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Wnt/β-catenin signaling interacts differentially with Ihh signaling in controlling endochondral bone and synovial joint formation

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Both the Wnt/β-catenin and Ihh signaling pathways play essential roles in crucial aspects of endochondral ossification: osteoblast differentiation, chondrocyte proliferation and hypertrophy. To understand the genetic interaction between these two signaling pathways, we have inactivated the β-catenin gene and upregulated Ihh signaling simultaneously in the same cells during endochondral skeletal development using β-catenin and patched 1 floxed alleles. We uncovered previously unexpected roles of Ihh signaling in synovial joint formation and the essential function of Wnt/β-catenin signaling in regulating chondrocyte survival. More importantly, we found that Wnt and Ihh signaling interact with each other in distinct ways to control osteoblast differentiation, chondrocyte proliferation, hypertrophy, survival and synovial joint formation in the developing endochondral bone. β-catenin is required downstream of Ihh signaling and osterix expression for osteoblast differentiation. But in chondrocyte survival, β-catenin is required upstream of Ihh signaling to inhibit chondrocyte apoptosis. In addition, Ihh signaling can inhibit chondrocyte hypertrophy and synovial joint formation independently of β-catenin. However, there is a strong synergistic interaction between Wnt/β-catenin and Ihh signaling in regulating synovial joint formation.

KEY WORDS: Wnt, β-catenin, Ihh, Patched, Cartilage, Endochondral bone, Joint, Chondrocyte hypertrophy, Osteoblast differentiation

INTRODUCTION

Vertebrate skeletal development is mainly controlled by endochondral ossification, during which mesenchymal progenitor cells first differentiate into chondrocytes that form cartilage shafts for the future bone. In the developing cartilage, chondrocytes sequentially go through a tightly controlled program of proliferation and differentiation. Eventually, proliferative chondrocytes exit the cell cycle and differentiate into hypertrophic chondrocytes. In the meantime, osteoblasts differentiate in the perichondrium. These events are followed by the invasion of blood vessels and osteoblasts to the hypertrophic cartilaginous region to replace it with trabecular bone. Chondrocyte hypertrophy is required for ossification because hypertrophic chondrocytes secrete factors such as Indian hedgehog (Ihh) and vascular endothelial growth factors (Vegfs), which are required for osteoblast differentiation (reviewed by de Crombrugghe et al., 2001; Karsenty and Wagner, 2002; Kronenberg, 2003; Zelzer and Olsen, 2003). Wnt and hedgehog signaling pathways control cell proliferation, differentiation, survival and migration in both developing embryos and adult animals. In the developing skeletal systems, several aspects of endochondral bone formation are critically regulated by both Wnt/β-catenin and Ihh signaling pathways. Inactivation of β-catenin or Ihh signaling leads to ectopic chondrocyte differentiation at the expense of bone formation (Day et al., 2005; Hill et al., 2005; Hu et al., 2005; Long et al., 2004). Wnt/β-catenin and Ihh signaling pathways also regulate chondrocyte hypertrophy and maturation (Akiyama et al., 2004; Day et al., 2005; St-Jacques et al., 1999; Vortkamp et al., 1996). In the developing cartilage, *Ihh* is expressed in prehypertrophic chondrocytes. It controls osteoblast differentiation in the perichondrium by controlling the expression of *Runx2*, which encodes a transcription factor required for osteoblast differentiation (St-Jacques et al., 1999). Ihh is also a major driving force for chondrocyte proliferation (Karp et al., 2000), and it sets the pace for chondrocyte hypertrophy by activating the expression of parathyroid hormone related peptide (*Pthrp*; *Pthlh* – Mouse Genome Informatics), which inhibits chondrocyte hypertrophy (Lanske et al., 1996; St-Jacques et al., 1999; Vortkamp et al., 1996). In contrast to what is known about the functional mechanism of Ihh signaling, the molecular mechanism underlying the Wnt/β-catenin functions in endochondral skeletal development remains to be elucidated. Deciphering the genetic relationship between Wnt/β-catenin and Ihh signaling pathways will provide significant new insight into the molecular mechanisms by which both signaling pathways control endochondral bone formation.

It has been proposed that Ihh may signal upstream of Wnt signaling because the expression of *Wnt7b* and *Tcf1* in the perichondrium is lost in the *Ihh* mutant (Hu et al., 2005). However, instead of being regulated by *Ihh* directly, the expression of *Wnt7b* and *Tcf1* may be simply associated with osteoblast differentiation in the perichondrium, which is abolished in the *Ihh* mutant. Consistent with this, *Tcf1* is not required for osteoblast differentiation in mice (Glass et al., 2005) and there is no evidence to support that *Wnt7b* is required for osteoblast differentiation in vivo. Another unsettled issue is the role of Wnt/β-catenin signaling in regulating the expression of the transcription factor osterix (*Osx*; *Sp7* – Mouse Genome Informatics) that acts downstream of *Runx2* in controlling osteoblast differentiation (Nakashima et al., 2002). Although *Osx* expression was clearly detected in the perichondrium/periosteum when β-catenin was inactivated by Cre-mediated recombination (Day et al., 2005), its expression was missing in another similar

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study (Hu et al., 2005). Therefore, the control of *Osx* expression by the Wnt/β-catenin signaling, and the genetic interactions between Wnt/β-catenin and Ihh signaling await further investigation by rigorous genetic tests.

Another important aspect of endochondral skeletal development is synovial joint formation, which is controlled by Wnt/β-catenin signaling (Guo et al., 2004; Hartmann and Tabin, 2001). Although joint formation is not disrupted in *Ihh* mutant mice (St-Jacques et al., 1999), it is likely that Ihh signals to the joint region, and it is possible that Ihh signaling needs to be kept at a low level to allow normal joint formation.

All mammalian hedgehogs (Hhs), including Ihh, transduce their signals through two multipass transmembrane proteins, smoothened (Smo) and patched 1 (Ptch1) (reviewed by Huangfu and Anderson, 2006; Lum and Beachy, 2004). The current model proposes that, in the absence of the Hh ligand, the signaling activity of Smo is inhibited by Ptch1. Hh binding to Ptch1 relieves its inhibition of Smo, allowing Smo to transduce the Hh signal to the intracellular components. It is predicted that Hh signaling can be maximally activated in a cell-autonomous manner by inactivating *Ptch1*. The Wnt/β-catenin signaling is the canonical Wnt signaling transduced through stabilizing β-catenin (reviewed by Logan and Nusse, 2004; Moon, 2005). In the absence of Wnt ligands, β-catenin is phosphorylated by glycogen synthase kinase 3 (Gsk3) and then targeted for degradation in the proteasome. When Wnt ligands bind to their receptors frizzled and low-density lipoprotein receptor-related protein 5/6 (Lrp5/6), β-catenin phosphorylation is inhibited. This causes the cytoplasmic accumulation and nuclear translocation of β-catenin, which activates downstream gene transcription by binding to Lef/Tcf factors. Therefore, the strength of the canonical Wnt signal can be manipulated by altering the protein levels of β-catenin. Canonical Wnt signaling has been blocked cell autonomously by tissue-specific inactivation of β-catenin in many developmental processes.

