbation of these processes with even a heterozygous *Mef2c* mutation. (2) MEF2C is sufficient to induce chondrocyte hypertrophy and bone formation in vivo, as revealed by the stimulation of these processes by MEF2C-VP16 in vivo. (3) MEF2D augments the prohypertrophic actions of MEF2C in chondrocytes. (4) MEF2C is a direct regulator of *Col10a1* transcription and is required for appropriate temporal and spatial expression of the genetic program of chondrocyte development, including expression of *Runx2* and *VEGF* in endochondral cartilage. (5) Chondrocyte hypertrophy and bone formation depend on the balance between the opposing actions of MEF2C and HDAC4. We conclude that MEF2C is a key regulator of chondrocyte development that orchestrates multiple steps in the transcriptional program for bone formation, as schematized in Figure 7.

Control of Chondrocyte Hypertrophy by MEF2C
Growth of endochondral bone requires a tightly regulated sequence of developmental steps in which proliferating chondrocytes in the growth plate exit the cell cycle and undergo hypertrophy. Hypertrophic chondrocytes secrete a calcified extracellular matrix and undergo apoptosis. Osteoblasts then invade the cartilaginous skeleton and deposit bone on the mineralized cartilaginous spicules of the primary spongiosa. Lengthwise bone growth is driven by the dynamics of chondrocyte proliferation, differentiation, and hypertrophy within the growth plates (reviewed in Olsen et al., 2000). *Mef2c* is expressed in prehypertrophic and hypertrophic chondrocytes, and its specific deletion from this cell population impairs hypertrophy, with consequent short-limb dwarfism. Deletion of *Mef2d* exacerbates the bone defects associated with a *Mef2c* mutation, and expression of a dominant-negative MEF2C-

Engrailed fusion protein in endochondral cartilage prevents hypertrophy and ossification. The importance of MEF2C as a regulator of chondrocyte hypertrophy and bone formation is underscored by the ability of the super-activating MEF2C-VP16 fusion protein to promote precocious ossification of endochondral bones and dwarfism due to premature growth plate fusion.

Runx2 mutant mice also display defects in chondrocyte hypertrophy and ossification, but the phenotypes of *Runx2* and *Mef2c* mutant mice differ in several important respects that highlight the distinct functions of these genes. For example, only a relatively small subset of chondrocytes displays defects in hypertrophy in *Runx2* mutant mice, whereas osteoblasts are missing in these mice, resulting in an absence of membranous and endochondral ossification (Komori et al., 1997). Deletion of *Mef2c* from multiple mesodermal lineages, including chondrocytes and osteoblasts, with *Twist2-Cre* or specifically from chondrocytes with *Col2-Cre* results in similar phenotypes, supporting the conclusion that MEF2C functions primarily to regulate chondrocyte hypertrophy. We interpret the differences between the *Mef2c* and *Runx2* mutant phenotypes to indicate a primary role of MEF2C in hypertrophy of endochondral cartilage.

How does MEF2 regulate chondrocyte hypertrophy and bone development? Many of the actions of MEF2 on these processes undoubtedly reflect the direct activation of downstream target genes, such as the *Col10a1* gene, a specific marker for chondrocyte hypertrophy, which serves as a direct transcriptional target of MEF2. It is noteworthy that *Runx2* expression was also dramatically diminished in the endochondral cartilage of *Mef2c* mutant mice, suggesting that MEF2C is required, either directly or indirectly, for *Runx2* expression, and that some of the defects of *Mef2c* mutant mice may be attributable to diminished expression of *Runx2*. MEF2C is also essential for normal expression of *VEGF* within the endochondral cartilage, which regulates angiogenesis in the late stages of chondrocyte development. In addition, *Ihh* fails to be upregulated in prehypertrophic chondrocytes of *Mef2c* mutant mice. Ihh plays multiple roles in endochondral bone development by enhancing chondrocyte proliferation (St-Jacques et al., 1999; Long et al., 2001) and stimulating PTHrP synthesis, thereby regulating a negative feedback loop to govern the timing of hypertrophy (St-Jacques et al., 1999; Vortkamp et al., 1996). The failure of *Ihh* to be upregulated in prehypertrophic chondrocytes of *Mef2c* mutant mice could contribute to the truncations of endochondral bones in these animals. MEF2C, therefore, influences numerous interwoven steps of signaling and gene activation during bone development.

