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# Histone Deacetylase 4 Controls Chondrocyte Hypertrophy during Skeletogenesis

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## Summary

**Histone deacetylases (HDACs) modulate cell growth and differentiation by governing chromatin structure and repressing the activity of specific transcription factors. We showed previously that HDAC9 acts as a negative regulator of cardiomyocyte hypertrophy and skeletal muscle differentiation. Here we report that HDAC4, which is expressed in prehypertrophic chondrocytes, regulates chondrocyte hypertrophy and endochondral bone formation by interacting with and inhibiting the activity of Runx2, a transcription factor necessary for chondrocyte hypertrophy. HDAC4-null mice display premature ossification of developing bones due to ectopic and early onset chondrocyte hypertrophy, mimicking the phenotype that results from constitutive Runx2 expression in chondrocytes. Conversely, overexpression of HDAC4 in proliferating chondrocytes in vivo inhibits chondrocyte hypertrophy and differentiation, mimicking a Runx2 loss-of-function phenotype. These results establish HDAC4 as a central regulator of chondrocyte hypertrophy and skeletogenesis and suggest general roles for class II HDACs in the control of cellular hypertrophy.**

## Introduction

Regulated changes in chromatin structure play a central role in the control of gene transcription. Posttranslational modifications of nucleosomal histones have been proposed to influence chromatin structure and to create a code that is interpreted by positive and negative transcriptional regulators that recognize specific histone modifications. Histone acetylation, catalyzed by histone acetyltransferases (HATs), promotes gene transcription by relaxing chromatin structure, thereby facilitating access of the transcriptional machinery to DNA target sequences (Jenuwein and Allis, 2001). The transcription-activating effect of histone acetylation is counterbalanced by histone deacetylation, which favors chromatin condensation and transcriptional repression.

There are two major classes of histone deacetylases (HDACs) (Grozinger and Schreiber, 2002). The class I HDACs (HDAC1, 2, 3, and 8) are widely expressed and consist mainly of a catalytic domain. In contrast, the class II HDACs (HDAC 4, 5, 7, and 9) display cell type-restricted patterns of expression and contain an N-terminal extension that links them to specific transcription factors and confers responsiveness to a variety of signal transduction pathways, thereby connecting the genome with the extracellular environment (Verdin et al., 2003). Phosphorylation of conserved serine residues in class II HDACs by calcium/calmodulin-dependent kinase (CaMK), and other kinases, in response to specific stimuli creates docking sites for the 14-3-3 family of protein chaperones (Grozinger and Schreiber, 2000; McKinsey et al., 2000a, 2000b). Binding of 14-3-3 results in the export of HDACs from the nucleus and derepression of specific HDAC target genes.

While the consequences of histone acetylation and deacetylation have been analyzed extensively in cultured cells and cell-free systems, relatively little is known of the specific functions of HDACs in vivo. HDAC5 and 9 are the only class II HDACs to have been inactivated in the mouse. Mice lacking HDAC9 are viable, but their hearts respond to stress by profound hypertrophic growth (Zhang et al., 2002; Chang et al., 2004), suggesting that HDAC5 and 9 act as a transcriptional repressor of cardiac hypertrophy. Consistent with this notion, HDAC9 interacts with and suppresses the activity of the MEF2 transcription factor (Zhang et al., 2001), an activator of hypertrophic gene expression. The role of HDAC9 (and other class II HDACs) as antagonists of cardiac hypertrophy is further supported by the findings that hypertrophic signals lead to the phosphorylation of these HDACs and their nuclear export, and mutant HDAC proteins that are signal resistant prevent cardiomyocyte hypertrophy (Zhang et al., 2002).

During embryonic development, the majority of bones form through a process of endochondral ossification in which mesenchymal cells first form a cartilaginous template of the future bone (Karsenty and Wagner, 2002). Chondrocytes in the center of these elements undergo hypertrophy and secrete an extracellular matrix that becomes mineralized and allows vascular invasion to occur. Thereafter, osteoblasts produce an extracellular matrix rich in type I collagen that replaces the cartilaginous template. Chondrocyte hypertrophy is essential for vascular invasion, osteoblast differentiation, and endochondral ossification (Kronenberg, 2003; Olsen et al., 2000). To date, the only transcription factor shown to be required for chondrocyte hypertrophy is runt-related transcription factor-2 (Runx2), which is also necessary for osteoblast differentiation (Karsenty and Wagner, 2002).

In the present study, we generated mice lacking HDAC4; the mice display a remarkable phenotype characterized by inappropriate chondrocyte hypertrophy leading to ectopic bone formation, abnormalities analo-

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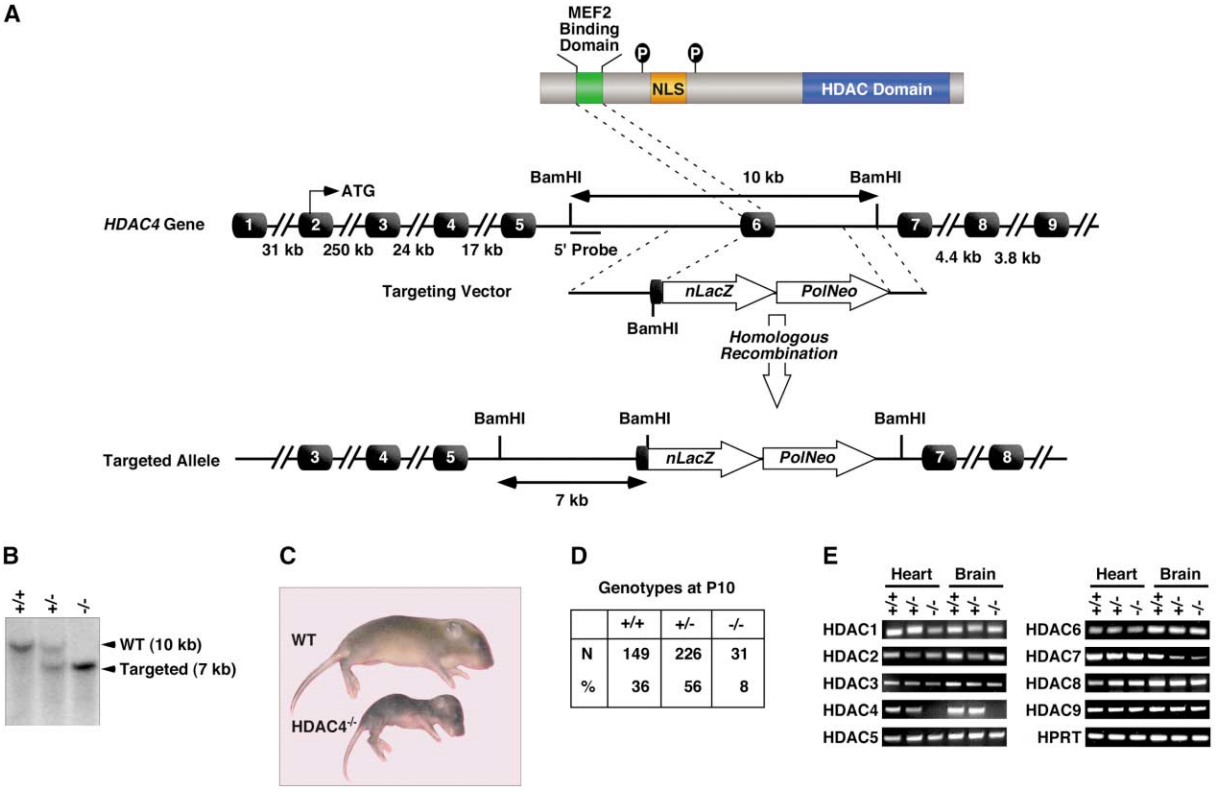


Figure 1. Generation of HDAC4-Null Mice

(A) Schematic representation of the *HDAC4* gene, the targeting vector, and the targeted gene. The first nine exons of the *HDAC4* gene are shown with the 5′ and 3′ genomic regions used for homologous recombination. A nuclear-localized lacZ expression cassette was inserted in-frame into exon 6.

