

Nkx3.2/Bapx1 acts as a negative regulator of chondrocyte maturation

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Parathyroid hormone-related protein (PTHrP) is essential to maintain a pool of dividing, immature chondrocytes in the growth plate of long bones. In chick and mouse, expression of Nkx3.2/Bapx1 in the growth plate is restricted to the proliferative zone and is downregulated as chondrocyte maturation begins. Nkx3.2/Bapx1 expression is lost in the growth plates of mice engineered to lack PTHrP signaling and, conversely, is maintained by ectopic expression of PTHrP in developing bones. Artificially preventing Nkx3.2/Bapx1 downregulation, by forced expression of either retroviral-encoded PTHrP or Nkx3.2 inhibits chondrocyte maturation. Although wild-type Nkx3.2 blocks chondrocyte maturation by acting as a transcriptional repressor, a 'reverse function' mutant of Nkx3.2 that has been converted into a transcriptional activator conversely accelerates chondrocyte maturation. Nkx3.2 represses expression of the chondrocyte maturation factor Runx2, and Runx2 misexpression can rescue the Nkx3.2-induced blockade of chondrocyte maturation. Taken together, these results suggest that PTHrP signals block chondrocyte hypertrophy by, in part, maintaining the expression of Nkx3.2/Bapx1, which in turn represses the expression of genes required for chondrocyte maturation.

KEY WORDS: Chondrogenesis, Runx2, Nkx3.2, Bapx1, PTHrP

INTRODUCTION

Vertebrate long bones are formed through a process of endochondral ossification (Erlebacher et al., 1995; Kronenberg, 2003). During this process, chondrocytes differentiate within an aggregated mesenchyme, generating distinct cartilage primordia that begin longitudinal growth. Cells lying within the central regions of the cartilage undergo a further maturation process, withdrawing from the cell cycle, enlarging in size and initiating synthesis of a new extracellular matrix containing collagen X (Erlebacher et al., 1995; Kronenberg, 2003). The resulting cells are termed hypertrophic chondrocytes, which eventually die off to be replaced by bone and bone marrow. The production and maturation of chondrocytes is eventually restricted to the epiphysis of the bone in a structure termed the growth plate. The rate of chondrocyte maturation in the growth plate is tightly regulated by a PTHrP/Indian hedgehog (Ihh) signaling loop. PTHrP is normally produced by the most distal perichondrium, at the articular surface, and its receptor is synthesized in the proliferative and prehypertrophic zones of the growth plate (Lanske et al., 1996; Vortkamp et al., 1996). Ectopic expression of either PTHrP or an activated form of the Parathyroid Hormone (PTH)/PTHrP receptor results in a dramatic blockade of cartilage maturation (Schipani et al., 1997; Weir et al., 1996). Conversely, mice lacking either PTHrP or its receptor, exhibit dwarfism of their long bones due to premature chondrocyte maturation (Karaplis et al., 1994; Lanske et al., 1996). PTHrP expression in turn is dependent upon the expression of Ihh, which is transiently produced by chondrocytes as they initiate the maturation process (Bitgood and McMahon, 1995; St-Jacques et al., 1999; Vortkamp et al., 1996).

The early steps of chondrogenesis, including mesenchymal condensation and expression of chondrocyte-specific extracellular matrix proteins is crucially dependent upon Sox-family transcription factors, including Sox9, Sox5 and Sox6 (de Crombrughe et al., 2001; Lefebvre, 2002). By contrast, the latter steps of chondrogenesis appear to be regulated by Runx family transcription factors. Runx2 is expressed in chondrocytes as they initiate chondrocyte hypertrophy, and loss of this factor in genetically engineered mice severely delays chondrocyte maturation in a number of developing bones (Inada et al., 1999; Kim et al., 1999). Furthermore, forced expression of Runx2 in immature chondrocytes drives premature maturation of chondrocytes by inducing expression of collagen X (col X) and other hypertrophic markers (Enomoto et al., 2000; Stricker et al., 2002; Takeda et al., 2001; Ueta et al., 2001). It has recently been demonstrated that Runx3, which is also expressed in maturing chondrocytes, works in combination with Runx2 to promote chondrocyte maturation (Yoshida et al., 2004), and that HDAC4 restricts ectopic and early onset chondrocyte hypertrophy, and associates with Runx2 (Vega et al., 2004).

In the current study, we explore the role of the transcriptional repressor Nkx3.2/Bapx1 in modulating the rate of chondrocyte maturation. Mice lacking the Nkx3.2/Bapx1 gene exhibit major defects in the axial skeleton, characterized by a lack of ventromedial elements in the vertebral column, and by hypoplasia of the neural arches (Akazawa et al., 2000; Lettice et al., 2001; Lettice et al., 1999; Tribioli and Lufkin, 1999). Consistent with this phenotype, we have found that signals that induce somitic chondrogenesis, including sequential Shh and BMP signals, induce the expression of Nkx3.2, and that forced expression of Nkx3.2 can activate chondrogenesis in somites (Kim et al., 2003; Murtaugh et al., 2001; Zeng et al., 2002). Nkx3.2 induces somitic chondrogenesis by acting as a transcriptional repressor (Murtaugh et al., 2001; Zeng et al., 2002). In the course of characterizing the role of Nkx3.2 in endochondral bone development, we observed that Nkx3.2 expression in the limb skeleton was restricted to proliferative immature chondrocytes, and that its expression in this region of the growth plate was dependent upon PTHrP signals. On the basis of these results, we hypothesized

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that *Nkx3.2/Bapx1* might negatively regulate chondrocyte maturation. However, analysis of the long bones of mice lacking *Nkx3.2/Bapx1* revealed that the limb bones of these mice display relatively normal formation of hypertrophic chondrocytes and bony tissue by Hematoxylin/Eosin staining (data not shown; S.P., A.B.L. and Hans-Henning Arnold, unpublished) (Akazawa et al., 2000; Lettice et al., 2001; Lettice et al., 1999; Tribioli and Lufkin, 1999). To explore the possibility that *Nkx3.2/Bapx1* might negatively regulate chondrocyte maturation, but that this role may not have been revealed in *Nkx3.2/Bapx1* deficient mice due to redundant/compensatory mechanisms that may modulate chondrocyte maturation in the growth plate, we have employed a gain-of-function approach in chick embryos to elucidate a potential role for *Nkx3.2* in the regulation of chondrocyte maturation.

MATERIALS AND METHODS

Materials

RCAS(A)-*Nkx3.2* and RCAS(B)-*Nkx3.2* were produced as previously described (Murtaugh et al., 2001; Zeng et al., 2002), and RCAS(A) retroviruses encoding deletion mutants of *Nkx3.2* have been described elsewhere (Kim et al., 2003; Murtaugh et al., 2001). RCAS(A)-*Runx2* and RCAS(B)-*Runx2* viruses, encoding mouse *Runx2*, were supplied by Masahiro Iwamoto (Thomas Jefferson University, Philadelphia, USA), and have been described previously (Enomoto et al., 2000). RCAS(A)-chick *Runx2* (Stricker et al., 2002) was supplied by Stefan Mundlos (Max Planck Institute for Molecular Genetics, Berlin, Germany). Details for the generation of RCAS(A)-PTHrP are available on request.

In situ hybridization (ISH)

Details of the probes employed for ISH are available upon request. Frozen sections of embryonic chick tissue, and paraffin sections of mouse, were prepared exactly as described previously (Murtaugh et al., 1999). Non-radioactive section ISH, with digoxigenin (DIG)-labeled probes, was performed as described previously (Murtaugh et al., 2001). ISH with ³⁵S-labeled riboprobes and Hematoxylin and Eosin staining were performed as described previously (Chung et al., 1998).

Retroviral misexpression

Virus preparation was as described elsewhere (Morgan and Fekete, 1996). Concentrated viral supernatant was injected into the nascent wing buds of late E3 [Hamburger-Hamilton (HH) stage 17-19] chick embryos. To ensure thorough infection, each wing bud was injected in several anteroposterior regions along its distal margin. Following injection, eggs were reincubated up to E8-E10, and processed for Alcian Blue and Alizarin Red staining (Murtaugh et al., 1999), or ISH as described above.

Chick embryo explant culture, retroviral infection, and RT-PCR analysis

Dissection and culture of somitic or pre-somitic mesoderm (psm) explants was previously described (Munsterberg et al., 1995). Retrovirus infection was performed essentially as described by Zeng et al. (Zeng et al., 2002), with slight modifications (available upon request). Reverse transcriptase (RT) reactions and polymerase chain reaction (PCR) analysis were carried out as previously described (Munsterberg et al., 1995; Zeng et al., 2002). PCR conditions and primer sequences have either been previously published or are available on request.