Control of Bone Development by the Balance of MEF2C and HDAC4
Impairment of chondrocyte hypertrophy resulting from deletion of *Mef2c* or expression of MEF2C-Engrailed in endochondral cartilage is the opposite of the phenotype resulting from genetic deletion of *Hdac4* (Vega et al., 2004), which results in premature ossification and fusion
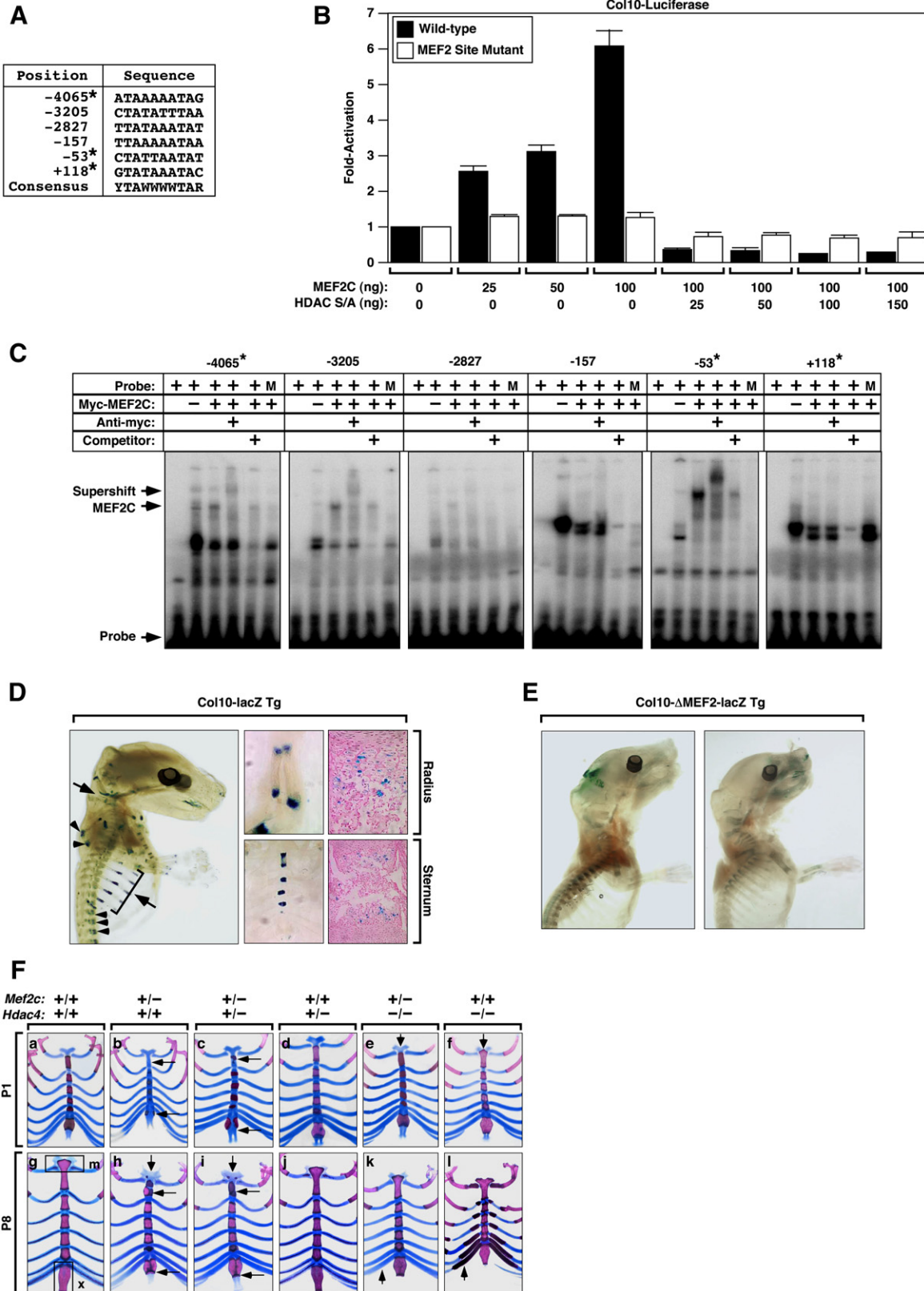
Figure 6. Regulation of the *Col10a1* Promoter by MEF2