(B) Representative Southern blot of genomic DNA from wild-type and *HDAC4* heterozygous and homozygous null animals digested with BamHI. The position of the probe is shown in (A).

(C) Photograph of postnatal day 8 (P8) wild-type and HDAC4-null pups.

(D) Genotypes of 10-day-old pups from matings of HDAC4$^{+/-}$ mice.

(E) RNA from hearts and brains of mice of the indicated genotypes was analyzed by RT-PCR for transcripts of the indicated HDACs. Transcripts for HPRT were detected as a control for RNA loading and integrity.

gous to the phenotype observed in mice with constitutive expression of Runx2 in chondrocytes (Takeda et al., 2001; Ueta et al., 2001). We demonstrate that HDAC4, which is expressed in prehypertrophic chondrocytes, associates with and inhibits the activity of Runx2. Furthermore, ectopic overexpression of HDAC4 in proliferating chondrocytes in vivo inhibits hypertrophy and, thus, ossification of the developing bone, mimicking the Runx2 loss-of-function phenotype (Inada et al., 1999; Komori et al., 1997). These results establish HDAC4 as a key regulator of chondrocyte hypertrophy and thereby skeletal development and suggest a general role for class II HDACs as negative regulators of cellular hypertrophy during development and disease.

## Results

### Generation of HDAC4$^{-/-}$ Mice

To investigate the function of HDAC4 in vivo, we disrupted the mouse *HDAC4* gene by homologous recombination. The *HDAC4* gene contains 31 exons encompassing approximately 425 kilobases of DNA on mouse chromosome 1. The targeting strategy is depicted in Figure 1A in the context of the first nine exons of the *HDAC4* locus. The targeting vector was designed to insert a nuclear-localized lacZ cassette into exon 6 such that lacZ was in-frame with amino acid 162 of HDAC4. This mutation resulted in deletion of the MEF2 binding domain of HDAC4, as well as the nuclear localization sequence and the complete deacetylase catalytic domain. Homologous recombination was confirmed by Southern blot analysis of ES cells (Figure 1B), which were used to generate HDAC4 mutant mice.

Intercrosses of HDAC4$^{+/-}$ mice in either the isogenic 129Sv/EV background or in a 129Sv/EV-C57BL/6 mixed genetic background yielded HDAC4-null offspring at Mendelian ratios at birth (data not shown). Within the first few days after birth, homozygous mutants were readily identifiable by their "dome-shaped" heads and misshaped spines (Figure 1C). A subset of HDAC4 mutants also displayed exencephaly (data not shown). By postnatal day (P) 10, HDAC4-null mice were severely runted and markedly underrepresented (Figure 1D), and no HDAC4-null mice survived to weaning. As discussed

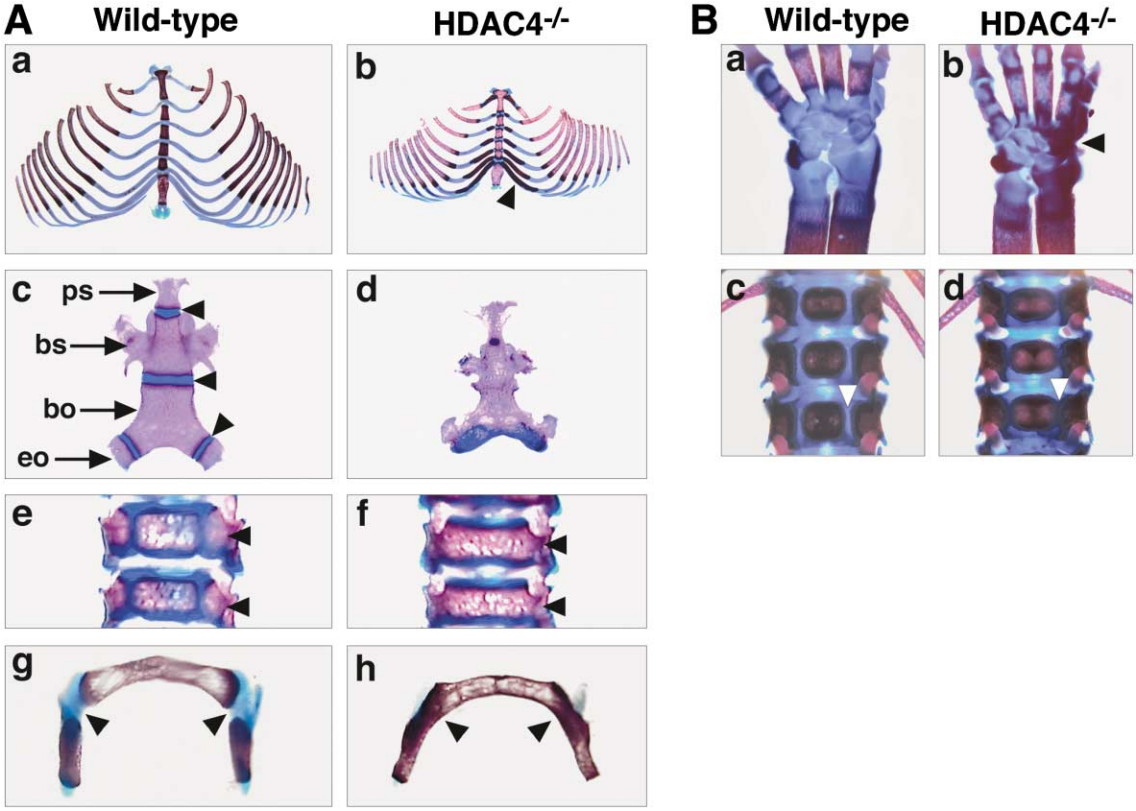


Figure 2. Premature Ossification in HDAC4$^{-/-}$ Mice

(A) Skeletons from wild-type and HDAC4$^{-/-}$ mice at P8 were stained with Alizarin red and Alcian blue as described in Experimental Procedures. Images show ribcages (a and b), the ventral chondrocranium (c and d), vertebrae (e and f), and the hyoid bone (g and h). Bones in (c) are marked in the wild-type sample as follows: ps, presphenoid; bs, basisphenoid; bo, basioccipital; and eo, exoccipital. Arrowheads mark synchondroses in the wild-type skull, which are completely ossified in the HDAC4 mutant. Arrowheads also mark regions of mineralized chondrocostal cartilage in the HDAC4$^{-/-}$ mice (b).

(B) Skeletons from E18 wild-type or HDAC4 mutant mice were stained with Alizarin red and Alcian blue as in (A). Aberrant ossification in the wrist (b) and the vertebrae (d) is evident in the HDAC4$^{-/-}$ skeletons as marked by the arrowheads.

below, the primary cause of growth retardation and eventual death of HDAC4 mutant mice appears to be the inappropriate ossification of cartilaginous skeletal elements, which impacts on mobility and ability to breathe.

To confirm that the targeted mutation eliminated functional HDAC4, we performed RT-PCR using primers for sequences upstream and downstream of the lacZ insertion site. There was a complete absence of wild-type HDAC4 transcripts in tissues from homozygous mutant mice (Figure 1E). We also examined the expression of HDACs 1, 2, 3, 5, 6, 7, 8, and 9 by RT-PCR in tissues from wild-type and HDAC4 mutant mice. Transcripts encoding these HDACs were expressed at normal levels in the mutant, indicating that other HDACs were not upregulated to compensate for the absence of HDAC4.