RESULTS

Nkx3.2 expression is restricted to immature proliferative chondrocytes during endochondral ossification

Although *Nkx3.2* transcripts are initially expressed in all cartilaginous cells in the limb bud soon after mesenchymal condensation (Murtaugh et al., 2001), as chondrocyte maturation begins, expression of these transcripts becomes restricted to the distal portion of the developing cartilage elements (Fig. 1). At

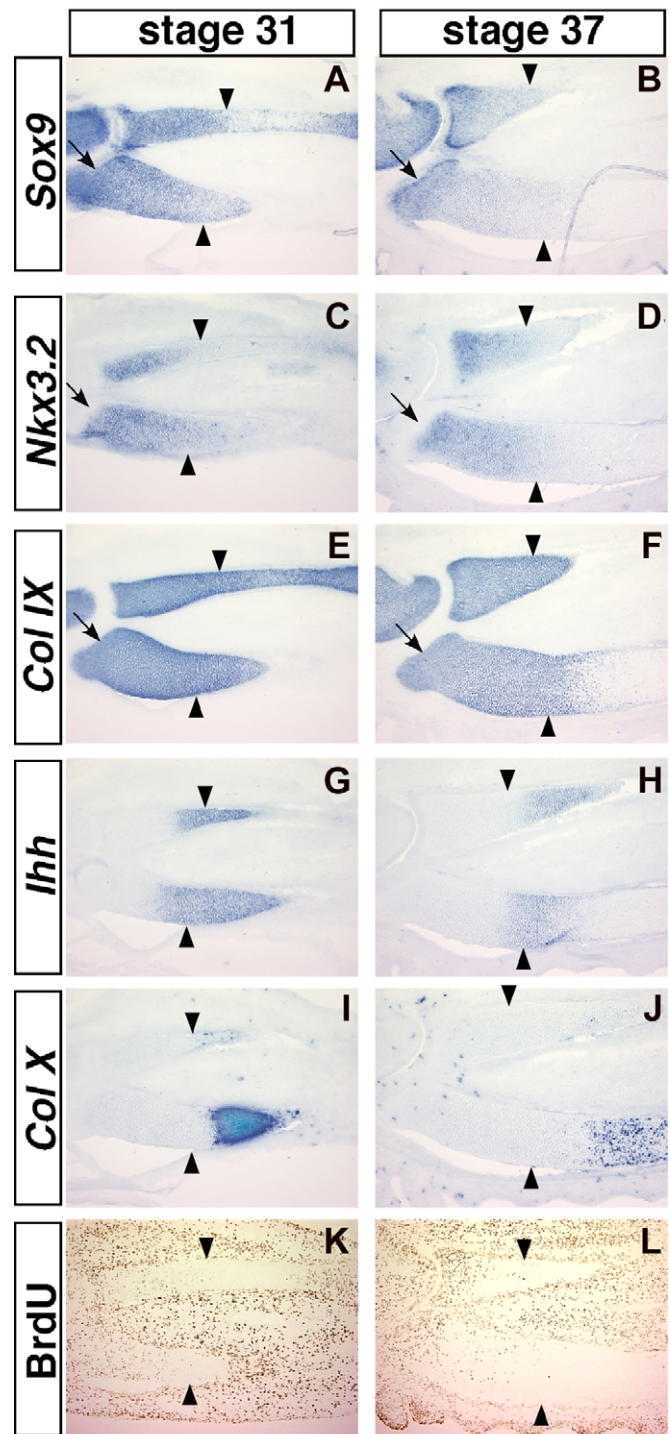


Fig. 1. Expression of chick *Nkx3.2* is restricted to immature proliferative chondrocytes during endochondral bone development. Chick embryos, stages as indicated, were pulse labeled with BrdU before harvesting and processing for section in situ hybridization (ISH; A-J) or stained with an anti-BrdU antibody (K,L). Arrowheads indicate approximate boundary of BrdU-positive zone in parallel sections. Arrows (A-F) indicate distal-most boundary of cartilage, marked by col IX and Sox9 expression.

Hamburger-Hamilton (HH) stages 31 and 37 (Fig. 1C,D), *Nkx3.2* expression appears to form a distal-to-proximal gradient within the cartilage, which resembles the expression of Sox 9 (Fig. 1A,B),

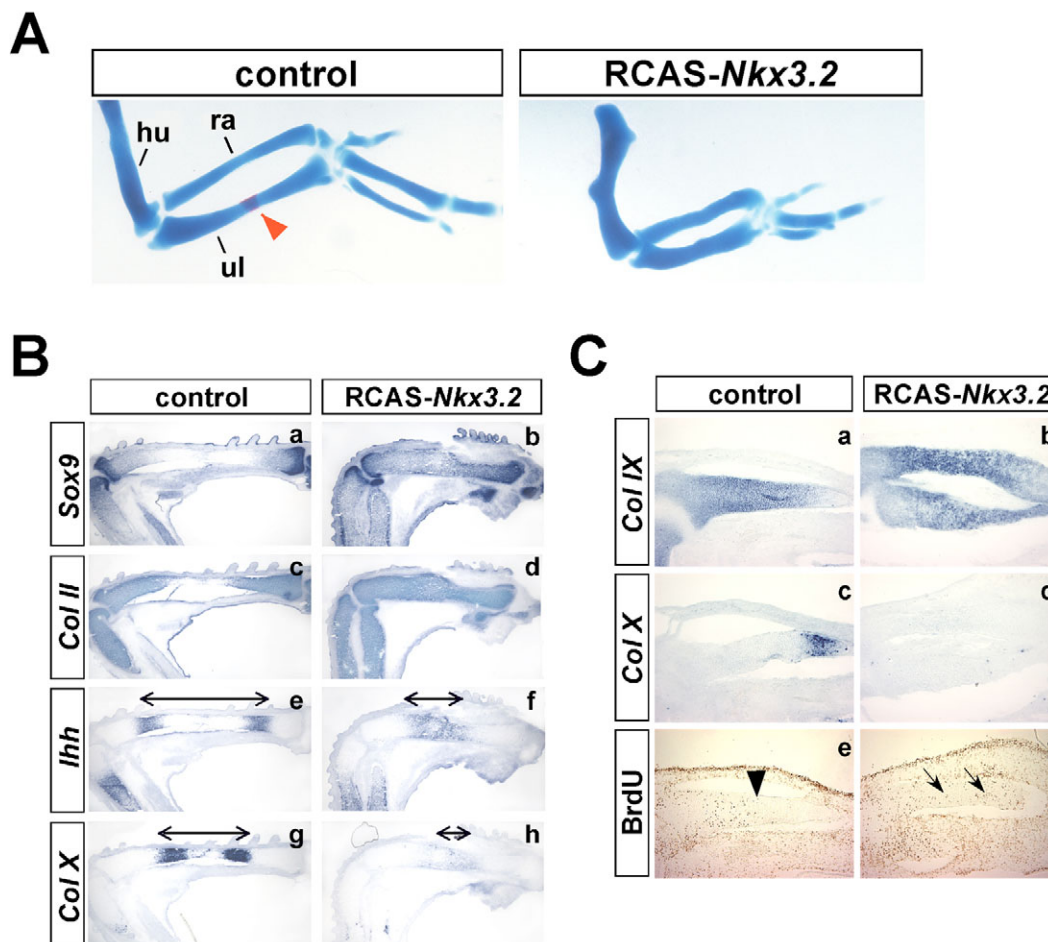


Fig. 2. Misexpression of Nkx3.2 inhibits chondrocyte maturation. The right wing buds of late E3 chick embryos (HH stage 16-18) were infected with RCAS(A)-Nkx3.2. The embryos were harvested on E9, and processed for Alcian Blue/Alizarin Red staining (A), or pulsed for three hours with BrdU prior to harvesting on E10 for ISH and BrdU incorporation (B,C). Red arrowhead (A) indicates the nascent bone collar. Double-headed arrows (B) compare the peripheral-most extent of *Ihh* and *col X* expression in the RCAS-Nkx3.2-infected and contralateral control wings. Arrowhead (C) indicates the approximate boundary between proliferative and non-dividing cells in the cartilage of the control wing; arrows indicate proliferative cells in central regions of the RCAS-Nkx3.2-infected wing cartilage. hu, humerus; ra, radius; ul, ulna.

collagen IX (*col IX*; Fig. 1E,F) and collagen II (*col II*; Fig. 4C, parts a,c), all markers of immature chondrocytes. Nkx3.2 expression declines in the cartilage cells expressing *Ihh*, a marker of prehypertrophic chondrocytes (Fig. 1G,H), and is expressed at trace levels in mature, hypertrophic chondrocytes, marked by *col X* expression (Fig. 1I,J). Comparison of Nkx3.2 expression with BrdU uptake, which marks active DNA synthesis (Fig. 1K,L), suggests that Nkx3.2 expression is restricted to the proliferative zone within the cartilage. It should be noted, however, that the greatest amount of cellular proliferation occurs in the most distal chondrocytes of the developing cartilage element, which do not express Nkx3.2 (arrows in Fig. 1A-F). Thus, not all proliferating chondrocytes express Nkx3.2. Its expression is restricted to the immature, proliferative chondrocytes within the developing cartilage element, and is specifically downregulated as cells begin to express the prehypertrophic marker *Ihh*, and to withdraw from the cell cycle. This expression pattern is consistent with that very recently described by Francis-West and colleagues, who noted that this gene is also expressed in soft tissues, such as developing tendons, muscle sheaths and surrounding mesenchyme, in developing chicken limbs (Church et al., 2005).