Here, we have generated a floxed allele of *Ptch1* and activated Hh signaling cell autonomously in osteochondral progenitor cells during endochondral bone formation. We found that upregulation of Hh signaling uncoupled chondrocyte hypertrophy and osteoblast differentiation. Endochondral ossification occurred in the absence of chondrocyte hypertrophy. In addition, upregulation of Hh signaling led to mild joint fusion. Furthermore, when canonical Wnt signaling was blocked at the same time that Ihh signaling was upregulated, we observed cell-context-dependent genetic interactions between the canonical Wnt and Ihh signaling pathways. In osteoblast differentiation, β-catenin acts, not only downstream of the Ihh pathway, but also downstream of *Osx*. In chondrocyte survival, Wnt/β-catenin is upstream of Ihh signaling when inhibiting chondrocyte apoptosis. Finally, during chondrocyte hypertrophy and synovial joint formation, Wnt/β-catenin and Ihh signaling pathways exert opposite activities and there is a synergistic interaction between them during synovial joint formation.

MATERIALS AND METHODS

Generation and genotyping targeted mouse lines

Generation of the conditional *Ptch1* mutant mice is described in Fig. 1. Genotyping was done by PCR using genomic DNA prepared from embryonic liver. Oligonucleotides Ptch1b (5′-GCAAGTTTTTGGTTGTG-GGTCTCC-3′) and PtclacZ2 (5′-GCGATTAAGTTGGGTAACGCC-3′) were used to genotype the *Ptch1* floxed allele (348 bp band size). Oligonucleotides Ptc2b (5′-CCTTCCCGCGAGCTGGATGTG-3′) and PtclacZ2 were used to genotype the $Ptch1^{\Delta loxP}$ allele (333 bp band size). Oligonucleotides Ptch1b and Ptch1f (5′-GCTACAAGGAGGCTCTAGG-TGC-3′) were used to genotype the *Ptch1* wild-type allele (200 bp band size). β-catenin alleles were genotyped as described (Guo et al., 2004).

Skeletal analysis

Embryos were dissected in PBS. The embryos were then skinned, eviscerated, and fixed in 95% ethanol. Skeletal preparations were performed as described (McLeod, 1980).

Histology, in situ hybridization, immunohistochemistry and TUNEL assay.

Embryos were fixed in 4% paraformaldehyde at 4°C overnight. Fixed samples were embedded in paraffin and sectioned at 5 μm thickness. Histological analysis, BrdU labeling, immunohistochemistry and radioactive ^{35}S-RNA in situ hybridization were performed as described (Yang et al., 2003). Primary antibodies included anti-p21 mouse monoclonal IgG (Santa Cruz) at 1:40, anti-p27 mouse monoclonal IgG (Santa Cruz) at 1:40, anti-p57 rabbit polyclonal IgG at 1:160 (Santa Cruz) and anti-cyclin D1 mouse monoclonal IgG (Santa Cruz) at 1:80. Signals were detected using biotin-conjugated secondary antibodies and the ABC Kit (Vectorstain). Cell death was detected as described (Barrow et al., 2003). Probes for in situ hybridization have been described previously: *ColX*, *ColII*, *Ihh* and *Runx2* (Yang et al., 2003); *Osx* (Nakashima et al., 2002); *Mmp13* (Day et al., 2005); *Gli1* (Lewis et al., 2001); and *Hip1* (Chuang and McMahon, 1999). An EST clone (Clone ID 5346064, from Invitrogen) was used to generate *Pthrp* probes.

Micromass and limb cultures

Micromass cultures and Cre-Adenovirus infection were performed as previously described (Guo et al., 2004). Recombinant mouse Bmp2 and noggin were purchased from R&D Systems. Bmp2 (0.5 μg/ml) and noggin (1.5 μg/ml) were added to the micromass culture 24 hours after the cells were plated at high density. In the limb culture, the BGJB (Invitrogen) medium, containing noggin (1.5 μg/ml), was changed every 2 days.

RESULTS

Cell autonomous activation of Hh signaling leads to abnormal cartilage, bone and synovial joint development during endochondral ossification

As both Ihh and canonical Wnt signaling control multiple aspects of chondrocyte and osteoblast development, testing the genetic epistasis of the Ihh and the canonical Wnt signaling pathways specifically in the developing skeletal tissue is essential for gaining more insight into the molecular regulatory mechanisms of both signaling pathways in vivo. To achieve this would require the upregulation of Ihh signaling and the inactivation of canonical Wnt signaling simultaneously in the same cells. For this purpose, we generated a floxed allele of *Ptch1* in mice (Fig. 1) that allowed cell autonomous activation of Hh signaling upon Cre-mediated recombination. *Ptch1* was inactivated using the *Col2a1-Cre* line in which Cre-mediated recombination was observed in all chondrocytes and many osteoblasts (Day et al., 2005). We found that in the $Ptch1^{c/-}$; *Col2a1-Cre* embryos, long bones in the limb were shorter, bone mineralization was enhanced and the posterior skull failed to form (Fig. 2L-N). These results are consistent with previous findings when *Ihh* was overexpressed during endochondral bone formation in mice (Long et al., 2004; Long et al., 2001). It appears that failure of posterior skull formation in the $Ptch1^{c/-}$; *Col2a1-Cre* embryos may be due to enhanced antichondrogenic Wnt/β-catenin signaling in the skull mesenchyme, which resulted from abnormal brain development. In support of this, loss of β-catenin completely rescued skull formation in the $Ptch1^{c/-}$; $Catnb^{c/-}$; *Col2a1-Cre* double mutant embryos (Fig. 3A). In the limb sections of the $Ptch1^{c/-}$; *Col2a1-Cre* embryos, we found that chondrocyte hypertrophy was inhibited (Fig. 2A,B) and *Pthrp* expression was upregulated (Fig. 2C,D), suggesting that Ihh signaling was activated in the developing cartilage. Indeed, the expression of Hh signaling transcriptional targets such as *Gli1* and *Hip1* was activated in both