### HDAC4-Null Mice Display Skeletal Defects

Tissue dissections and histological examination of HDAC4-null mice revealed no obvious abnormalities of the heart, skeletal muscle, or other major organs examined. However, numerous skeletal abnormalities were readily apparent. Staining of mineralized bone/cartilage and of nonmineralized cartilage with Alizarin red and Alcian blue, respectively, revealed premature mineralization of cartilage in skeletal elements ossifying through endochondral ossification in all mutant mice analyzed at P8. This was particularly evident in the chondrocostal cartilage (Figures 2Aa and 2Ab). In addition, the synchondroses in the base of the skull between the presphenoid and basisphenoid bones and between the basisphenoid and basioccipital bones were completely mineralized in HDAC4$^{-/-}$ but not in wild-type mice (Figures 2Ac and 2Ad). This fusion prevents longitudinal growth of the skull and results in dorsal and caudal displacement of the brain, producing a domed skull. In the vertebrae of HDAC4$^{-/-}$ mice, the ossification centers of the arch and body were fused such that no cartilage remained (Figures 2Ae and 2Af). The cartilaginous regions of the hyoid bone were also completely ossified in the HDAC4 mutant mice (Figures 2Ag and 2Ah). Bones that form through intramembranous ossification, i.e., without a cartilaginous intermediate, such as bones of the cranium and the clavicle, were unaffected in the mutant mice. These phenotypic abnormalities were already present at E18 of embryonic development. In-

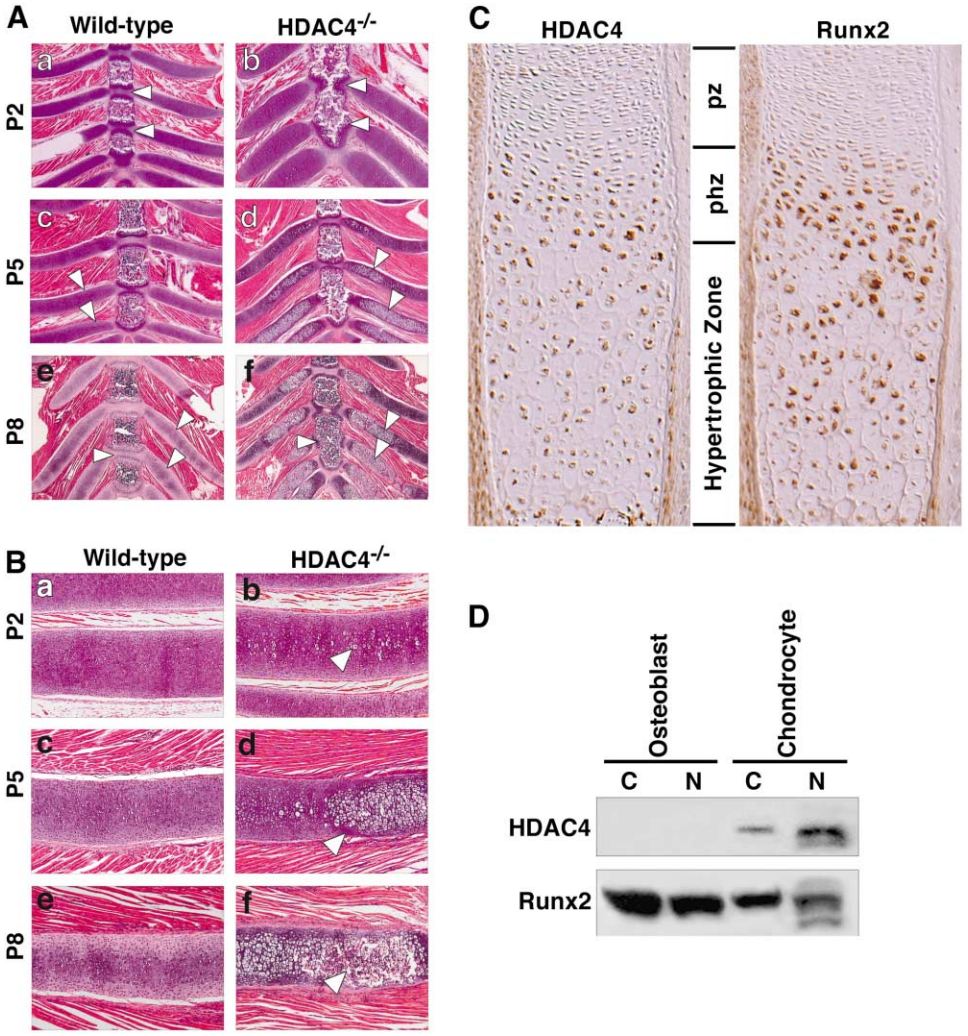


Figure 3. Ectopic Chondrocyte Hypertrophy in HDAC4$^{-/-}$ Mice

(A) Premature chondrocyte hypertrophy in HDAC4$^{-/-}$ mice. Hematoxylin and eosin staining was performed on sections of ribs isolated from wild-type (a, c, and e) and HDAC4$^{-/-}$ (b, d, and f) mice at P2, P5, and P8. Arrowheads in (a) and (b) mark sternum, which is abnormally ossified in the HDAC4 mutant. Arrowheads in (c)–(f) mark the ribs in which chondrocytes are prematurely hypertrophied in the HDAC4 mutant.

(B) Higher magnification of ribs from wild-type (a, c, and e) and HDAC4$^{-/-}$ (b, d, and f) mice. Arrowheads point to ectopic chondrocyte hypertrophy at P2 and P5 (b and d) and ossification at P8 (f) in the HDAC4 mutant.

(C) Immunohistochemistry of HDAC4 and Runx2 was performed on E18.5 ribs. HDAC4 expression is localized to the prehypertrophic and hypertrophic zones paralleling the staining pattern of Runx2. No HDAC4 staining is observed in the proliferating chondrocytes or trabeculated bone. Phz, prehypertrophic zone; pz, proliferative zone.

(D) Western blot of whole-cell (C) and nuclear extracts (N) from primary chondrocytes and osteoblasts for Runx2 and HDAC4. Runx2 is expressed in both cell types, whereas HDAC4 is expressed only in chondrocytes.

deed, examination of the developing wrist revealed premature mineralization in HDAC4$^{-/-}$ animals (Figure 2B). Fusion of the ossification centers of the arch and body of the vertebrae was also beginning at this stage.

Chondrocyte hypertrophy is a mandatory process during endochondral bone formation. The cartilage growth plate of developing bones consists of proliferating chondrocytes that exit the cell cycle and transition to become prehypertrophic and then hypertrophic chondrocytes (Karsenty and Wagner, 2002). Hypertrophic chondrocytes then undergo apoptosis and are eventually replaced by osteoblasts that lay down the bone-specific matrix. The specificity of the bone abnormalities in HDAC4 mutant mice suggested that the primary defect might be in the control of chondrocyte hypertrophy .

### Ectopic Chondrocyte Hypertrophy in HDAC4-Null Mice

Since the chondrocostal cartilage never mineralizes in wild-type mice, whereas it was almost completely mineralized in HDAC4-null animals, we focused our attention on this skeletal element. To pinpoint the onset of skeletal abnormalities in HDAC4-null mice, we examined the chondrocostal cartilage at time points before (E18, P0,

P2, and P5) and after (P8) extensive ectopic ossification was observed. No abnormalities in the chondrocostal cartilage were observed at E18 or P0 (data not shown). By P2, hypertrophic chondrocytes were visible in the chondrocostal cartilage of mutant mice but not in their wild-type littermates (Figures 3A and 3B). At P5, hypertrophic chondrocytes were present throughout the chondrocostal cartilage of the mutants, and by P8, a mature ossification center was established and hypertrophic cartilage was replaced by trabeculated bone (Figures 3A and 3B). These phenotypic abnormalities were not restricted to the chondrocostal cartilage and were also evident in other skeletal elements such as the vertebrae, occipital sutures, and the sternum, which was ossified already at P2 (data not shown and Figure 3A).