Misexpression of Nkx3.2 inhibits chondrocyte maturation

The close correlation between the loss of Nkx3.2 expression and the onset of chondrocyte maturation is consistent with a role for Nkx3.2 in negatively regulating this transition. To explore this possibility, we infected wing buds with a retrovirus encoding Nkx3.2 to prevent

its downregulation during cartilage growth. Chick wing buds (HH stage 17-19) were infected with a replication-competent RCAS(A) virus (Hughes et al., 1987) programmed to express Nkx3.2 (RCAS-Nkx3.2) (Murtaugh et al., 2001), and the embryos were allowed to develop through embryonic day 9-10 (E9-E10), at which point cartilage maturation should normally be well underway. Nkx3.2-infected wings exhibited considerably shorter and thicker cartilage elements than did their contralateral control counterparts, and displayed a lack of mineralized bone, as detected by Alizarin Red staining (Fig. 2A). This phenotype was observed in 98% of RCAS-Nkx3.2-infected wings ($n > 80$); in some of these wings the bones were sometimes severely misshapen, but we never observed any patterning defects.

To further characterize this phenotype, we sectioned RCAS-Nkx3.2-infected and control wings and subjected them to in situ hybridization (ISH) analysis. The contralateral control wings exhibited the ordinary pattern of chondrocyte maturation, as evidenced by sequential expression domains of collagen IX (*col IX*), *col II* and *Sox9* in immature chondrocytes, *Ihh* in prehypertrophic chondrocytes, and *col X* in hypertrophic chondrocytes (Fig. 2B, parts a,c,e,g, and 2C, parts a,c). By contrast, RCAS-Nkx3.2-infected wings displayed uniform expression of *Sox9* throughout the entire cartilage, with greatly reduced *Ihh* expression and barely, if any, detectable expression of *col X* (Fig. 2B, parts b,d,f,h, and 2C, parts b,d). Moreover, the usual separation between the two *col X* expression domains, comprising late hypertrophic chondrocytes and trabecular bone (Fig. 2B, part g), is completely absent from RCAS-Nkx3.2-infected limbs (Fig. 2B, part h). Thus, forced expression of Nkx3.2

inhibits the expression of both prehypertrophic and hypertrophic chondrocyte marker genes. The expression of osteopontin (OP), which marks late hypertrophic chondrocytes, was barely detectable in RCAS-Nkx3.2-infected wings (Fig. 7A, compare parts m and n), indicating that the Nkx3.2-induced depletion of *Ihh*- and *col X*-expressing cells is not due to an accelerated maturation of such cells into late hypertrophic chondrocytes, but rather reflects a blockade in the maturation of immature chondrocytes. Consistent with this result, BrdU labeling of RCAS-Nkx3.2-infected wings indicated that the normal boundary between proliferative and quiescent chondrocytes present in the control wing was absent in the infected cartilage; instead, proliferative cells were distributed uniformly throughout the infected skeletal elements (Fig. 2C, parts e,f). These results are consistent with the normal expression pattern of Nkx3.2, as described above, and strongly suggest that downregulation of this gene is required for chondrocyte maturation to proceed.

Both DNA binding and transcriptional repression by Nkx3.2 are required to inhibit chondrocyte maturation

To further clarify how Nkx3.2 expression blocks chondrocyte maturation, we investigated whether DNA binding or transcriptional repression activity was necessary for this effect. Nkx3.2-NQ contains a glutamine substitution of a conserved asparagine (amino acid 200) located in the homeodomain; this mutant is defective in DNA-binding activity but still retains an intrinsic transcriptional repressor activity (Kim et al., 2003). Viral misexpression of Nkx3.2-NQ, unlike wild-type Nkx3.2, failed to alter the morphology of the limb skeleton (zero abnormal wings, $n=11$; Fig. 3A,D). Furthermore, analysis of *col II* and *col X* expression revealed a normal distribution of mature chondrocytes in RCAS-Nkx3.2-NQ infected limbs, in spite of an efficient viral infection throughout the limb (Fig. 3G,J, and data not show). Thus, the DNA-binding activity of Nkx3.2 is required to block chondrocyte maturation. As the

Nkx3.2-NQ protein is as stable as wild-type Nkx3.2 (Nkx3.2-WT; D.-W.K. and A.B.L., unpublished), this result suggests that the blockade of chondrocyte maturation observed with wild-type Nkx3.2 was not simply due to the titration (by protein-protein interaction) of important factors necessary for chondrocyte hypertrophy, as a consequence of Nkx3.2 overexpression. Instead, it appears that Nkx3.2 represses chondrocyte maturation by binding to the regulatory element of a gene(s) involved in this process.

Prior work has indicated that Nkx3.2 functions as a transcriptional repressor, and that regions of the protein C-terminal to the homeodomain are critical for this activity (Kim and Lassar, 2003; Murtaugh et al., 2001). While an Nkx3.2 mutant lacking this C-terminal domain, Nkx3.2 Δ C, fails to repress transcription, fusion of the repression domain of the *Drosophila* Engrailed protein onto this protein, to generate Nkx3.2 Δ C-En, restores transcriptional repressor activity to this fragment of Nkx3.2 (Murtaugh et al., 2001). Viral misexpression of Nkx3.2 Δ C had no morphologically detectable effect on the limb skeleton (zero abnormal wings, $n=23$; Fig. 3B,E). When the infected cartilage was examined by ISH, chondrocyte maturation appeared normal, in spite of a thorough viral infection of the cartilage (Fig. 3H,K, and data not shown). By contrast, misexpression of Nkx3.2 Δ C-En yielded shortened and thickened limb cartilage elements (80% abnormal wings, $n=20$; Fig. 3C,F) similar to those observed following infection with RCAS-Nkx3.2-WT. As expected, these cartilage elements failed to undergo hypertrophy (Fig. 3I,L). We conclude that the ability of ectopic Nkx3.2 to block chondrocyte maturation correlates with its ability to repress transcription of a gene(s) normally required to promote this process.

A reverse-function form of Nkx3.2 that activates gene expression promotes chondrocyte maturation

Because Nkx3.2 is a transcriptional repressor, one would expect that a derivative of Nkx3.2 that is a transcriptional activator might induce the expression of genes that are normally repressed by

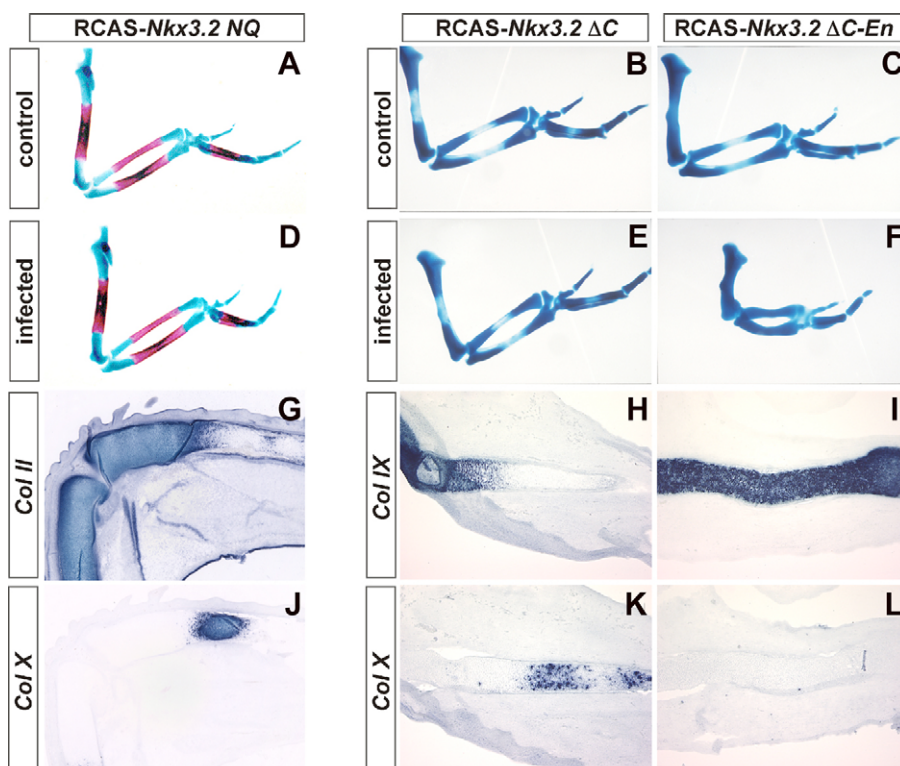


Fig. 3. DNA binding and transcriptional repression by Nkx3.2 are required to block chondrocyte maturation. The right forelimb buds of day 3 chick embryos were infected with RCAS viruses encoding mutant forms of Nkx3.2, as indicated. The embryos were harvested at E10 for skeletal preparation (A-F), or section ISH (G-L) with the indicated probes.