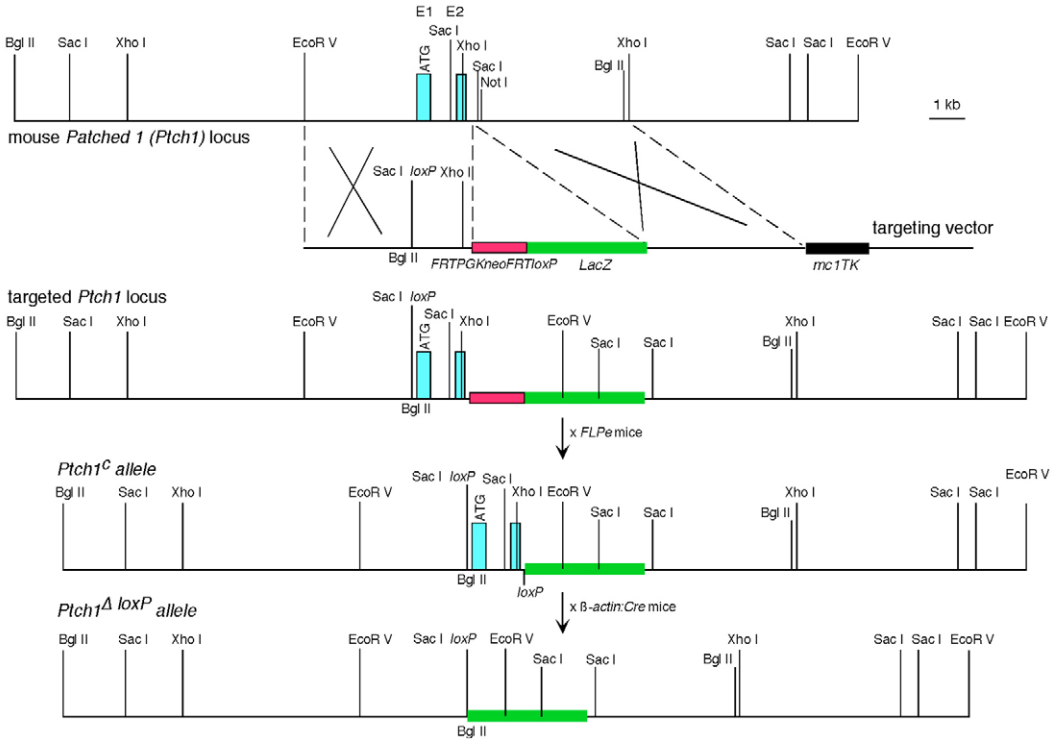

Fig. 1. Generation of a conditional allele of mouse *Ptch1*. Schematic diagram showing the mouse patched 1 (*Ptch1*) genomic locus, the targeting vector and the mutant alleles. The top line shows a partial restriction map of the mouse *Ptch1* genomic locus on chromosome 13. The mouse *Ptch1* genomic locus consists of 23 exons (E1-E23) and only the first two exons are shown for simplicity. The translation start ATG is predicted to reside in the first exon (E1). The regions between the dotted lines represent the 5′ and 3′ regions of homology used in gene targeting, respectively, and 'X' indicates events of homologous recombination. Germline-transmitting chimeric males carrying the targeted *Ptch1* locus were mated with *FLPe* (Rodriguez et al., 2000) mice to remove the *PGKneo* selection cassette, which is flanked by two FRT sites. The resulting allele is a conditional allele of *Ptch1*, denoted as $Ptch1^{c}$. $Ptch1^{c/+}$ animals were mated with a *Cre* deleter strain [e.g. β-actin::Cre (Meyers et al., 1998)] to remove sequences between the two loxP sites and to generate a null allele, designated as $Ptch1^{-}$ or $Ptch1^{\Delta loxP}$ in this study. Matings were set up between $Ptch1^{\Delta loxP/+}$ animals, and homozygous $Ptch1^{\Delta loxP/\Delta loxP}$ embryos display characteristic phenotypes that are indistinguishable from those of the published null alleles of *Ptch1* (Goodrich et al., 1997). When the *Ptch1* conditional allele is converted into the null allele, β-galactosidase is brought under the control of the *Ptch1* regulatory elements. It should be noted that a small percentage of homozygous $Ptch1^{c/c}$ animals display embryonic/neonatal lethality, suggesting that the inclusion of *lacZ* may have affected the expression from the *Ptch1* locus.

chondrocytes and perichondrium, with the strongest sites of activation observed in the perichondrium and joint region (Fig. 3B). These results demonstrate that the *Ptch1* allele was inactivated by Cre-mediated recombination, which led to Hh signaling activation in the $Ptch1^{c/-}$; *Col2a1-Cre* embryos. As a result of Hh signaling activation, we found that mineralized periosteum extended ectopically to the joint region, where mineralization was never found in wild-type controls (Fig. 2E,F). Mild joint fusion was also observed in the $Ptch1^{c/-}$; *Col2a1-Cre* embryo (Fig. 2G,H), which is consistent with previous observation that *Shh* overexpression in the cartilage resulted in joint fusion (Tavella et al., 2004). These results suggest that, to maintain joint integrity, Ihh signaling in the joint needs to be kept sufficiently low not to allow ectopic osteoblast differentiation and cartilage fusions.

Wnt/β-catenin signaling is required downstream of Ihh and Osx in controlling endochondral ossification

Ectopic osteoblast differentiation in the perichondrium of $Ptch1^{c/-}$; *Col2a1-Cre* embryos allowed us to directly test whether Ihh signaling requires canonical Wnt signaling for inducing osteoblast differentiation, by simultaneously blocking canonical Wnt signaling and activating Ihh signaling cell autonomously. We generated $Ptch1^{c/-}$; $Catnb^{c/-}$; *Col2a1-Cre* double conditional mutant embryos in which upregulation of Ihh signaling occurred in *Col2a1*-expressing cells and their descendants, where canonical Wnt signaling was abolished. We found that ossification and mineralization, as indicated by Alizarin red staining, were more severely inhibited in $Ptch1^{c/-}$; $Catnb^{c/-}$; *Col2a1-Cre* mutant embryos than in $Catnb^{c/-}$; *Col2a1-Cre* single mutant embryos (Fig. 3A). This was due to the blockage of both osteoblast differentiation and chondrocyte hypertrophy in $Ptch1^{c/-}$; $Catnb^{c/-}$; *Col2a1-Cre* double conditional mutant embryos. In the limb sections, mature osteoblasts, indicated by von Kossa staining, were almost completely missing, except for in a very small spot in the $Ptch1^{c/-}$; $Catnb^{c/-}$; *Col2a1-Cre* double mutant embryo at 16.5 days post-coitum (dpc; Fig. 4B). However, when the activity of Hh signaling was assessed by examining expression of the hedgehog transcriptional targets *Gli1* and *Hip1*, both were still ectopically activated in the perichondrium and the joint in $Ptch1^{c/-}$; $Catnb^{c/-}$; *Col2a1-Cre* double mutant embryos at similar levels to those in $Ptch1^{c/-}$; *Col2a1-Cre* single mutants, although the perichondrium in the $Ptch1^{c/-}$; $Catnb^{c/-}$; *Col2a1-Cre* double mutant was slightly thinner (Fig. 3B). These results indicate that Hh target gene