Premature chondrocyte hypertrophy in HDAC4-null animals was also demonstrated molecularly. Indian hedgehog (Ihh) secretion from prehypertrophic chondrocytes tightly controls chondrocyte hypertrophy and differentiation (Vortkamp et al., 1996, Kronenberg, 2003). Recently, the *Ihh* gene was shown to be a direct transcriptional target gene of Runx2 (Yoshida et al., 2004). At E18.5, a developmental stage when histological abnormalities were not yet noticeable, Ihh expression was dramatically increased in the chondrocostal cartilage of the mutant compared to WT littermates (Figure 4A). Runx2 also regulates its own expression by binding its own gene promoter, thereby fulfilling a positive autoregulatory loop (Ducy et al., 1999). Consistent with the possible enhancement of Runx2 activity in developing skeletal elements of HDAC4 mutant mice, Runx2 expression was upregulated in the chondrocostal cartilage of the mutant (Figure 4A). Following this increase in Ihh and Runx2 expression, $\alpha$1(X) collagen, a marker of hypertrophic chondrocytes, began to be expressed in chondrocostal chondrocytes of HDAC4 mutants at P2 (Figure 4B). By P8, expression of the osteoblast marker, $\alpha$1(I) collagen, confirmed the invasion of osteoblasts and the presence of bone trabeculae, following the presence of an established growth plate in HDAC4$^{-/-}$ chondrocostal cartilage (Figure 4B).

The above results suggested that the skeletal abnormalities in HDAC4 mutant mice arose from a primary defect in the regulation of chondrocyte hypertrophy. To determine if HDAC4 was expressed in a pattern compatible with such a phenotype, immunohistochemical analysis was performed. HDAC4 was not expressed at detectable levels in proliferating chondrocytes but was upregulated in prehypertrophic chondrocytes in the growth plate at E18.5 (Figure 3C). Expression of HDAC4 diminished through the area of hypertrophic chondrocytes. HDAC4 expression was not visible by immunostaining of the bone or osteoblasts. Expression of HDACs 5, 7, and 9 was not detected in developing bones (data not shown), suggesting that HDAC4 is the sole class II HDAC involved in chondrocyte hypertrophy and skeletogenesis during embryogenesis.

Western blot analysis of whole-cell and nuclear extracts of isolated osteoblasts and chondrocytes also confirmed the specific expression of HDAC4 in chondrocytes (Figure 3D). In contrast, Runx2 was readily detectable in both cell types as described previously (Ducy et al., 1997; Takeda et al., 2001).

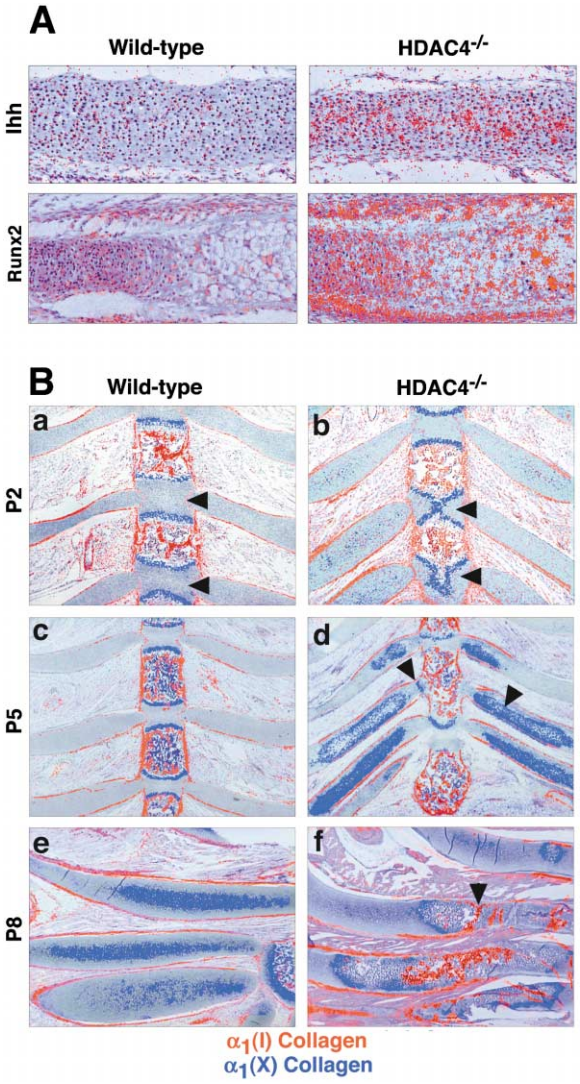


Figure 4. Increased Expression of Markers of Hypertrophic Chondrocytes and Bone in HDAC4$^{-/-}$ Mice

(A) Expression of Indian hedgehog (Ihh) and Runx2 (shown in red) was detected by in situ hybridization of chondrocostal cartilage from E18.5 and E16.5 specimens, respectively. Ihh and Runx2 expression was upregulated precociously in the mutant.

(B) Expression of $\alpha$1(I) collagen (shown in red) and $\alpha$1(X) collagen (shown in blue) was detected by in situ hybridization of ribs from wild-type (a, c, and e) and HDAC4$^{-/-}$ (b, d, and f) mice at P2, P5, and P8. Arrowheads in (a) and (b) mark chondrocostal cartilage, which is abnormally ossified in the HDAC4 mutant. Arrowheads in (c)–(f) mark the ribs in which chondrocytes are prematurely hypertrophied in the HDAC4 mutant. Extensive ossification can be seen in the mutant by P8, whereas no evidence of ossification is detected in these developing skeletal elements of the wild-type animal at this stage.

## HDAC4 Is a Runx2 Corepressor

The phenotypic abnormalities noted in HDAC4$^{-/-}$ mice are reminiscent of those observed in transgenic mice with prolonged expression of Runx2 in prehypertrophic cartilage (Takeda et al., 2001; Ueta et al., 2001). Given the expression of HDAC4 in prehypertrophic chondrocytes and the abnormal chondrocyte differentiation seen in HDAC4$^{-/-}$ mice, we speculated that chondrocyte hyper-