endogenous Nkx3.2. To this end, we generated a ‘reverse function’ derivative of Nkx3.2, termed Nkx3.2 Δ C-VP16, by appending the strong transcriptional activation domain of the Herpes Simplex Virus VP16 protein onto the C terminus of Nkx3.2 Δ C. This derivative of Nkx3.2 no longer represses reporter gene activity, but instead acts as a strong activator of gene expression *in vitro* (Kim and Lassar, 2003). Viral misexpression of Nkx3.2 Δ C-VP16 resulted in an extended zone of mineralization (Alizarin Red staining in Fig. 4A), which is likely to be the result of accelerated chondrocyte maturation. To confirm that Nkx3.2 Δ C-VP16 accelerates chondrocyte maturation, we analyzed by ISH the distribution of chondrogenic markers at two different stages after viral infection. At E8.5 (Fig. 4B), the Nkx3.2 Δ C-VP16-infected wing seems slightly longer than the control wing (Fig. 4B, parts a,b). Although col X is just beginning to be expressed within the core of the control wing skeletal element (Fig. 4B, part c), col X expression is considerably stronger and more broadly expressed within the core of the RCAS-Nkx3.2 Δ C-VP16-infected skeletal element (Fig. 4B, part d). At E10 (Fig. 4C), in RCAS-Nkx3.2 Δ C-VP16-infected wings, col X expression is upregulated and the distance between the peripheral-most extent of the two col X

expression domains is expanded relative to the control wing (Fig. 4C, parts e,f). In addition, the distance between the two col II expression domains, which consist of both early and late hypertrophic chondrocytes, is broader than in the control uninfected wing (Fig. 4C, parts c,d). Thus, Nkx3.2 Δ C-VP16 accelerates chondrocyte maturation *in vivo*. This finding is consistent with the notion that Nkx3.2 inhibits chondrocyte maturation by transcriptionally repressing a gene(s) that directly or indirectly promotes chondrocyte maturation, and that a reverse function form of Nkx3.2 (i.e. Nkx3.2 Δ C-VP16) should activate the expression of such a gene(s) and therefore accelerate chondrocyte maturation.

Nkx3.2/Bapx1 acts downstream of the Ihh-PTHrP signaling pathway

Because the phenotype observed in Nkx3.2-infected wings is remarkably similar to that seen in mouse cartilage in which the PTHrP pathway has been artificially stimulated (Schipani et al., 1997; Weir et al., 1996), we initially investigated whether PTHrP might be upregulated by Nkx3.2 misexpression. As shown in Fig. 5A, expression of PTHrP is in fact downregulated in articular

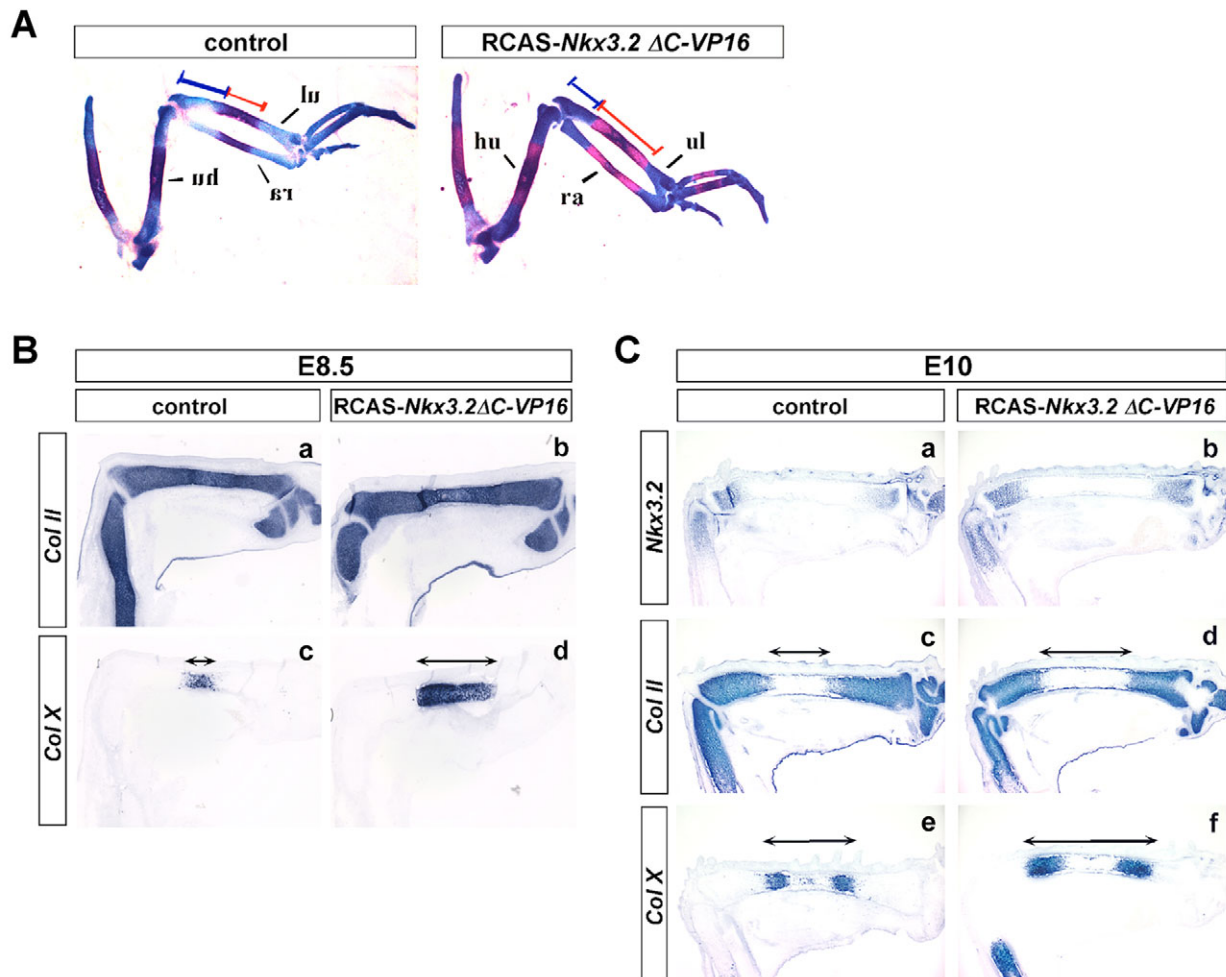


Fig. 4. Nkx3.2- Δ C-VP16 acts as a reverse function form of Nkx3.2 and accelerates chondrocyte maturation. The right wing buds of day 3 chick embryos were infected with RCAS(A)-Nkx3.2- Δ C-VP16 virus. Embryos were harvested at either E10 (A,C) or E8.5 (B), and processed for Alcian Blue/Alizarin Red staining (A) or section ISH (B,C). The extent of mineralized bone (red bar in A) is increased in limbs infected with RCAS-Nkx3.2- Δ C-VP16 relative to the contralateral control limb. Double-headed arrows (B,C) compare either the peripheral-most extent of col X expression or the peripheral-most extent lacking col II expression.