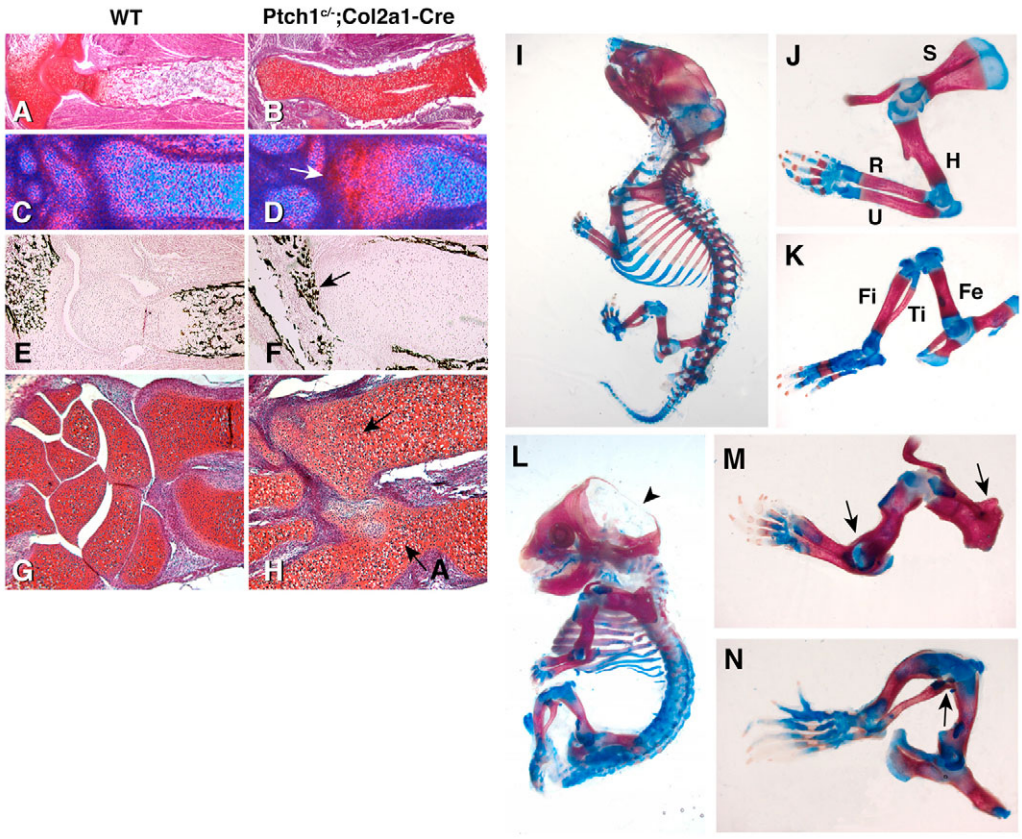

Fig. 2. Inactivation of *Ptch1* during endochondral skeletogenesis resulted in multiple defects. (**A-H**) Sections of developing long bones. (A,B) Safranin O staining of sections of humerus from 18.5 dpc embryos at low magnification. Proliferative chondrocytes were bright red, whereas hypertrophic chondrocytes were light red. Hypertrophic chondrocytes were not detected in the *Ptch1$^{c/-}$; Col2a1-Cre* mutant. (C,D) Joint regions between radius and carpel bones at 14.5 dpc are shown at higher magnification. Robust upregulation of *Pthrp* expression in the articular cartilage was observed in the mutant (arrow, D). (E,F) von Kossa staining of the distal humerus at 18.5 dpc. Ectopic ossification was observed in the joint of the mutant (arrow, F). (G,H) Safranin O staining of the radius/ulna/carpel junction at 18.5 dpc. Radius and ulna were fused with carpel bones in the mutant (arrows). (**I-N**) Skeletal preparations of 18.5 dpc wild-type (I-K) and *Ptch1$^{c/-}$; Col2a1-Cre* (L-N) mouse embryos. (I,L) Whole embryos; (J,M) forelimbs; (K,N) hindlimbs. Inactivation of *Ptch1* resulted in extensive ossification. Ectopic ossification at the joint is indicated by arrows (M,N). Skull formation was defective in the mutant (arrowhead, L). S, scapula; H, humerus; R, radius; U, ulna; Fe, femur; Fi, fibula; Ti, tibia.

expression in endochondral bone development does not require β-catenin and suggest that β-catenin may be required for perichondrial cell proliferation. Consistent with this, we found that the expression of *Pthrp*, a cartilage-specific transcriptional target of Ihh signaling, was similarly upregulated (Fig. 3B), and chondrocyte hypertrophy was inhibited, as shown by the lack of *ColX* expression that marks hypertrophic chondrocytes (Fig. 4A,B), in both *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* and *Ptch1$^{c/-}$; Col2a1-Cre* mutant embryos. Taken together, our results show that Wnt/β-catenin signaling is required for osteoblast differentiation downstream of, or in parallel to, Ihh signaling.