(A) Comparison of potential MEF2-binding sites in the *Col10a1* promoter to the MEF2-binding consensus. All of the selected sites match the core TAWWWWTA of the MEF2-binding consensus. MEF2-binding sites conserved between the mouse and human genomes are indicated with an asterisk (*).
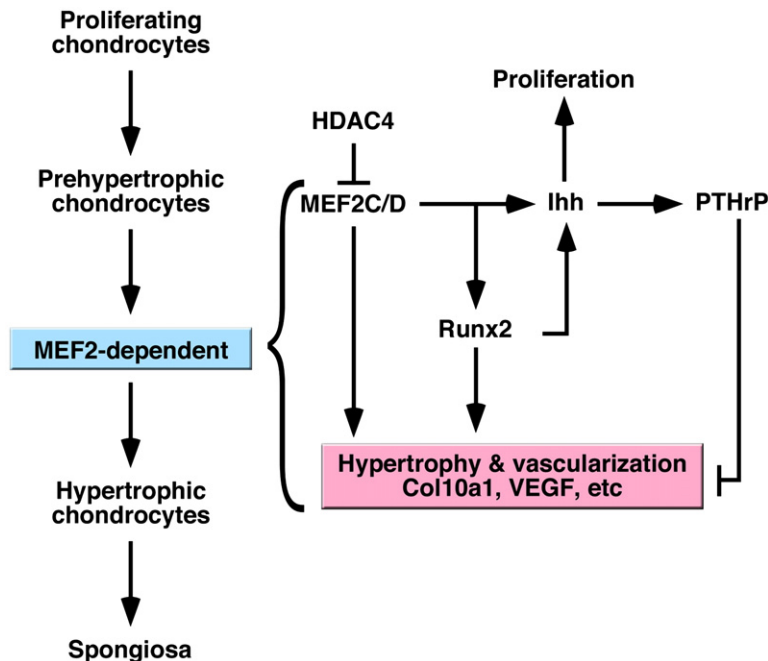
Figure 7. A Model for Transcriptional Regulation of Bone Development by MEF2C and HDAC4

HDAC4 repression in chondrocytes is mediated by MEF2 and Runx transcription factors. MEF2 factors function to regulate chondrocyte hypertrophy, while Runx factors are required for both chondrocyte hypertrophy and osteoblast development.

of the growth plates. Conversely, the premature and ectopic ossification of endochondral bones resulting from expression of MEF2C-VP16 is similar to, but more severe than, that of *Hdac4* null mice. Altering the balance between the opposing actions of MEF2C and HDAC4 can dictate the extent of chondrocyte hypertrophy and ossification in predictable ways. Failure of endochondral ossification resulting from heterozygosity of *Mef2c*, for example, can be rescued in a dose-dependent manner by removal of *Hdac4* alleles, whereas the premature and ectopic ossification in *Hdac4* mutant mice can be reversed by deletion of a *Mef2c* allele. The rescue of the *Hdac4* mutant phenotype with a heterozygous *Mef2c* allele provides the strongest genetic evidence to date for the opposing actions of MEF2 and class II HDACs in vivo.