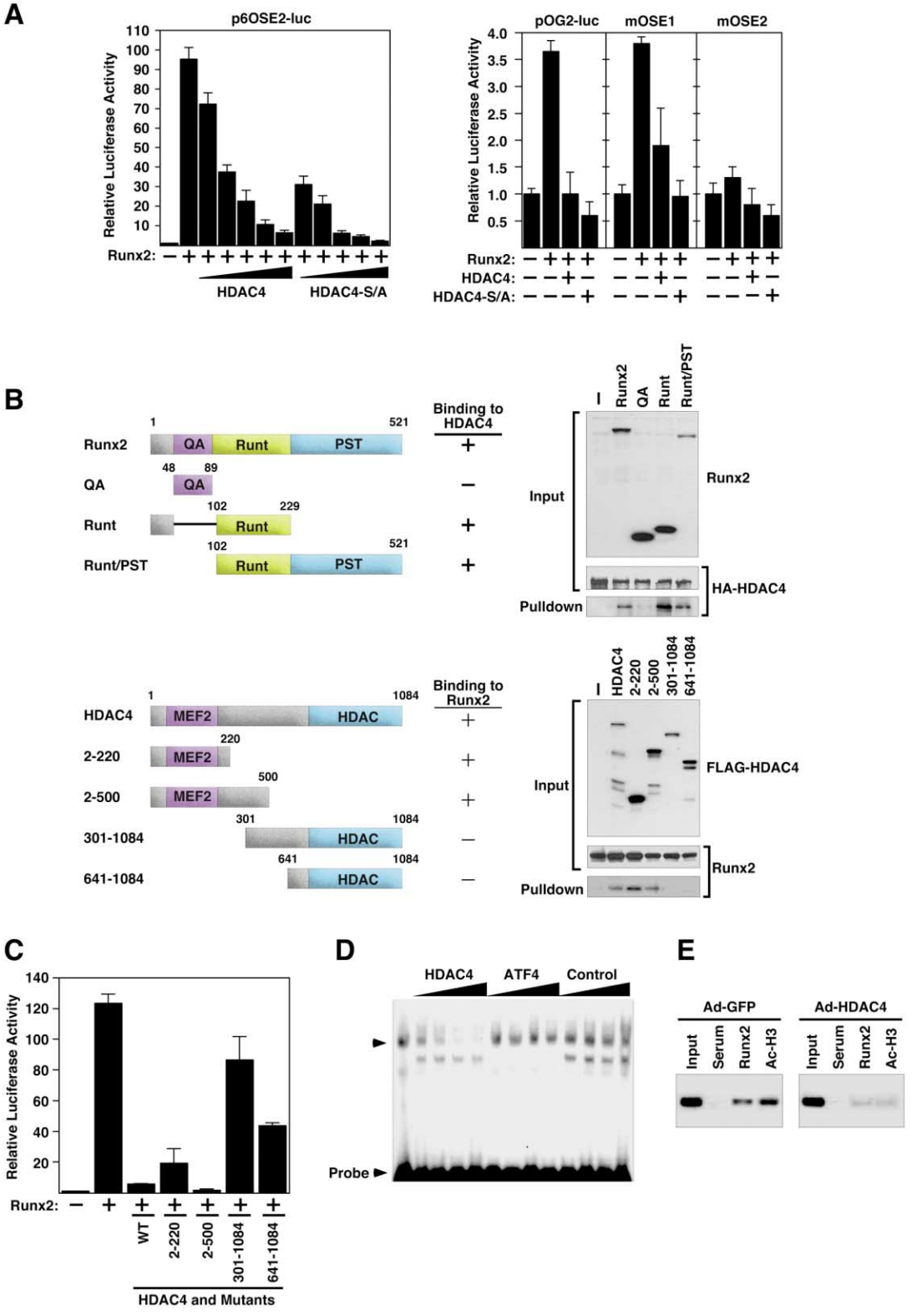


Figure 5. Repression of Runx2 Activity by Association with HDAC4

(A) Left panel: COS cells were transfected with the p6OSE2-Luc reporter containing six copies of the Runx2 binding site with or without expression plasmids encoding Runx2 (100 ng) and wild-type HDAC4 or signal-resistant HDAC4 mutant (HDAC4-S/A) in increasing amounts (10, 25, 50, 100, or 200 ng). Right panel: COS cells were transfected with the pOG2-Luc reporter containing a portion of the osteocalcin promoter with or without expression plasmids encoding Runx2 (100 ng) and wild-type HDAC4 or signal-resistant HDAC4 (100 ng). Transfections were also performed with pOG2-Luc constructs containing mutations in the Runx2 binding sites, mOSE1-Luc and mOSE2-Luc. The data represent the mean ± the SEM.

trophy might require the release of Runx2 from a repressive influence of HDAC4. To address this possibility, we tested the effect of HDAC4 on the transcriptional activity of Runx2. Indeed, the ability of Runx2 to activate a luciferase reporter controlled by tandem copies of the Runx2 binding site was repressed by HDAC4 in a dose-dependent manner (Figure 5A). A signal-resistant and constitutively nuclear mutant of HDAC4 in which three serines responsible for nuclear export were mutated to alanines (HDAC4-S/A) was an even more potent repressor of Runx2. HDAC4 repression was dependent on Runx2 DNA binding, as a reporter construct containing a mutant Runx2 site was largely unaffected by HDAC4 (Figure 5A). In contrast to the complete inhibition of Runx2 activity by HDAC4, the activity of Sox9, a transcription factor required for commitment to the chondrocyte lineage (Akiyama et al., 2002), was only modestly repressed by HDAC4 (data not shown). These results suggest that Runx2 is a key target for the repressive influence of HDAC4 on chondrocyte hypertrophy and mineralization of developing bones.

### HDAC4 Interacts Directly with Runx2

To explore the mechanism for HDAC4-mediated repression of Runx2 activity, we tested whether HDAC4 and Runx2 could physically interact. As shown in Figure 5B, coimmunoprecipitation experiments demonstrated the association of HDAC4 and Runx2. This interaction was localized to the Runt DNA binding domain of Runx2. The Runx2 interaction domain of HDAC4 was localized to the first 220 amino acids of the protein, which also contains the MEF2 binding region. HDAC4 binding to Runx2 was necessary for full repression of Runx2 activity (Figure 5C). However, we observed residual repressive activity with two HDAC4 deletion mutants (301–1084 and 641–1084) that did not display detectable binding to Runx2 (Figure 5C). Whether the latter mutants bind Runx2 weakly or act through a mechanism distinct from that of the Runx2 binding domain of HDAC4 is unclear.

The observation that HDAC4 interacts with the Runx2 DNA binding domain suggested that it might also inhibit Runx2 DNA binding activity. Indeed, as shown in Figure 5D, HDAC4 inhibited the ability of Runx2 to bind DNA in vitro. In contrast, ATF4, another transcription factor important in osteoblast differentiation (Yang et al., 2004), had no effect on Runx2 binding. We conclude that the association of HDAC4 with the Runt domain of Runx2 interferes with DNA binding by Runx2 and, consequently, inhibits activation of Runx2 target genes.

### Detection of HDAC4-Runx2 Interaction by Chromatin Immunoprecipitation

To further explore the potential role of HDAC4 in repression of Runx2 activity, we examined the effect of HDAC4 on Runx2 association with the *Runx2* promoter by chromatin immunoprecipitation (ChIP) of extracts from mouse primary chondrocytes infected with adenovirus expression vectors encoding HDAC4 or GFP as a negative control. As shown in Figure 5E, the association of Runx2 with the *Runx2* promoter was readily detected by ChIP assay, and this interaction was diminished in the presence of HDAC4. Adenoviral expression of HDAC4 also specifically suppressed the acetylation of histone H3 on the *Runx2* promoter. These results support the conclusion that HDAC4 negatively influences Runx2 activity in vivo and provide a potential explanation for the increase in Runx2 expression in HDAC4 mutant mice.

### HDAC4 Is Sufficient to Inhibit Chondrocyte Hypertrophy In Vivo

The above findings suggested that HDAC4 suppressed Runx2 activity, which would be expected to prevent chondrocyte hypertrophy and, therefore, bone formation in skeletal elements that ossify through endochondral bone formation. To determine whether HDAC4 could inhibit chondrocyte hypertrophy in vivo, we generated mice bearing a transgene in which the signal-resistant HDAC4 mutant was overexpressed in proliferating chondrocytes under control of the *α1(II) collagen* promoter. Skeletons of at least 12 α1(II)-HDAC4 transgenic animals were analyzed at E18.5. As shown in Figure 6A, α1(II)-HDAC4 transgenic mice displayed a severe skeletal phenotype, mimicking that of Runx2-deficient mice, characterized by the lack of mineralized bone as detected by Alizarin red staining (Komori et al., 1997; Otto et al., 1997). All bones ossifying through an endochondral process were affected. This was particularly evident in the ribcage, in vertebrae, and at the base of the skull. In contrast, bones that form through intramembranous ossification, such as the clavicle, were unaf-

(B) Top panel: COS cells were transfected with expression plasmids encoding Flag-epitope-tagged Runx2 deletion mutants (shown to the left) and HA-tagged HDAC4. Flag-Runx2 was immunoprecipitated with an anti-Flag antibody, followed by Western blot analysis to detect HA-HDAC4 binding. Bottom panel: COS cells were transfected with expression plasmids encoding Flag-epitope-tagged HDAC4 deletion mutants (shown to the left) and Runx2. Flag-HDAC4 was immunoprecipitated with an anti-Flag antibody, followed by Western blot analysis to detect Runx2 binding.