We found in our previous studies that the expression of *Runx2* and *Osx*, two early osteoblast differentiation markers, was present, but at a much reduced level, in the perichondrium of *Catnb$^{c/-}$; Col2a1-Cre* mouse embryos (Day et al., 2005). It was not clear whether the residual expression of *Runx2* and *Osx* was a direct consequence of residual β-catenin-positive cells caused by incomplete recombination, or an indirect result of reduced Ihh signaling and thinner perichondrium caused by reduced *Ihh* expression in the *Catnb$^{c/-}$; Col2a1-Cre* embryo (Day et al., 2005), as *Ihh* is required for cell proliferation and the expression of *Runx2* and *Osx* (Karp et al., 2000; Long et al., 2004). To clarify this issue, we took advantage of the thicker perichondium and the ligand-independent cell autonomous upregulation of Hh signaling in *Ptch1$^{c/-}$; Col2a1-Cre* mouse embryos and investigated whether the removal of Wnt signaling in these embryos would affect *Runx2* and *Osx* expression. In the *Ptch1$^{c/-}$; Col2a1-Cre* mouse embryo at 14.5 dpc, we found that *Runx2* expression was significantly upregulated throughout the thickened perichondrium, including the joint region, in a similar pattern to *Gli1* and *Hip1* expression (Fig. 4A). Importantly, in the *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* double mutant embryo, we still observed strong ectopic expression of *Runx2* and *Osx* in the perichondrium, although the perichondrium was slightly thinner (Fig. 4A). The persistent and strong expression of *Runx2* and *Osx* cannot be due to β-catenin expression in the perichondrium because ossification, which requires β-catenin, was almost completely inhibited in the *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* double mutant (Fig. 3A, Fig. 4B). These results indicate that like *Gli1* and *Hip1* expression, *Runx2* expression may be directly controlled by Ihh signaling. Furthermore, β-catenin is not required for the expression of *Runx2* and *Osx*, two downstream targets of Ihh signaling, in early osteoblasts in the developing long bones. Our results demonstrate that β-catenin is required downstream of Ihh signaling and *Osx* expression during early osteoblast differentiation.

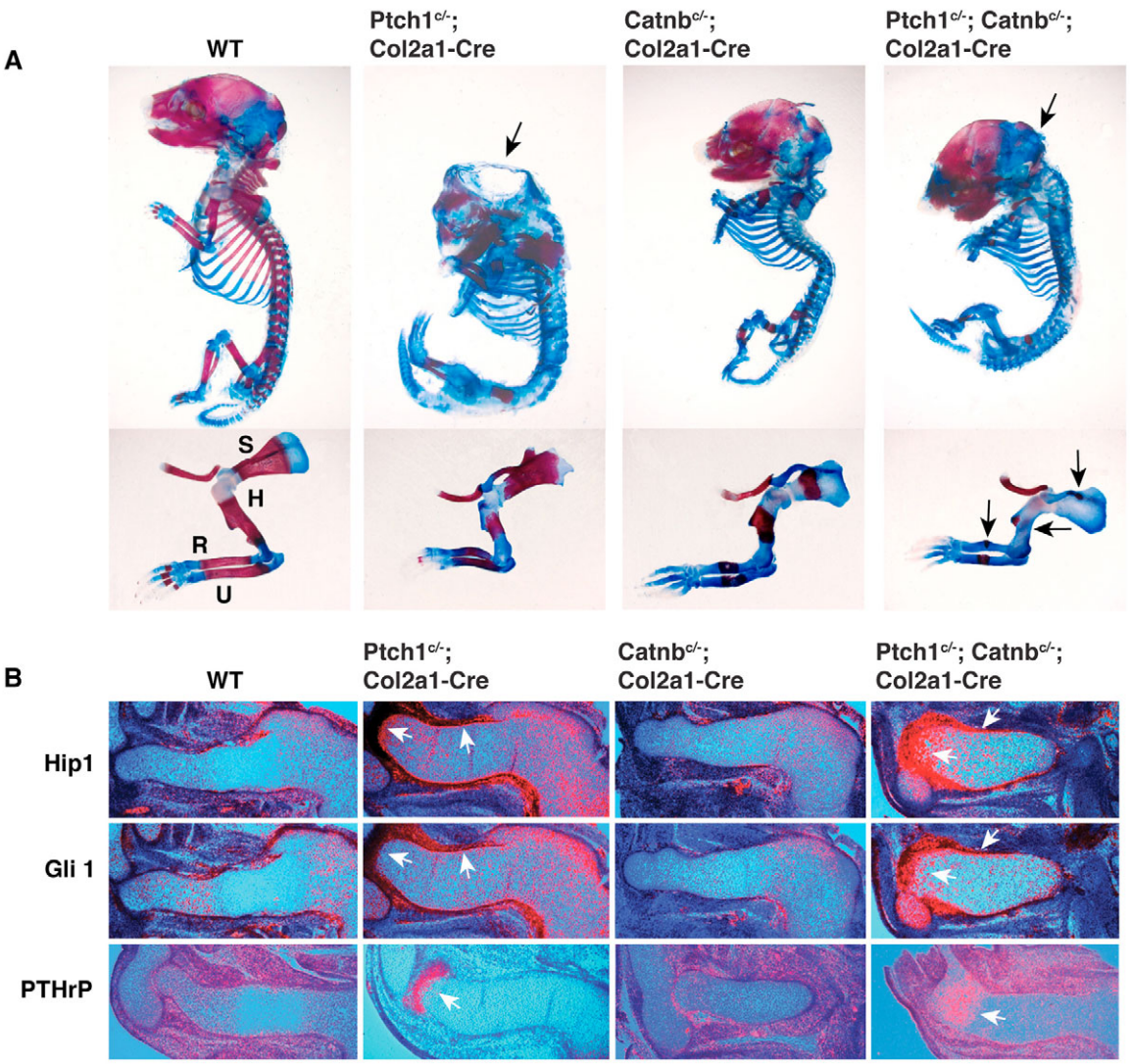

Fig. 3. Analysis of *Ptch1* and β-catenin mutant skeletons. (**A**) Skeletal preparations of 16.5 dpc mouse embryos. A forelimb of each embryo is shown in the lower panel. The posterior skull in the *Ptch1$^{c/-}$; Col2a1-Cre* mutant did not form (arrow). Skull formation in the *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* double mutant was rescued (arrow). Mineralization (arrows, lower panel) in the long bones was more severely reduced in the *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* double mutant than in either of the single mutants. S, scapular; H, humerus; R, radius; U, ulna. (**B**) β-catenin is not required for Ihh signaling. Consecutive sections of the developing humerus at 14.5 dpc were examined by in situ hybridization with the indicated ^{35}S-labelled riboprobes. In both *Ptch1$^{c/-}$; Col2a1-Cre* and *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* mutant embryos, expression of the Hh signaling targets *Hip1*and *Gli1*, and of *Pthrp*, was similarly upregulated in cartilage, perichondrium and joints (arrows). The perichondrium was slightly thinner in the *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* mutant than in the *Ptch1$^{c/-}$; Col2a1-Cre* mutant.