Misregulation of signaling pathways that influence growth plate dynamics results in dwarfisms and bone deformations due to either accelerated maturation of the growth plate cartilage, characteristic of achondroplasia,

or delayed or absent chondrocyte maturation, characteristic of many chondrodysplastic syndromes (Zelzer and Olsen, 2003; Kronenberg, 2003). Our results demonstrate that growth plate dynamics can be modified by the balance of MEF2C and its repressor, HDAC4. The realization that bone formation is controlled by the partnership of MEF2C and HDAC4 suggests possibilities for therapeutically manipulating endochondral bone growth by modulating the signaling pathways that govern the association of HDAC4 with MEF2C. Moreover, our results suggest that HDAC inhibitors currently in clinical trials for cancer, neurodegenerative diseases, and other disorders may also promote bone formation.

Commonalities of Developmental Processes Controlled by MEF2 and Class II HDACs

MEF2 transcription factors are well known regulators of skeletal muscle differentiation and fiber type switching, as well as cardiovascular development, heart growth, and neuronal development (reviewed in McKinsey et al.,

(B) COS7 cells were transfected with a luciferase reporter controlled by the wild-type *Col10a1* upstream region or the same region in which the MEF2 sites were disrupted by point mutations and increasing amounts of MEF2C-myc or a constitutively nuclear HDAC4 mutant (HDAC S/A), as indicated. Error bars indicate one standard deviation.

(C) Electromobility shift assays of MEF2 binding to sites in the *Col10a1* promoter. Oligonucleotides containing MEF2 sites from the *Col10a1* promoter were labeled with ^{32}P and were assayed for myc-MEF2C binding. MEF2C was supershifted with an anti-myc antibody (Anti-myc) or competed with 100 molar excess of the *Srpk3* MEF2-binding site (cold competitor) and compared to reticulocyte lysate that did not contain MEF2C (−) or binding of a labeled probe containing the corresponding MEF2 site disrupted by point mutations (M). MEF2-binding sites conserved between the mouse and human genomes are indicated with an asterisk (*).

(D) β-galactosidase staining of E18.5 mice transgenic for Col10-lacZ. Note the strong expression of the transgene in the growth plates. H&E sections of the radius and sternum show specific expression of the transgene in hypertrophic chondrocytes.

(E) Mutation of the MEF2-binding sites in the Col10-lacZ transgene diminishes the expression of β-galactosidase in hypertrophic chondrocytes, demonstrating the requirement of MEF2 factors in the activation of the Col10-lacZ transgene.

(F) Opposing influences of MEF2C and HDAC4 on mineralization of sternal and rib cartilage. Anterior views of the sternum and ribs of mice stained for bone (red) and cartilage (blue) at (Fa–Ff) P1 and (Fg–Fl) P8. Genotypes are indicated at the top. The manubrium (m) and xiphoid process (x) are identified in (Fg). Arrows mark sites of ossification that are altered by the balance of *Mef2c* and *Hdac4*.

2002). Class II HDACs play key roles in each of these processes by governing MEF2 transcriptional activity. The results of the present study indicate that these seemingly unrelated processes of cell differentiation, tissue growth, and remodeling share an underlying commonality with the regulatory mechanisms that control bone development. Specificity in each case is dictated by the association of MEF2 and class II HDACs with other cell type-specific and signal-responsive transcriptional regulators. Given the importance of MEF2 for vascular development (Lin et al., 1998) and bone formation, it will be of interest to determine whether the developmental mechanism described here is also operative in the settings of vascular calcification, which is among the most common cardiovascular disorders.

EXPERIMENTAL PROCEDURES

Creation of a Conditional *Mef2c* Mutant Allele
Methods for creation of the *Mef2c* allele and generation of mutant mice are described in Supplemental Data.