(C) COS cells were transfected with the p6OSE2-Luc reporter and expression plasmids encoding Runx2 and the various HDAC4 deletion mutants shown in (B). The data represent the mean ± the SEM.

(D) Electromobility shift assays were performed with a labeled OSE2 probe and GST-Runx2. Increasing amounts of recombinant HDAC4 or ATF4 translated in vitro were added to the DNA binding reaction, as indicated. As a control, equivalent amounts of unprogrammed lysate were used. The upper arrowhead depicts the OSE2/Runx2 complex. Free unbound probe is indicated at the bottom of the gel.

(E) ChIP assays were performed using soluble chromatin prepared from mouse primary chondrocytes infected with adenovirus expression constructs encoding HDAC4 or GFP, as indicated. Chromatin was immunoprecipitated with nonimmune rabbit IgG or antibodies specific for Runx2 or acetylated histone H3, as indicated, and precipitated genomic DNA was analyzed by PCR using primers for the *Runx2* promoter, which contains a Runx2 binding site. An input control in which PCR amplification was performed prior to immunoprecipitation is also shown. HDAC4 specifically perturbs the association of Runx2 with its target sequence in chromatin and suppresses acetylation of histone H3 on the *Runx2* promoter.

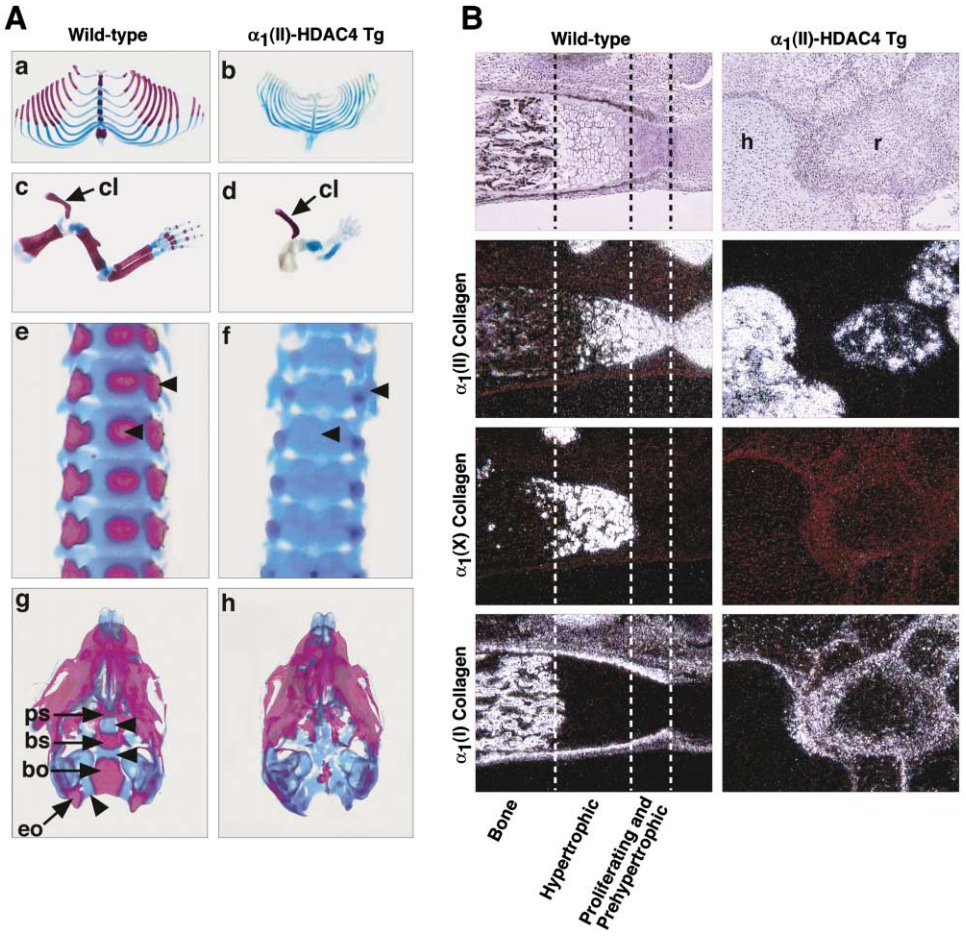


Figure 6. HDAC4 Inhibits Chondrocyte Hypertrophy In Vivo

(A) Skeletal preparations from α1(II)-HDAC4 transgenic or wild-type embryos at E18.5 were stained with Alizarin red and Alcian blue to visualize mineralized bone and cartilage, respectively. Skeletal elements shown are ribcage (a and b), forelimb (c and d), vertebrae (e and f), and the base of the skull (g and h). Note that the clavicle (cl) is unaffected in the transgenic mouse (d). The abbreviations in the bottom panel are as follows: ps, presphenoid; bs, basisphenoid; bo, basiocciptal; and eo, exoccipital. Arrowheads in (e) point to ossification centers and in (f) to the corresponding region which lacks ossification.

(B) Histological analysis and in situ hybridization were performed on forearms isolated from α1(II)-HDAC4 transgenic or wild-type control embryos at E18.5. The dashed lines depict the boundaries between the proliferating/prehypertrophic chondrocytes, hypertrophic chondrocytes, and bone. The structures in the top right panel are noted as the humerus (h) and radius (r).

fected by forced expression of HDAC4 (Figure 6A). The specificity of this phenotype provides strong support for the involvement of HDAC4 in the control of endochondral bone formation.

Certain transgenic animals also displayed severe foreshortening of the limbs. An example of this is shown in Figure 6Ad in which the growth of the forelimb is markedly blunted. Histological analysis of α1(II)-HDAC4 transgenic mice revealed a complete lack of hypertrophic chondrocytes and osteoblasts and an absence of bone trabeculae (Figure 6B).

We further defined the molecular defects associated with ectopic HDAC4 expression by analyzing the expression of collagen genes specific for the different phases of chondrocyte development. α1(II) collagen, a marker of proliferating chondrocytes, was expressed throughout the cartilaginous skeletal elements of the transgenic forelimb at E18.5 (Figure 6B). α1(X) collagen, a marker of hypertrophic chondrocytes, was not expressed in the cartilaginous skeletal elements of transgenic animals, consistent with the lack of chondrocyte hypertrophy. There was also a complete absence of expression of α1(I) collagen, a marker of trabeculated bone, in skeletal elements of transgenic mice.

## Discussion

Most bones ossify through endochondral ossification, which includes an intermediary step in which a cartilaginous template containing hypertrophic chondrocytes prefigures the future bone. The results presented here demonstrate that HDAC4 governs chondrocyte hypertrophy and thereby endochondral bone formation by negatively regulating the activity of Runx2. The following observations support this conclusion. (1) HDAC4 is expressed in prehypertrophic chondrocytes in vivo. (2) HDAC4 mutant mice display precocious and ectopic mineralization of endochondral bones, mimicking the

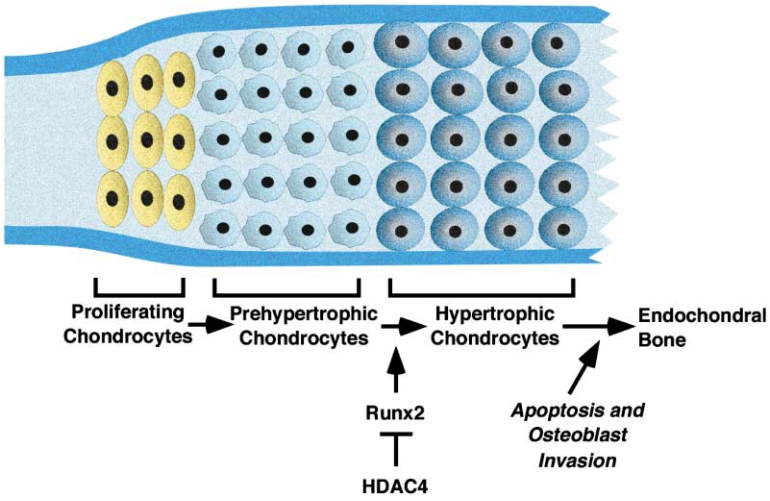


Figure 7. A Model for the Control of Chondrocyte Hypertrophy and Bone Development by HDAC4

A schematic of cellular transitions involved in endochondral bone development is shown at the top. The regulatory step controlled by HDAC4 is shown at the bottom.