Interestingly, osteoblast maturation, indicated by von Kossa staining and the expression of mature osteoblast markers osteopontin (*Opn*; *Spp1* – Mouse Genome Informatics) and matrix metalloproteinase 13 (*Mmp13*), was not detected in the *Ptch1$^{c/-}$; Col2a1-Cre* mouse embryo at 14.5 dpc when ossification had just started in the wild-type embryo, although the expression of *Runx2* and *Osx* was strongly activated (Fig. 4A). Osteoblast maturation caught up later at 16.5 dpc (Fig. 4B). Furthermore, in the humerus of the *Ptch1$^{c/-}$; Col2a1-Cre* embryo, chondrocyte hypertrophy indicated by *ColX* expression was missing, and yet osteoblast differentiation occurred at 16.5 dpc (Fig. 4B). Therefore, activated Ihh signaling is sufficient to induce osteoblast differentiation and maturation without chondrocyte hypertrophy. Because *Ihh* is also required for *Runx2* expression and osteoblast differentiation (St-Jacques et al., 1999), our results further indicate that during normal endochondral bone formation, the coupling of chondrocyte hypertrophy with osteoblast differentiation and maturation is mediated by Ihh signaling. The slight delay in osteoblast maturation in the *Ptch1$^{c/-}$; Col2a1-Cre* embryo is likely to be due to the severely delayed chondrocyte hypertrophy (Fig. 4A,B), because factors such as *Vegf* and *Mmp*s are expressed in hypertrophic chondrocytes and are required to promote osteoblast maturation (Inada et al., 2004; Stickens et al., 2004; Zelzer et al., 2002). We also observed that the *Mmp9* expression was ectopically activated in both the *Ptch1$^{c/-}$; Col2a1-Cre* and *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* double mutant mouse embryos in a pattern similar to that of *Gli1* and *Hip1* expression

(Fig. 4A), suggesting that *Mmp9*, but not *Mmp13*, expression may also be directly controlled by Hh signaling. In contrast to the short delay in osteoblast maturation in the *Ptch1$^{c/-}$; Col2a1-Cre* mutant, osteoblast differentiation was severely blocked in the *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* double mutant in which there was almost no von Kossa staining or expression of *Opn* and *Mmp13* at 16.5 dpc, although the expression of *Osx* was still stronger than that in the *Catnb$^{c/-}$; Col2a1-Cre* mutant (Fig. 4B). These results confirm that β-catenin is required downstream of *Osx* for the progression of osteoblast maturation.

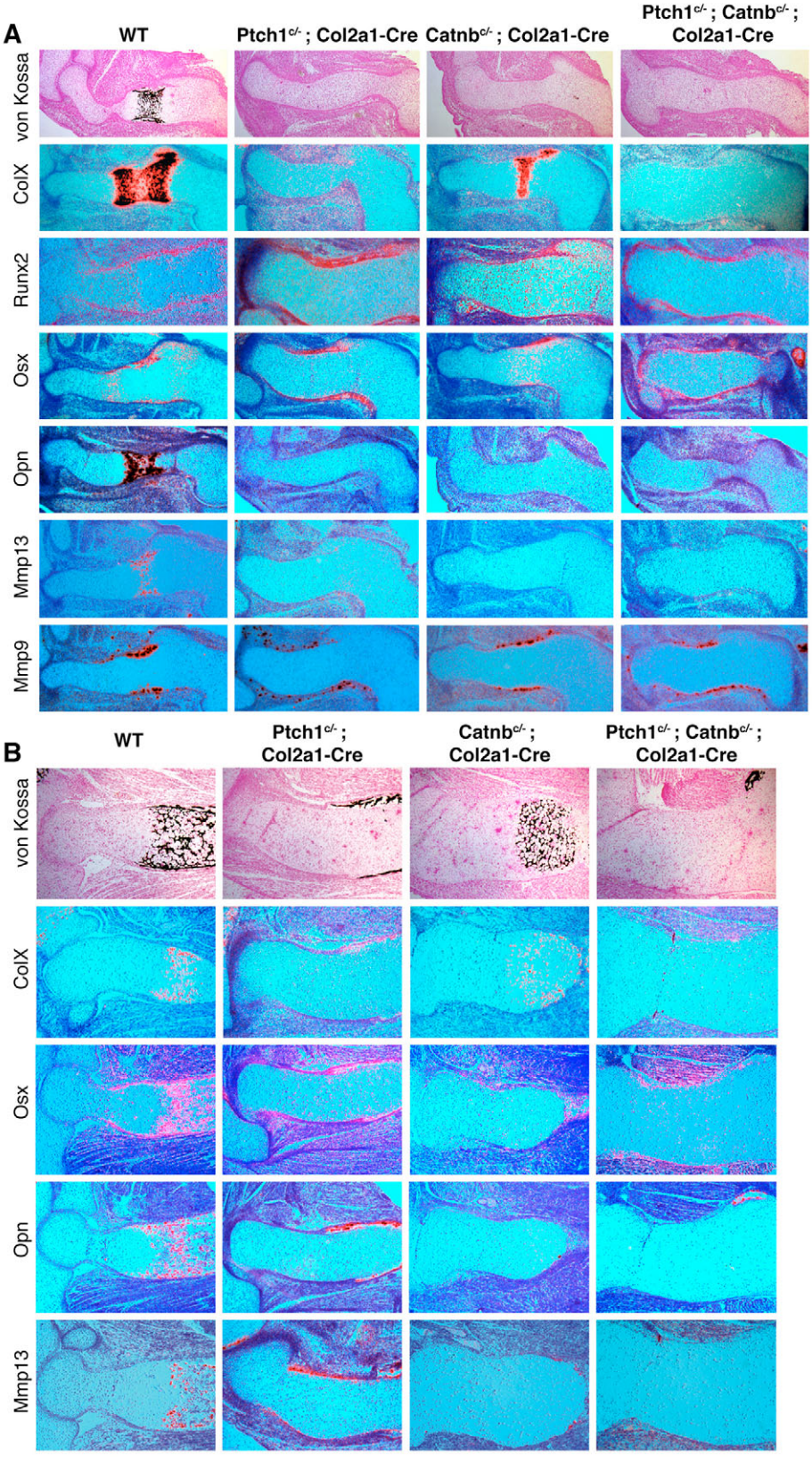

Fig. 4. Analysis of osteoblast differentiation in β-catenin and *Ptch1* mutant embryos. (**A**,**B**) Consecutive sections of the developing humerus at 14.5 dpc (A) and 16.5 dpc (B) were examined by von Kossa staining and in situ hybridization with the indicated ^{35}S-labelled riboprobes. (A) At 14.5 dpc, *ColX* expression was missing, and expression of the early osteoblast markers *Runx2* and *Osx* was activated in both *Ptch1$^{c/-}$; Col2a1-Cre* and *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* mutants. Ossification and expression of the late osteoblast markers *Opn* and *Mmp13* were only detected in the wild-type embryo. (B) At 16.5 dpc, *ColX* expression was still missing in the *Ptch1$^{c/-}$; Col2a1-Cre* and *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* mutants. *Osx* expression was still stronger in the *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* mutant than in the *Catnb$^{c/-}$; Col2a1-Cre* mutant. Ossification and expression of *Opn* and *Mmp13* were enhanced in the *Ptch1$^{c/-}$; Col2a1-Cre* mutant, but diminished in *Catnb$^{c/-}$; Col2a1-Cre* and *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* mutants.