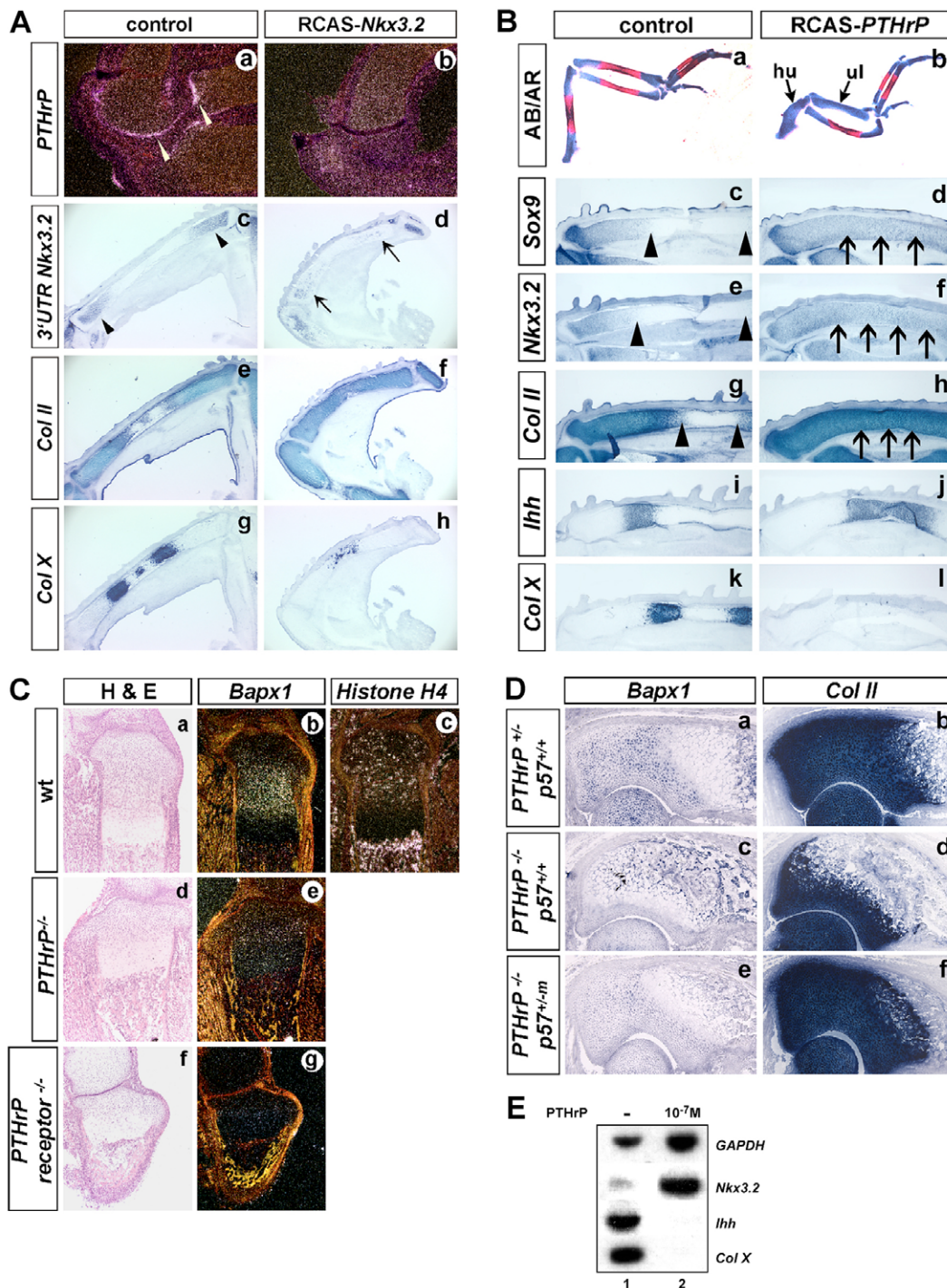


Fig. 5. PTHrP signals are necessary to drive *Nkx3.2* expression in the growth plate. (A) Infection of limbs with RCAS-*Nkx3.2* results in the loss of PTHrP and endogenous *Nkx3.2* expression. Arrowheads (part a) indicate strong PTHrP expression in the periarticular region of the control wing; arrowheads (part c) indicate normal expression of endogenous *Nkx3.2* in control wing, whereas arrows (d) indicate the absence of endogenous *Nkx3.2* expression in RCAS(A)*Nkx3.2*-infected cartilage. (B) Ectopic PTHrP delays chondrocyte maturation and maintains uniform *Nkx3.2* expression throughout the cartilage. Wing buds of E3 chick embryos were infected with RCAS(A)-PTHrP virus. Embryos were harvested at E10, and RCAS(A)-PTHrP-infected wings and contralateral control wings were analyzed by Alcian Blue/Alizarin Red staining (AB/AR, parts a,b) or section ISH (c-l). Arrowheads (c,e,g) indicate the central region of the ulna in which expression of *Sox9*, *Nkx3.2* and *col II* is downregulated in the control wing. Arrows (d,f,h) indicate the central region of the ulna in which expression of these markers is maintained in the RCAS-PTHrP infected wing. (C) *Nkx3.2*/*Bapx1* expression is lost in PTHrP and PTHrP-receptor null animals. Bright field (a,d,f) or ISH (b,c,e,g) of E18.5 mouse tibiae with the indicated probes, taken from mice of indicated genotypes. (D) Loss of *p57* expression rescues the absence of immature chondrocytes but not *Nkx3.2*/*Bapx1* expression in the absence of PTHrP signals. ISH of E17.5 mouse ulnae, taken from mice of indicated genotypes, with indicated probes. (E) PTHrP signals induce the expression of *Nkx3.2* in cultured chondrocytes. Upper sternal chondrocytes (USC) were isolated from the cephalic portion of 15-day-old chick embryo sterna and cultured for 3 days in either the absence (lane 1) or presence (lane 2) of 100nM PTHrP. Gene expression was assayed by RT-PCR.

chondrocytes following infection with RCAS-Nkx3.2 (Fig. 5A, compare parts a and b). This is not surprising, given that ectopic Nkx3.2 both diminishes the level of *Ihh* expression and blocks the progression of *Ihh* expression into regions of the cartilage that lie proximal to the articular perichondrium (Fig. 2B, parts e,f; Fig. 7A, parts g,h), where it is normally required to maintain expression of PTHrP (St-Jacques et al., 1999). We then considered the possibility that Nkx3.2 might actually be functionally downstream of PTHrP, and therefore mediate at least some of the effects of PTHrP on chondrocytes.

If PTHrP were required for Nkx3.2 expression, one would expect that the loss of PTHrP expression observed upon Nkx3.2 misexpression would lead, in turn, to a significant decrease of endogenous Nkx3.2 expression. To evaluate this possibility, we employed a riboprobe able to distinguish between endogenous versus retrovirus-encoded *Nkx3.2* mRNA. This probe was derived from sequences present in the 3' UTR of Nkx3.2, but not included in the viral construct. Whereas we detected normal expression of endogenous Nkx3.2 in the proliferating cartilage of control wings, endogenous Nkx3.2 expression was greatly reduced in RCAS-Nkx3.2-infected limbs (Fig. 5A, parts c,d). The repression of endogenous Nkx3.2 expression in RCAS-Nkx3.2-infected limbs stands in stark contrast to the maintained expression of other markers of immature chondrocytes, such as col II, col IX and Sox9, in these limbs (Fig. 5A, parts e,f; Fig. 2B, parts a-d; Fig. 2C, parts a,b). These findings are consistent with the notion that RCAS-Nkx3.2-mediated repression of *Ihh* expression in chondrocytes that lie adjacent to the epiphysis results in the loss of PTHrP expression, which itself is necessary to maintain the expression of endogenous Nkx3.2.

To confirm that expression of Nkx3.2 in growth plate cartilage depends on PTHrP signaling, we analyzed its expression under conditions where PTHrP signaling had been either artificially activated or suppressed. To examine the first case, we infected wing buds of chicken embryos with an RCAS construct encoding PTHrP. As expected, viral PTHrP misexpression led to a phenotype similar to that of Nkx3.2 misexpression; cartilage elements within the limb skeleton were shorter and displayed a decrease in Alizarin Red staining (Fig. 5B, parts a,b, see humerus and ulna marked by arrows). We analyzed sections of RCAS-PTHrP infected limbs by in situ hybridization and observed that chondrocyte maturation was severely delayed. Although expression of both Sox9 and col II was extinguished in the central region of the control uninfected ulna (Fig. 5B, parts c,g; between arrowheads), expression of both of these genes was maintained within the central region of the contralateral RCAS-PTHrP-infected ulna (Fig. 5B, parts d,h; see arrows). In addition, in contrast to the control wing, the RCAS-PTHrP-infected ulna displayed a single *Ihh* expression domain in the middle of this cartilage element and a loss of col X expression (Fig. 5B, parts i-l). Interestingly, although Nkx3.2 expression was diminished in the central region of the control uninfected ulna (Fig. 5B, part e; between arrowheads), viral misexpression of PTHrP maintained the expression of Nkx3.2 in the central region of the contralateral infected ulna (Fig. 5B, part f; see arrows). Thus, increased levels of PTHrP signaling prolong the expression of Nkx3.2 throughout the entire cartilage template, concomitant with a delay in chondrocyte maturation.

To evaluate Nkx3.2 expression in the absence of PTHrP signaling, we examined mice embryos deficient for either the PTHrP (PTHrP^{-/-}) or the PTH/PTHrP-receptor (PTHrP-receptor^{-/-}) gene. Mice lacking PTHrP or its receptor exhibit accelerated chondrocyte maturation (Karaplis et al., 1994; Lanske et al., 1996), essentially the opposite phenotype to that seen following Nkx3.2

misexpression. In growth plates of wild-type E18.5 mouse embryos, we found that Nkx3.2/Bapx1 is expressed in the columnar layer of proliferative chondrocytes, but not in pre-hypertrophic and hypertrophic cells (Fig. 5C, parts a-c; histone H4 mRNA marks proliferating cells). This expression pattern is consistent with the fact that the PTHrP-receptor is expressed in such proliferating cells (Lee et al., 1996), and would therefore be expected to activate target gene expression in this cellular compartment. In contrast to what we found in wild-type animals, Nkx3.2/Bapx1 expression is greatly reduced or undetectable in growth plates of both PTHrP^{-/-} and PTHrP-receptor^{-/-} mutant mice (Fig. 5C, parts d-g). Thus, PTHrP signaling is either directly required to maintain Nkx3.2/Bapx1 expression in chondrocytes, or indirectly required to maintain expression of this gene by augmenting either the proliferation or survival of the columnar chondrocytes that express this gene. To distinguish between these two possibilities we examined Nkx3.2/Bapx1 expression in mice embryos engineered to lack expression of both PTHrP and the cyclin-dependent kinase inhibitor p57.

Whereas PTHrP knockout mice embryos lack columnar proliferative chondrocytes within their growth plates, this cell population is restored in mice deficient for both PTHrP and p57 expression (MacLean et al., 2004). Bapx1/Nkx3.2 expression is confined to the immature chondrocytes in the growth plates of E17.5 PTHrP^{+/-}; P57^{+/+} mice (Fig. 5D, parts a,b). These Bapx1/Nkx3.2-expressing chondrocytes, which also express collagen II, are specifically deleted from the growth plate in PTHrP^{-/-}; P57^{+/+} mice (Fig. 5D, parts c,d). To examine if Bapx1/Nkx3.2 expression is regained under conditions that restore the presence of immature columnar chondrocytes despite the absence of PTHrP signaling, we examined the expression of Bapx1/Nkx3.2 in mice lacking expression of both PTHrP and p57. Because only the maternally inherited allele of p57 is expressed (Hatada and Mukai, 1995), P57^{+/-m} mice (which contain only a paternal wild-type p57 allele) lack expression of this gene. Interestingly, although the population of immature chondrocytes expressing col II is expanded in PTHrP^{-/-}; P57^{+/-m} mice, expression of Bapx1/Nkx3.2 is not restored in these cells (Fig. 5D, parts e,f). Thus PTHrP signals are required to maintain the expression of Bapx1/Nkx3.2 in immature chondrocytes of the growth plate.