Runx2 gain-of-function phenotype. (3) Expression of a signal-resistant HDAC4 mutant in prehypertrophic chondrocytes prevents hypertrophy and ossification and phenocopies the Runx2 loss-of-function phenotype. (4) HDAC4 physically associates with Runx2 with consequent repression of Runx2 DNA binding and transcriptional activity. Taken together, these results establish HDAC4 as a key regulator of chondrocyte differentiation and endochondral bone formation.

### Regulation of Chondrocyte Hypertrophy by HDAC4

Endochondral bone formation begins with the condensation of mesenchymal cells and their subsequent differentiation into chondrocytes, which organize into a template of the eventual bone comprised of a succession of proliferating, prehypertrophic, and hypertrophic zones (Erlebacher et al., 1995). The extracellular matrix surrounding hypertrophic chondrocytes is permissive to vascular invasion, which allows the ingression of the skeletal cells of the osteoblast lineage (Figure 7).

Our results suggest that the primary defect in the HDAC4$^{-/-}$ mice occurs in the control of chondrocyte hypertrophy. The formation of cartilaginous templates of endochondral bones occurs normally in HDAC4 mutant mice. However, the onset of chondrocyte hypertrophy is accelerated and, consequently, endochondral mineralization occurs precociously. These findings suggest that a primary function of HDAC4 is to delay chondrocyte hypertrophy and thereby to determine the timing and extent of ossification of endochondral bones.

The role of HDAC4 as a repressor of chondrocyte hypertrophy appears to be unique and distinct from the functions of other class II HDACs based on the lack of expression of HDACs 5, 7, or 9 in developing bones. In addition, knockout mice lacking these class II HDACs show distinct phenotypes. Mice lacking HDACs 5 or 9 are viable and show no skeletal abnormalities (Zhang et al., 2002, Chang et al., 2004), and mice lacking HDAC7 die during mid-gestation from cardiovascular defects (S. Chang and E.N.O., unpublished data). We have also generated mice in which the HDAC4-null mutation has been combined with null mutations in the genes encoding HDACs 5 and 9 and have not observed any exacerbation of the HDAC4 skeletal phenotype, further indicating the lack of functional overlap of these class II HDACs with HDAC4 (unpublished data).

### Repression of Runx2 Activity by HDAC4

Runx2 is a member of the runt family of transcription factors originally isolated on the basis of its ability to activate transcription of the osteoblast-specific *osteocalcin* gene (Ducy et al., 1997). Runx2 is necessary for osteoblast differentiation in vivo (Komori et al., 1997; Otto et al., 1997) and is a critical mediator of chondrocyte hypertrophy (Inada et al., 1999; Kim et al., 1999; Takeda et al., 2001). Runx2 is expressed in prehypertrophic and hypertrophic chondrocytes during skeletogenesis. Several lines of evidence indicate that Runx2 is a physiological regulator of chondrocyte hypertrophy. (1) Hypertrophic chondrocytes are absent in multiple skeletal elements in Runx2-null mice (Inada et al., 1999; Kim et al., 1999). (2) Constitutive expression of Runx2 in prehypertrophic chondrocytes leads to premature and ectopic chondrocyte hypertrophy in a manner strikingly similar to what we observed in HDAC4-null mice (Takeda et al., 2001; Ueta et al., 2001). (3) Expression of a dominant-negative Runx2 mutant inhibits chondrocyte hypertrophy and ossification in vivo (Ueta et al., 2001). The precise timing of chondrocyte hypertrophy during skeletogenesis suggests that Runx2 function must be regulated during this process so that bone formation does not occur prematurely. Our results identify HDAC4 as such a regulator. While we cannot rule out the possible involvement of transcription factors in addition to Runx2 as targets for repression by HDAC4 in the developing skeleton, the remarkable resemblance of the HDAC4 mutant and Runx2 gain-of-function phenotypes and of the Runx2 mutant and HDAC4 gain-of-function phenotypes in vivo, coupled with the direct interaction of these proteins, argues strongly for the central role of the Runx2-HDAC4 interaction in the control of chondrocyte development.

The expression pattern of Runx2 in prehypertrophic chondrocytes (Takeda et al., 2001) overlaps with that of HDAC4. Based on the similarity between HDAC4 loss-

of-function and Runx2 gain-of-function phenotypes, both of which show premature chondrocyte hypertrophy and aberrant endochondral ossification, we tested whether HDAC4 might inhibit Runx2 activity. HDAC4 physically associated with Runx2 and inhibited its ability to transactivate target promoters. Likewise, chondrocyte overexpression of HDAC4 produced a similar phenotype to the dominant-negative Runx2, i.e., inhibition of chondrocyte hypertrophy and subsequent ossification. These results suggest that HDAC4 tightly controls Runx2 activity during chondrocyte differentiation, possibly until certain signals release Runx2 from the repressive influence of HDAC4. In HDAC4 mutant mice, Runx2 expression is elevated in regions of developing skeletal elements that display precocious chondrocyte hypertrophy, suggesting that the *Runx2* gene is itself a target for repression by HDAC4. Consistent with this conclusion, Runx2 has been shown to bind and activate its own promoter via a positive feedback loop (Ducy et al., 1999). We show here by ChIP assays that HDAC4 associates with the *Runx2* promoter and reduces histone acetylation and Runx2 binding to the promoter.

Our results indicate that association of HDAC4 with the Runt domain of Runx2 inhibits Runx2 DNA binding. These findings suggest that the inhibitory influence of HDAC4 on Runx2 occurs independently of HDAC catalytic activity. It is notable in this regard that other class II HDACs have been shown to repress transcription in the absence of a functional catalytic domain (Zhang et al., 2001). It is curious that two HDAC4 mutant proteins lacking the Runx2 binding domain retained residual repressive activity toward Runx2. We are uncertain of the mechanism of action of these mutants, but their partial repressive activity suggests that HDAC4 may have transcriptional targets in addition to Runx2 in the pathway for chondrocyte differentiation.

We have previously demonstrated that class II HDACs act as signal-responsive repressors of cardiac hypertrophy (Zhang et al., 2002; Chang et al., 2004). The activity of class II HDACs is influenced by phosphorylation of specific serine residues, which stimulates their nuclear export and derepression of their target genes (Chang et al., 2004; McKinsey et al., 2000a, 2000b). We observed nuclear staining of HDAC4 in both prehypertrophic and, to a lesser extent, hypertrophic chondrocytes. No evidence was seen of cytoplasmic-localized HDAC4 in the chondrocytes of the growth plate. Thus, it appears that loss of HDAC4 expression triggers activation of Runx2 and chondrocyte maturation.

Recently, the Twist-1 and -2 transcription factors were shown to repress Runx2 activity (Bialek et al., 2004). However, the mechanism involved in this form of repression is distinct from that of HDAC4 in one important respect. Twist expression is restricted to osteoblasts, whereas the phenotype of the HDAC4 mutant mouse reflects a primary defect in chondrocyte maturation and differentiation. Thus, Twist and HDAC4 act in entirely different steps of the skeletogenic process to regulate Runx2 function.