Differential relationship between Wnt/β-catenin and Ihh signaling in regulating chondrocyte hypertrophy, proliferation and survival

As Ihh and canonical Wnt signaling both control chondrocyte hypertrophy, we tested whether they act in the same pathway. In contrast to the humerus, where chondrocyte hypertrophy was not detected in the *Ptch1$^{c/-}$; Col2a1-Cre* embryos, chondrocyte hypertrophy did occur in the radius, ulna and tibia, which allowed us to compare the effects Ihh and Wnt/β-catenin signaling in regulating chondrocyte hypertrophy. The differential inhibition of chondrocyte hypertrophy in different long bones in the *Ptch1$^{c/-}$; Col2a1-Cre* embryos was not due to different Cre activities, as Hh target genes were expressed at similar levels in the long bones (data not shown). In the tibia, the expression domains of *Ihh* and *ColX*, which mark prehypertrophic and hypertrophic chondrocytes, respectively, were smaller in both *Ptch1$^{c/-}$; Col2a1-Cre* and *Catnb$^{c/-}$; Col2a1-Cre* mutants than in the wild-type control (Fig. 5), indicating that chondrocyte hypertrophy was delayed in each single mutant. In the *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* double mutant, *Pthrp* expression was similarly upregulated when compared with that in the *Ptch1$^{c/-}$; Col2a1-Cre* mutant, but chondrocyte hypertrophy was more delayed than that in either of the single mutants, because *Ihh* and *ColX* expression was completely missing (Fig. 5). These results reveal that Ihh signaling inhibits chondrocyte hypertrophy independently of canonical Wnt signaling.

Because the regulation of chondrocyte hypertrophy is coupled with proliferation in that chondrocyte hypertrophy is delayed when proliferation is prolonged by removing cell cycle inhibitors (Scheijen et al., 2003; Yan et al., 1997; Zhang et al., 1997; Cobrinik et al., 1996), we examined the status of chondrocyte proliferation by BrdU labeling in *Ptch1* and β-catenin mutant embryos. In the developing cartilage, chondrocytes are organized into two zones with distinct proliferation rates before hypertrophy (Long et al., 2001; Yang et al., 2003). The resting zone chondrocytes are located at the articular end and proliferate slowly. The proliferative zone is composed of fast proliferating chondrocytes, and is located between the resting zone and the prehypertrophic region where *Ihh* is expressed (Fig. 6A). We found that, as expected, in the *Ptch1$^{c/-}$; Col2a1-Cre* embryo, chondrocyte proliferation in the presumed resting zone was significantly increased to a level similar to that in the proliferative zone by activated Hh signaling (Fig. 6A,B). In contrast to a previous study (Akiyama et al., 2004), we did not detect a reduction in chondrocyte proliferation in the *Catnb$^{c/-}$; Col2a1-Cre* mouse embryo (Fig. 6B). Interestingly, chondrocyte proliferation in *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* double mutant embryos resembles that in the *Catnb$^{c/-}$; Col2a1-Cre* mouse embryo (Fig. 6A,B), particularly in the resting zone, indicating that Wnt/β-catenin signaling may be required for the activity of Hh signaling in promoting cell proliferation in resting zone chondrocytes. As both Hh and Wnt/β-catenin signaling can regulate cell proliferation through regulating cyclin D1 expression (Long et al., 2001; Tetsu and McCormick, 1999), we next examined the expression of cyclin D1, and p21, p27 and p57, members of the Cip/Kip family of cyclin-cyclin dependent kinase (CDK) inhibitors, in mutant embryos at 14.5 dpc.

Consistent with the status of cell proliferation revealed by BrdU labeling, cyclin D1 expression is normally expressed highly in proliferative chondrocytes and we found that its expression was greatly upregulated in the presumed resting chondrocytes in the *Ptch1$^{c/-}$; Col2a1-Cre* mutant embryo (Fig. 7A). In addition, we did not observe a reduction in cyclin D1 expression in the *Catnb$^{c/-}$; Col2a1-Cre* mutant embryo, and the cyclin D1 expression pattern was similar in *Catnb$^{c/-}$; Col2a1-Cre* and *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* mutant embryos. These results suggest that although β-catenin is required for cell proliferation and cyclin D1 expression downstream of Ihh signaling in the resting zone, there may be a β-catenin-independent pathway in the proliferative zone to promote cyclin D1 expression and cell proliferation. P27 was expressed in chondrocytes of all zones and its expression was not altered by Hh or Wnt/β-catenin signaling (Fig. 7A). P57 is strongly expressed in prehypertrophic chondrocytes and is required to promote chondrocyte hypertrophy (Yan et al., 1997; Zhang et al., 1997). The expression pattern of p57 was not altered in the *Catnb$^{c/-}$; Col2a1-Cre* mutant embryo. But in the *Ptch1$^{c/-}$; Col2a1-Cre* and especially the *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* mutant embryo, strong expression of p57 was detected throughout the cartilage even though chondrocyte hypertrophy was significantly delayed (Fig. 7A). Interestingly, p21 was expressed almost exclusively in prehypertrophic chondrocytes in the wild-type embryo. Only very few cells in the proliferative zone expressed p21 at both 14.5 and 18.5 dpc (Fig. 7A,B). In the *Ptch1$^{c/-}$; Col2a1-Cre* double mutant embryo, few chondrocytes expressed p21, similar to what was observed in the proliferative region of the wild-