To evaluate if PTHrP signals are sufficient to induce the expression of Nkx3.2 in chondrocytes, we treated cultures of hypertrophic upper sternal chondrocytes derived from 15-day-old chick embryos with PTHrP in vitro. After 3 days of treatment with PTHrP, we noted that both *Ihh* and collagen X expression was extinguished in these cultures, whereas Nkx3.2 expression was significantly induced (Fig. 5E). Taken together, these data indicate that expression of Nkx3.2/Bapx1 in the growth plate lies downstream of PTHrP signaling, and suggest that Nkx3.2/Bapx1 may mediate at least some of the effects of this signaling pathway on chondrocyte maturation.

Nkx3.2 represses Runx2 expression

We wondered whether Nkx3.2 blocks chondrocyte hypertrophy by blocking the expression of Runx2, which is a positive regulator of this process. To evaluate this possibility, we first compared the expression of endogenous Nkx3.2 and Runx2 during cartilage maturation in the chick limb. We found that Nkx3.2 and Runx2 are expressed in a reciprocal pattern within the developing cartilage element; Runx2 expression is most intense in the central region of the cartilage element where Nkx3.2 expression is most diminished (see arrowheads in Fig. 6A, parts a,b). To test whether Nkx3.2 might

directly or indirectly modulate Runx2 expression, we infected chicken wing buds with RCAS viruses encoding either wild-type Nkx3.2 or the 'reverse function' Nkx3.2- Δ C-VP16, and examined Runx2 expression by ISH. RCAS-Nkx3.2 infection reduced Runx2 expression specifically in the central region of the developing cartilage that would ordinarily mature into hypertrophic chondrocytes and express col X (outlined by orange boxes in Fig. 6B, parts a,b). By contrast, infection with RCAS-Nkx3.2- Δ C-VP16 increased Runx2 transcript levels principally in the peripheral regions of the cartilage (outlined by blue boxes in Fig. 6B, parts e,f), while accelerating col X expression in the central region of the cartilage (Fig. 6B, parts g,h). Quantification of Runx2 expression indicated that misexpression of Nkx3.2 led to a twofold decrease of Runx2 expression in the central region of the skeletal element that would normally become hypertrophic (Fig. 6C, left panel, orange bars). Conversely, forced expression of Nkx3.2- Δ C-VP16 increased Runx2 expression approximately twofold in the peripheral regions of the developing cartilage (Fig. 6C, right panel, blue bars).

In addition to assaying the effects of Nkx3.2 on Runx2 expression in vivo, we also examined these issues in a chondrogenic culture system in vitro. Presomitic mesoderm (psm) dissected from HH stage 10 chicken embryos can initiate a chondrogenic program

following either sequential treatment with Shh and Bmp4 (Murtaugh et al., 1999), or following infection with RCAS-Nkx3.2 in the presence of Bmp4 signals (Murtaugh et al., 2001). Psm explants cultured with Shh for 2 days and Bmp4 for an additional 6 days begin to differentiate into mature chondrocytes, as indicated by the expression of both Runx2 and col X (Fig. 6D, lane 1). Culture of such explants in Bmp4 for 12 days increased the expression of both Runx2 and col X (Fig. 6D, lane 3). In striking contrast, psm infected with RCAS-Nkx3.2 and exposed to Bmp4 signals for either 6 or 12 days expressed very robust levels of the immature chondrocyte marker epiphygan, but only trace levels of Runx2 and col X (Fig. 6D, lanes 2 and 4). Thus, retroviral-encoded Nkx3.2 induced chondrogenesis in explants of presomitic mesoderm while blocking chondrocyte maturation in these cultures by either directly or indirectly repressing the expression of both Runx2 and col X.

Misexpression of Runx2 rescues the Nkx3.2-induced delay of chondrocyte maturation

If Nkx3.2 blocks chondrocyte hypertrophy by repressing Runx2 expression, one would expect that Runx2 overexpression would rescue this inhibition. To test this hypothesis, we examined the phenotype of embryonic chick limbs co-infected with either

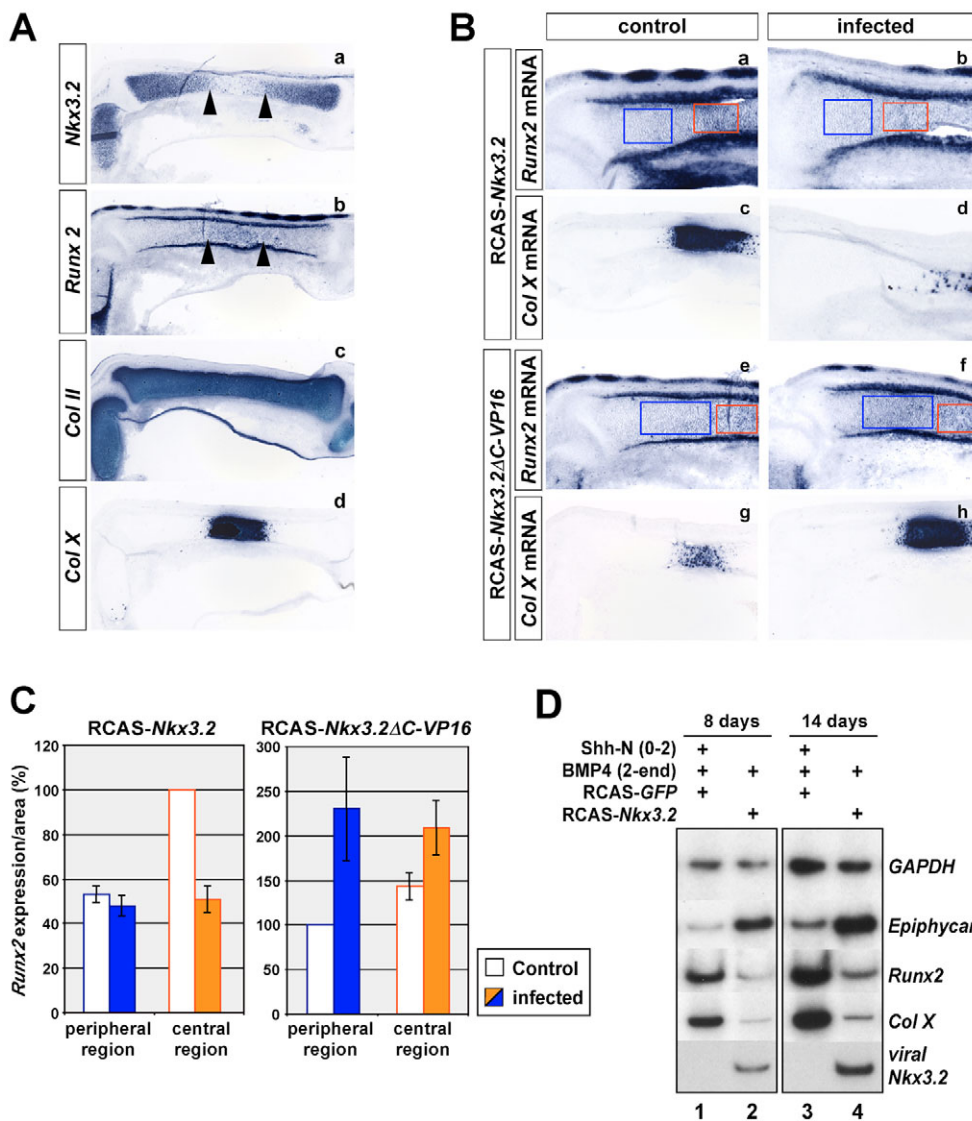


Fig. 6. Nkx3.2 represses Runx2 expression. (A) Nkx3.2 and Runx2 are expressed in a reciprocal pattern in the developing cartilage elements of the limb. ISH for the indicated probes is displayed for E8 chick limbs. In the cartilage element, Runx2 expression is most intense where Nkx3.2 expression is diminished (marked by arrowheads in a,b). (B) Forced expression of Nkx3.2 represses Runx2 expression in vivo. The right wing buds of E3 chick embryos were infected with either RCAS(A)-Nkx3.2 (b,d) or RCAS(A)-Nkx3.2- Δ C-VP16 (f,h) viruses. Embryos were harvested at either E8.5 (a-d) or E8 (e-h), and sections of the infected or contralateral control wings were hybridized with the indicated probes. (C) The intensity of the Runx2 ISH signal displayed in B and in two other experiments was quantified, in both the peripheral (blue rectangles in B) and central regions (orange rectangles in B) of the cartilage in both control and infected wings, as indicated. (D) Forced expression of Nkx3.2 represses Runx2 expression in presomitic mesoderm explants in vitro. Explants of presomitic mesoderm were either cultured for 2 days in either Shh (lanes 1 and 3) or infected with RCAS-Nkx3.2 (lanes 2 and 4). The explants were cultured in Bmp4 for either an additional 6 (lanes 1 and 2) or 12 (lanes 3 and 4) days. Expression of indicated markers was assayed by RT-PCR.