### Regulation of Ihh Expression by HDAC4

Longitudinal bone growth is driven primarily by the rate of production of hypertrophic chondrocytes. Ihh-deficient mice display severe skeletal abnormalities characterized by a marked decrease in chondrocyte proliferation and osteoblast differentiation (St-Jacques et al., 1999). Ihh secretion by prehypertrophic chondrocytes positively regulates chondrocyte proliferation and induces expression of parathyroid hormone-related peptide (PTHrP) in the articular perichondrium, which inhibits its differentiation of prehypertrophic to hypertrophic chondrocytes (Vortkamp et al., 1996). Cells no longer expressing Ihh, and released from the inhibitory effects of PTHrP, become hypertrophic. In addition, Ihh may promote hypertrophic chondrocyte maturation after the cells are outside the inhibitory range of PTHrP (Akiyama et al., 1999; Stott and Chuong, 1997). The *Ihh* gene is a direct target gene of Runx2 (Yoshida et al., 2004), and Runx2 expression in proliferating chondrocytes induces Ihh expression in prehypertrophic chondrocytes (Takeda et al., 2001). A similar induction of Ihh expression is seen in prehypertrophic chondrocytes appearing in the chondrocostal cartilage of HDAC4$^{-/-}$ mice (Figure 4A) before any histologic changes are evident. These data suggest a model in which HDAC4 repression of Runx2 activity controls proper temporal and spatial Ihh expression. Therefore, in the HDAC4$^{-/-}$ mice, ectopic chondrocyte maturation leads to increased Ihh expression with subsequent chondrocyte hypertrophy. Aberrant Ihh expression also activates osteoblast differentiation in the perichondrium leading to subsequent osteoblast invasion and bone trabeculation (St-Jacques et al., 1999).

### Control of Cellular Hypertrophy by Class II HDACs

The bone phenotype of HDAC4 knockout mice bears an intriguing resemblance to the cardiac phenotype of HDAC9 mutant mice, suggesting common mechanisms of action of these HDACs. Mice lacking HDAC9 display a heightened sensitivity to signals that promote cardiac hypertrophy, reflecting the enhanced activation of the MEF2 transcription factor. HDAC5 knockout mice display the same phenotype as HDAC9 mutants, and HDAC5/9 double mutants show an even greater degree of cardiac hypertrophy (Chang et al., 2004). Thus, we propose that class II HDACs may act within diverse cell types to control hypertrophic growth in response to developmental or pathological signals. Hypertrophic growth of different cell types depends on different stimuli and is controlled by distinct sets of transcription factors—MEF2 in the case of cardiomyocytes and Runx2 in the case of chondrocytes. The realization that cellular hypertrophy is governed by class II HDACs through their association with specific target transcription factors suggests possible strategies for therapeutically manipulating this mechanism in the settings of development and disease.

## Experimental Procedures

### Generation of HDAC4-Null Mice

The *HDAC4* targeting vector was created by cloning a 4-kb genomic fragment between exons 5 and 6 and a 1.3-kb fragment between exons 6 and 7 into the pN-Z-TK$_2$ vector (kindly provided by R. Palmiter). This vector encodes a nuclear LacZ and contains a neomycin resistance cassette. The *HDAC4* targeting vector was linearized and electroporated into ES cells. Resulting clones were screened for correct homologous recombination by Southern blot analysis. ES

clones with a targeted disruption of the *HDAC4* locus were injected into C57BL/6 blastocysts to produce chimeric mice. Chimeric mice obtained from one targeted ES clone were bred to C57BL/6 mice to obtain germline transmission. All experiments described here were performed with mice of a C57BL/6/129 mixed background.

### RNA Isolation and Analysis

Total RNA was isolated from tissues with Trizol reagent and used as a template for reverse transcriptase with primers specific for each HDAC transcript (Invitrogen) according to manufacturer's instructions. Primer sequences are available upon request.

### Cartilage and Bone Staining

Embryonic or newborn mice were skinned, eviscerated, and fixed in ethanol. Skeletal preparations were stained using Alcian blue and Alizarin red, respectively, as described previously (McLeod, 1980). Soft tissue was removed by incubation in 2% KOH.

### Histological Analysis

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.

### Immunohistochemical and Western Blot Analysis of HDAC4 Expression

Bone sections were deparaffinized using a Sakura Tissue-Tek robotic stainer. Antigen retrieval was performed by microwaving at 87°C twice for 9 min. Slides were then equilibrated in PBS followed by permeabilization in 0.3% Triton X-100. Rabbit polyclonal anti-HDAC4 antibody (Santa Cruz Biotechnology, Santa Cruz, California) was used at a 1:20 dilution followed by biotinylated goat anti-rabbit antibody at a 1:200 dilution. Bound antibody was visualized by incubation with peroxidase streptavidin (1:500) followed by DAB chromagen (Dako, Glostrup, Denmark). Slides were washed in $H_2O$, dehydrated, and coverslips mounted.

For Western blot analysis of HDAC4 and Runx2 expression, 10 μg of cytosolic or nuclear extracts from chondrocytes or osteoblasts was used. Primary chondrocytes were isolated from rib cartilage of 2- to 4-day-old mice as described by Lefebvre et al. (1994). Osteoblast extracts were prepared as described (Ducy and Karsenty, 1995).

### In Situ Hybridizations

Embryos or tissue samples were fixed overnight in DEPC-treated 10% phosphate-buffered formalin prepared freshly from paraformaldehyde. Riboprobes were labeled with $^{35}$S-UTP using the MAXIscript in vitro transcription kit (Ambion, Austin, Texas). In situ hybridization of sectioned tissues was performed as previously described (Shelton et al., 2000). Signals were pseudocolored as described in figure legends.

### Cell Culture and Transfections

COS7 cells were grown in DMEM with 10% FBS. Lipofectamine (Invitrogen, Carlsbad, California) was used for transient transfections according to manufacturer's instructions. Expression constructs for HDAC4 and Runx2 deletions were generated by PCR. Reporter plasmids used here have been described previously (Ducy et al., 1997). Reporter plasmid (250 ng) and indicated amounts of expression plasmids were transfected with 25 ng of an RSV-β-galactosidase expression plasmid to control for transfection efficiency. Assays were performed as previously described (Ducy et al., 1997). For immunoprecipitations, 500 μg of nuclear extracts from transfected COS7 cells were rotated with anti-Flag beads (Sigma-Aldrich, St. Louis, Missouri) for 2 hr in 500 μl of NTN buffer (200 mM NaCl, 50 mM Tris [pH8.0], 0.5% NP-40) at 4°C. Beads were subsequently washed four times with NTN buffer and boiled in SDS-PAGE loading buffer. Eluates were separated by SDS-PAGE and immunoblotted using the indicated antibody.

### Electromobility Shift Assays

In vitro translated HDAC4 or ATF4 proteins were prepared using TNT Coupled Wheat Germ Extract Systems (Promega). GST-Runx2 was prepared using glutathione-Sepharose (Amersham) per the manufacturer's instructions. For electromobility shift assay (EMSA), double-stranded oligonucleotides corresponding to the *osteocalcin* OSE2 site containing the Runx2 binding site were $^{32}$P-labeled. EMSA was performed as previously described (Ducy et al., 1997).

### ChIP Assays

ChIP assays were performed using soluble chromatin prepared from primary mouse chondrocytes infected with adenovirus expression vectors encoding HDAC4 or GFP at a multiplicity of infection of 10. Primer sequences are available upon request.

### Transgenic Mice

A chondrocyte-specific HDAC4 transgene was constructed by subcloning the signal-resistant HDAC4-S/A mutant, in which serines 246, 467, and 632 were changed to alanines (McKinsey et al., 2000b), between a 3-kb fragment of the α1(II) promoter and its 3-kb chondrocyte-specific enhancer region (Zhou et al., 1998). Linearized transgenic construct was injected into the pronuclei of fertilized oocytes as described previously (Cheng et al., 1993). At least 12 $F_0$ transgenic offspring were harvested and analyzed for changes in skeletal development. The severity of the skeletal phenotype in these mice correlated with transgene copy number.

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