Transgenic Mice
Chondrocyte-specific transgenes were constructed by subcloning either an in-frame fusion of the MEF2C DNA-binding domain and the Engrailed repressor or an in-frame fusion of MEF2C and the VP16 activation domain, between a 3 kb fragment of the *Col2a1* promoter and its 3 kb chondrocyte-specific enhancer region (Zhou et al., 1998). Linearized transgenic constructs were injected into the pronuclei of fertilized oocytes. Embryos were harvested and analyzed for changes in skeletal development. The severity of the skeletal phenotype in these mice correlated with transgene copy number.

Tissue Culture and Transfection
COS7 cells were grown in DMEM with 10% FBS. Fugene 6 (Roche) was used for transient transfection according to the manufacturer's instructions. A reporter plasmid containing 4.5 kb of the *Col10a1* locus was generated by PCR and was cloned into pGL3-basic (Promega). Expression constructs for MEF2C, HDAC4, and Runx2 have been previously described (McKinsey et al., 2000; Vega et al., 2004). Plasmids were cotransfected with 10 ng of a CMV-β-galactosidase reporter plasmid to control for transfection efficiency.

Electromobility Shift Assays
MEF2C was translated in vitro in a coupled transcription-translation T7 reticulocyte lysate system (Promega). In vitro binding analysis was performed as previously described (Wang et al., 2001), by using oligonucleotide fragments that contained a MEF2-binding site from the *Col10a1* promoter, which competed with a MEF2-binding site from the *Srpk3* promoter (Nakagawa et al., 2005). Oligonucleotide sequences are available upon request.

Tissue-Specific Deletion of *Mef2c*
To generate mice that lack MEF2C in specific tissues, mice heterozygous for the null allele of *Mef2c* were mated to mice bearing the designated Cre transgene or knockin. The resulting mice were mated to mice homozygous for the *Mef2c^{loxP}* allele to produce conditional null mice. Mesoderm deletion of *Mef2c* was performed by using the *Twist2-Cre* knockin (Yu et al., 2003). Chondrocyte deletion was performed by using *Col2-Cre* (Long et al., 2001). All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees at the University of Texas Southwestern Medical Center.

Cartilage and Bone Staining
Euthanized mice were skinned, eviscerated, and fixed overnight in 100% ethanol. Adult animals were additionally incubated overnight in acetone to clear fatty tissues. All specimens were then stained with Alcian blue for 48 hr. Soft tissues were removed by incubation in 2% KOH as needed, and specimens were stained with Alizarin red for 30–90 min as needed. Tissues were cleared in 1% KOH, 20% glycerol and were photographed in 50% ethanol, 50% glycerol. Staining reagents were prepared as described previously (McLeod, 1980).

Histology and β-Galactosidase Staining
Tissues were fixed in 10% phosphate-buffered formalin at 4°C. Samples were then embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin or with von Kossa's method. Briefly, sections were deparaffinized and immersed in 5% silver nitrate solution for 30 min while being exposed to a 100W light bulb. Slides were rinsed in distilled water and immersed in 5% sodium thiosulfate for 3 min. Slides were rinsed again in distilled water and counterstained with nuclear fast red for 5 min. Methods for β-galactosidase staining of transgenic embryos are described in Supplemental Data.

In Situ Hybridization
Tissue samples were fixed overnight in DEPC-treated 4% paraformaldehyde. Riboprobes were labeled with ^{35}S-UTP by using the MAXIscript in vitro transcription kit (Ambion; Austin, Texas). In situ hybridization of sectioned tissues was performed as previously described (Vega et al., 2004).

Supplemental Data
Supplemental Data include the strategy for creation of the conditional *Mef2c* mutant allele and analysis of markers of bone development in mutant embryos and are available at http://www.developmentalcell.com/cgi/content/full/12/3/377/DC1/.

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