RCAS(B)-Nkx3.2 alone or co-infected with RCAS(B)-Nkx3.2 plus RCAS(A)-Runx2. In agreement with our previous results obtained with RCAS(A)-Nkx3.2, embryos infected with RCAS(B)-Nkx3.2 alone displayed a short wing phenotype with decreased mineralization (60% embryos with abnormal wings, $n=17$; data not shown). In contrast, none of the wings co-infected with RCAS(B)-Nkx3.2 plus RCAS(A)-Runx2 displayed this phenotype (0% embryos with abnormal wings, $n=12$; data not shown). We also analyzed the distribution of chondrogenic markers in limbs co-infected with RCAS(A)-Nkx3.2 plus either RCAS(B) encoding alkaline phosphatase (RCAS(B)-AP) or RCAS(B)-Runx2. Although the control contralateral uninfected wing displayed normal Alizarin Red staining and the typical nested expression pattern of *Ihh*, *col X* and osteopontin (OP; Fig. 7A, parts a,d,g,j,m), co-infection of wings with RCAS(A)-Nkx3.2 plus RCAS(B)-AP led to a severe delay of chondrocyte maturation, as evidenced by a deficiency in Alizarin Red staining and a decreased expression of *Ihh*, *col X* and OP in 88% of infected wings ($n=16$; Fig. 7A, parts b,e,h,k,n). In striking contrast, infection of wings with RCAS(A)-Nkx3.2 plus RCAS(B)-

Runx2 led to no discernable deficiency of Alizarin Red staining, or drop in either *Ihh*, *col X* or OP expression (0% abnormal wings, $n=31$; Fig. 7A, parts c,f,i,l,o), in spite of high viral Nkx3.2 misexpression (data not shown). Thus, co-expression of exogenous Runx2 rescues an Nkx3.2-induced delay of chondrocyte maturation in vivo.

The reversal of an Nkx3.2-induced blockade of chondrocyte maturation in the limb skeleton by the forced expression of retrovirally encoded Runx2 is consistent with the notion that Nkx3.2 blocks chondrocyte maturation by decreasing endogenous Runx2 gene expression. This scenario would predict that, although forced Nkx3.2 expression would be able to block the endogenous program of chondrocyte hypertrophy, it would not affect such a program of gene expression when induced by ectopic Runx2. To explore this issue, we examined whether misexpression of Nkx3.2 would affect the ability of RCAS-Runx2 to induce chondrocyte hypertrophy in explants of presomitic mesoderm (psm). Although psm explants exposed to *Shh/Bmp4* signals for greater than 8 days activated *col X* expression (Fig. 6D, lanes 1 and 3), psm explants exposed to such

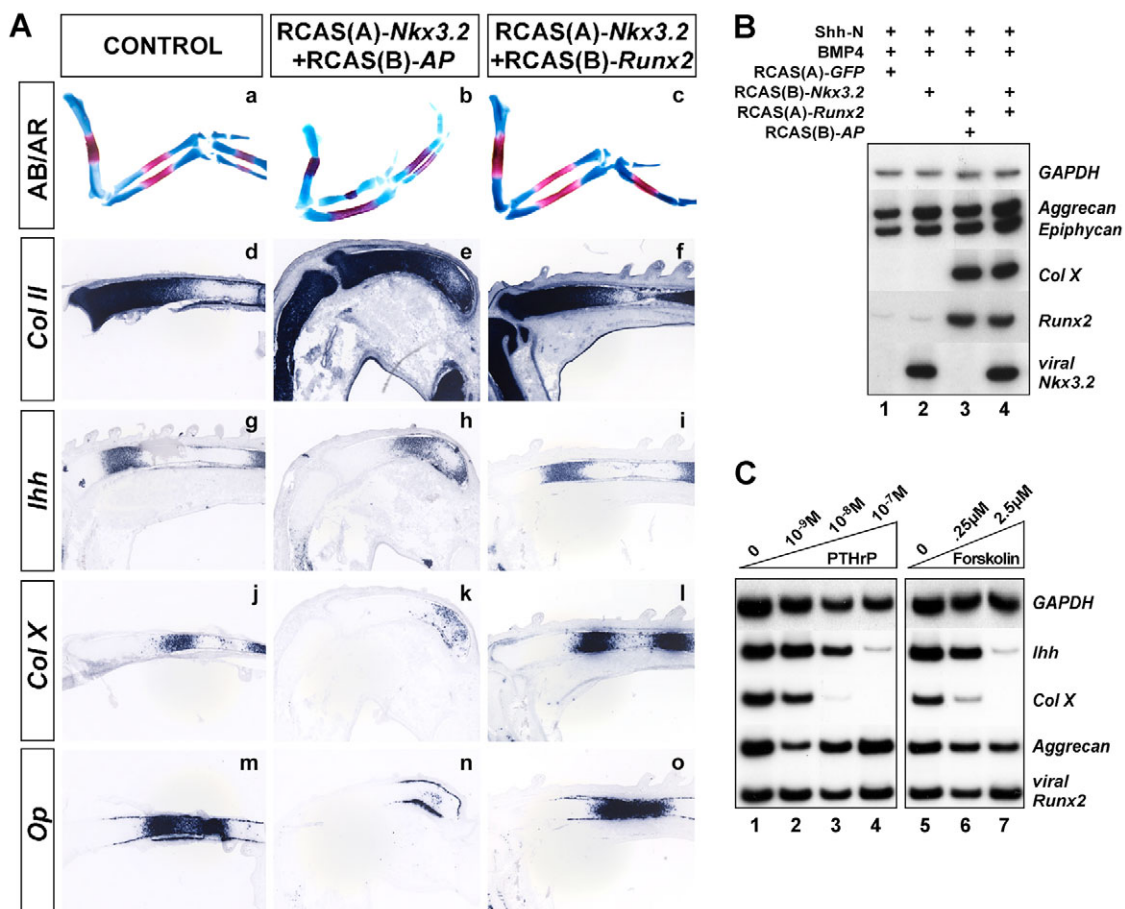


Fig. 7. Forced Runx2 expression rescues an Nkx3.2-induced but not a PTHrP-induced blockade of chondrocyte maturation. (A) Forced Runx2 expression rescues an Nkx3.2-induced blockade of chondrocyte maturation in vivo. The right wing buds of E3 chick embryos were co-infected with RCAS(A)-Nkx3.2 plus RCAS(B)-Alkaline Phosphatase (AP) viruses (b,e,h,k,n), or RCAS(A)-Nkx3.2 plus RCAS(B)-Runx2 viruses (c,f,i,l,o), and reincubated until E10. The control contralateral uninfected wing (a,d,g,j,m) and the co-infected wings were analyzed either by Alcian Blue/Alizarin Red staining (AB/AR, a-c), or by section ISH with the indicated probes (d-o). (B) Nkx3.2 does not block chondrocyte hypertrophy induced by ectopic Runx2 in vitro. Explants of presomitic mesoderm were co-infected with the indicated viruses and cultured sequentially in *Shh* for 2 days and *Bmp4* for 3 days. Gene expression was assayed by RT-PCR. (C) PTHrP and forskolin can block chondrocyte hypertrophy induced by ectopic Runx2 in vitro. Explants of presomitic mesoderm were infected with RCAS(A)-Runx2 and cultured as described in part B, in either the absence (lanes 1 and 5) or in the presence of increasing amounts of either PTHrP (lanes 2-4) or forskolin (lanes 6 and 7), as indicated. Gene expression was assayed by RT-PCR.

signals for only 5 days expressed robust levels of the early chondrocyte makers aggrecan and epiphygan, but only trace levels of endogenous Runx2 and no detectable col X (Fig. 7B, lane 1). By contrast, infection of such short-term psm cultures with RCAS(A)-Runx2 induced robust levels of col X after only 5 days culture (Fig. 7B, lane 3). Thus, ectopic Runx2 expression can accelerate the rate of chondrocyte maturation in psm explants exposed to prochondrogenic signals. Interestingly, psm explants infected with the combination of RCAS(A)-Runx2 and RCAS(B)-Nkx3.2 expressed high levels of col X, identical to that observed with RCAS(A)-Runx2 alone (Fig. 7B, compare lanes 3 and 4). Thus, Nkx3.2 fails to block chondrocyte maturation in the presence of exogenous Runx2, both in vivo in limbs, and in vitro in psm explants, suggesting that Nkx3.2 may delay chondrocyte maturation by repressing endogenous Runx2 expression.

Misexpression of Runx2 does not rescue a PTHrP-induced blockade of chondrocyte maturation

Ectopic expression of either PTHrP or Nkx3.2 in limb cartilage blocks chondrocyte hypertrophy (Figs 2 and 5) and simultaneously represses the expression of Runx2 (Fig. 6) (Guo et al., 2006). In light of the inability of Nkx3.2 to block chondrocyte maturation in the presence of exogenous Runx2, we wondered if this would also be the case for PTHrP signals. To evaluate this issue, we analyzed col X and Ihh expression in psm explants infected with RCAS(A)-Runx2, in either the absence or presence of increasing levels of PTHrP. Although 10^{-8} M PTHrP repressed col X expression and only slightly dampened the expression of Ihh, 10^{-7} M PTHrP significantly decreased the expression of both col X and Ihh in psm cultures infected with RCAS(A)-Runx2 (Fig. 7C, lanes 1–4), despite the continued expression of retrovirally encoded *Runx2* RNA in these cultures. Thus, PTHrP signals can blunt the ability of exogenous Runx2 to promote chondrocyte maturation. As PTHrP signals are thought to block chondrocyte maturation by increasing cAMP levels and protein kinase A (PKA) activity in the cell (Guo et al., 2002), we evaluated whether the elevation of cAMP levels by forskolin would similarly block the ability of retroviral Runx2 to induce chondrocyte hypertrophy. Administration of increasing levels of forskolin phenocopied the effects of PTHrP administration and blocked the expression of col X (but not Ihh) at lower concentrations, and extinguished the expression of both col X and Ihh at higher concentrations (Fig. 7C, lanes 5–7). These findings indicate that elevated cAMP levels are sufficient to block chondrocyte maturation downstream of *Runx2* gene expression. Together with our observation that Nkx3.2 expression requires PTHrP signals in the growth plate, these results indicate that PTHrP signaling can repress chondrocyte hypertrophy both by affecting Runx2 transcription (via Nkx3.2 induction), and by another mechanism that blocks chondrocyte maturation by acting either downstream or in parallel to *Runx2* mRNA expression.

DISCUSSION

Nkx3.2 represses chondrocyte maturation

Nkx3.2/Bapx1 expression, in the skeletal elements of chick wings and mouse limbs, coincides with regions of immature and proliferative chondrocytes; its expression declines in chondrocytes that become prehypertrophic, and is either undetectable or is only detected at trace levels in hypertrophic chondrocytes (see also Church et al., 2005). Retroviral misexpression of Nkx3.2 in chicken wings resulted in a severe delay of chondrocyte maturation, indicating that Nkx3.2 expression needs to be downregulated to allow chondrocyte maturation to proceed. Both the DNA binding

and the transcriptional repressor activities of Nkx3.2 were required to inhibit chondrocyte hypertrophy, suggesting that Nkx3.2 represses the transcription of a gene(s) necessary for chondrocyte maturation. Consistent with this notion, we found that ectopic expression of a 'reverse function' mutant of Nkx3.2 that has been converted into a transcriptional activator hastens chondrocyte maturation, presumably by activating the expression of a Nkx3.2 target gene(s) that is necessary to promote chondrocyte hypertrophy. Taken together, these findings strongly suggest that Nkx3.2 either directly or indirectly represses the expression of a gene(s) that is normally required for chondrocyte maturation to proceed.

Nkx3.2/Bapx1 delays chondrocyte maturation at least in part by repressing Runx2 expression

We observed that Nkx3.2 and Runx2 are expressed in reciprocal patterns during chondrocyte maturation, such that Runx2 expression is most intense in the central region of the cartilage element where Nkx3.2 expression is most diminished. Forced expression of Nkx3.2 represses Runx2 expression in the regions of the chick limb cartilage that would normally become hypertrophic in vivo, and dramatically represses Runx2 expression in cultured somite explants in vitro. Most significantly, we found that infection of limbs or somite cultures with RCAS-Runx2 restored chondrocyte maturation in tissues infected with RCAS-Nkx3.2. Thus, forced expression of Runx2, which promotes chondrocyte maturation, is epistatic to the effects of forced Nkx3.2 expression. Interestingly, infection of chick wings with RCAS-Nkx3.2 leads to a profound blockade of chondrocyte maturation but only a partial loss of Runx2 expression in the developing cartilage, resulting in an approximately 50% decreased expression of Runx2 in cells located in the position of normally hypertrophic chondrocytes. These findings suggest either that chondrocyte hypertrophy requires a threshold level of Runx2 or that Nkx3.2 represses other factors necessary for the activity of this residual level of Runx2. Indeed chondrocyte maturation is completely absent in mice lacking both Runx2 and Runx3, but is also considerably delayed in mice that lack Runx3 but have only one intact allele of Runx2 (Yoshida et al., 2004), suggesting that a threshold level of Runx factors are indeed necessary to promote chondrocyte hypertrophy. Although these findings are consistent with the notion that Nkx3.2 blocks chondrocyte maturation by dropping Runx2 levels below a threshold that is required for this process to take place, it is also plausible that Nkx3.2 blocks the expression of co-factors required for Runx2 activity, and that overexpression of Runx2 somehow bypasses the need for such co-factors. Nkx3.2 has recently been shown to bind to a Runx2 promoter in gel shift assays, and can repress transcription driven by this regulatory element in vitro (Lengner et al., 2005), suggesting that Nkx3.2 may directly repress Runx2 expression.

Nkx3.2/Bapx1 acts downstream of the PTHrP signaling pathway

Two crucial and well-characterized paracrine regulators in endochondral bone development are Indian hedgehog and PTHrP, which form a feedback loop to control the rate at which chondrocytes undergo maturation (Lanske et al., 1996; Vortkamp et al., 1996). Here, we provide several lines of evidence that the *Nkx3.2/Bapx1* gene is a target of PTHrP signaling that regulates chondrocyte maturation in the growth plate. Chicken Nkx3.2 and mouse Bapx1 are expressed in proliferative chondrocytes, the site of active PTHrP signaling. Nkx3.2 expression is significantly expanded throughout the cartilage following the misexpression of PTHrP, and is lost in mouse limbs deficient for both PTHrP and PTHrP-R expression.

Because the proliferating columnar chondrocytes that express Nkx3.2 are specifically lost in the absence of PTHrP signals, we evaluated whether these signals were necessary for the expression of Nkx3.2 or only for the maintenance of this cell population. Although loss of expression of the cdk inhibitor p57 restored col II expressing columnar chondrocytes in PTHrP deficient mice, these cells failed to express Bapx1/Nkx3.2 in the absence of PTHrP signals, suggesting that PTHrP signals are indeed necessary to maintain the expression of Bapx1/Nkx3.2 in the growth plate. Consistent with this idea, we have found that application of PTHrP to cultures of hypertrophic chicken chondrocytes could induce the expression of Nkx3.2.

PTHrP signals block chondrocyte hypertrophy by at least two parallel pathways

In addition to maintaining the expression of Bapx1/Nkx3.2 in immature chondrocytes, our results indicate that PTHrP signals can also block chondrocyte maturation by a mechanism(s) that is independent of the suppression of Runx2 transcription. This conclusion stems from the observation that PTHrP or forskolin can block the ability of retroviral-encoded Runx2 to induce either *Ihh* or *col X* gene expression in somitic explants (this work), or mineralization in cultured chondrocytes (Iwamoto et al., 2003). Repression of chondrocyte maturation in RCAS-Runx2 infected somites by either PTHrP or forskolin administration occurs without an affect on viral Runx2 transcript levels. This observation is consistent with recent findings that PTH/PTHrP signals can block the ability of transgenic Runx2, driven by the *col II* regulatory regions, to induce chondrocyte hypertrophy (Guo et al., 2006). Thus, we think it is most likely that PTHrP signals block chondrocyte maturation in the growth plate by at least two parallel pathways, one transcriptional, involving the induction of Nkx3.2/Bapx1, which in turn blocks the transcription of Runx2 and/or a co-factor necessary for chondrocyte maturation, and another distinct pathway that blocks chondrocyte maturation downstream of Runx2 mRNA expression (outlined in Fig. 8). According to this scenario, PTHrP signals would continue to repress chondrocyte maturation (by a Nkx3.2/Bapx1-independent pathway) in mice lacking *Nkx3.2/Bapx1*, thus explaining the apparently normal development of the limb skeleton in such mice.

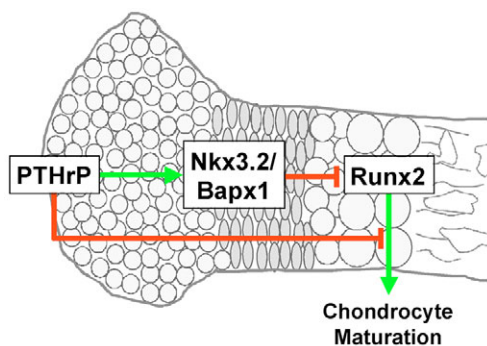


Fig. 8. PTHrP signals block chondrocyte hypertrophy by both Nkx3.2/Bapx1-dependent and -independent pathways. Our findings indicate that PTHrP signals are necessary and sufficient to maintain the expression of Nkx3.2/Bapx1 in the proliferative region of the growth plate, and suggest that Nkx3.2/Bapx1 blocks chondrocyte maturation in this region of the growth plate by repressing the expression of Runx2 mRNA and possibly other co-factors necessary for chondrocyte hypertrophy. In addition, PTHrP signals can signal via a cAMP-dependent pathway to block the ability of Runx2 to induce chondrocyte maturation in a post-transcriptional manner.

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