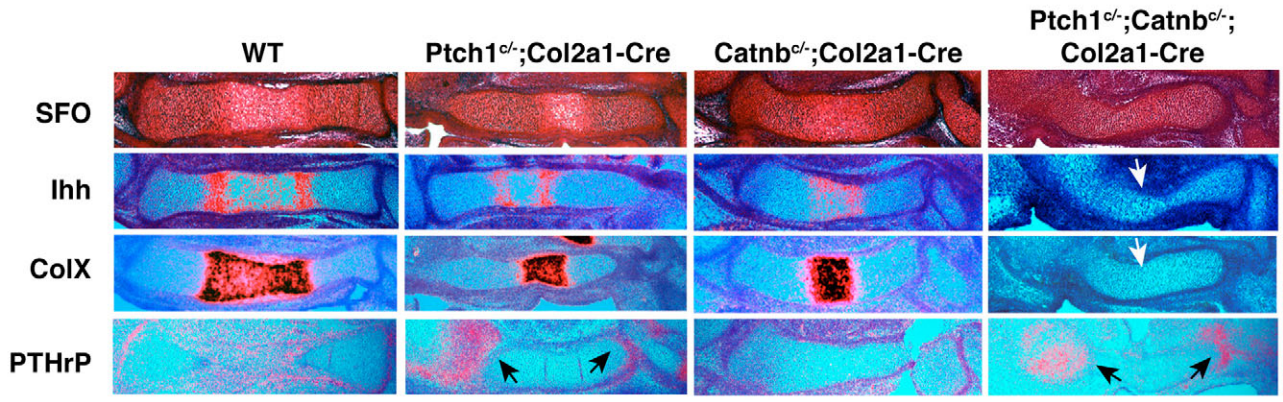

Fig. 5. Enhanced delay of chondrocyte hypertrophy in β-catenin and *Ptch1* double mutant embryos. Consecutive sections of the developing tibia at 14.5 dpc were examined by Safranin O staining and in situ hybridization with the indicated ^{35}S-labelled riboprobes. Hypertrophic chondrocytes are enlarged and stained light red by Safranin O. The expression domains of *Ihh* and *ColX* were smaller in both *Ptch1$^{c/-}$; Col2a1-Cre* and *Catnb$^{c/-}$; Col2a1-Cre* mutant embryos, and were missing in the *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* double mutant (arrows). *Pthrp* expression was similarly upregulated in *Ptch1$^{c/-}$; Col2a1-Cre* and *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* mutants (arrows).

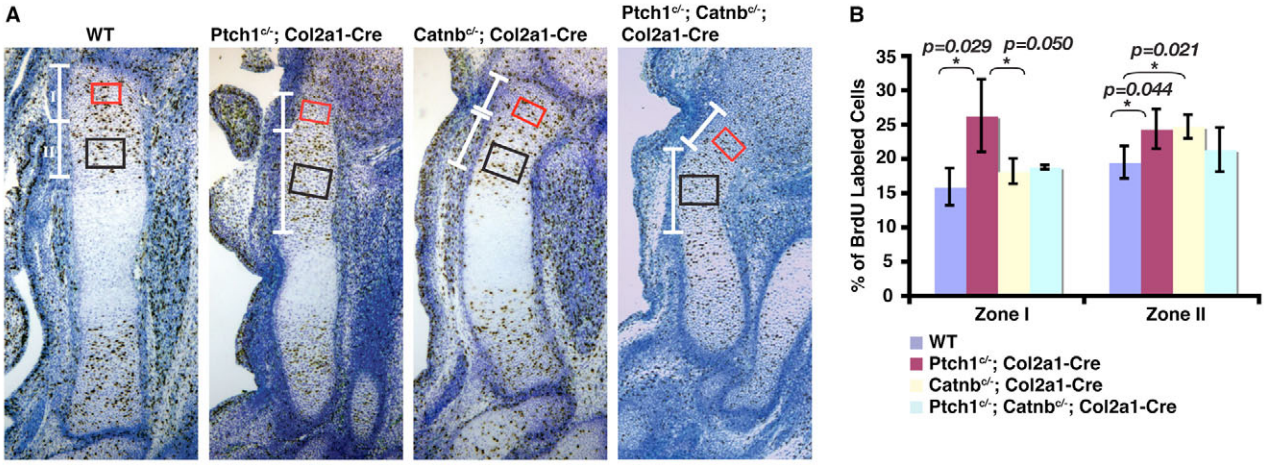

Fig. 6. Analysis of chondrocyte proliferation in β-catenin and *Ptch1* mutant embryos. (**A**) BrdU-labelled cells were detected by immunohistochemistry on sections of the developing tibia at 14.5 dpc. Zone I (resting zone) contains slow proliferating chondrocytes and Zone II (proliferative zone) contains fast proliferating chondrocytes. For each genotype, BrdU-labelled and total chondrocyte numbers in the boxed regions were counted from three different samples to find the mean. (**B**) Comparison of BrdU-labelled chondrocytes in Zone I and II in different mutants. Three samples were counted, and the mean±s.d. and statistically significant *P*-values are shown.

type cartilage at 14.5 and 18.5 dpc (Fig. 7A,B). In the *Catnb$^{c/-}$; Col2a1-Cre* mouse embryo, slightly more cells in the resting and proliferative zones expressed p21 at 14.5 dpc (Fig. 7A). Strikingly, at 18.5 dpc, significantly more cells in the proliferative and resting zones of the *Catnb$^{c/-}$; Col2a1-Cre* embryo expressed p21 (Fig. 7B). But in the double mutant embryo, p21 expression was reduced to a level similar to that in the *Ptch1$^{c/-}$; Col2a1-Cre* embryo at both 14.5 and 18.5 dpc (Fig. 7A,B), indicating that p21 expression is inhibited by both the Wnt/β-catenin and Hh signaling pathways in proliferative and resting chondrocytes, and that Hh signaling acts downstream of Wnt/β-catenin signaling. It should be noted here that, in contrast to cyclin D1 expression, changes in p57 and p21 expression did not correlate with those of chondrocyte proliferation in the mutants. p57 or p21 expressing cells were increased in either *Ptch1$^{c/-}$; Col2a1-Cre* or *Catnb$^{c/-}$; Col2a1-Cre* embryos, respectively, but there was no corresponding reduction in cell proliferation (Figs 6, 7). As p21 acts, not only as an inhibitor for cell proliferation, but also as a regulator of cell death with both anti- and pro-apoptotic function depending on the cellular context (Gartel and Radhakrishnan, 2005; Tsao et al., 1999; Wang et al., 2005; Yamamoto and Nishioka, 2005), we examined cell death by TUNEL assay. In the wild-type embryo, cell death was mostly observed in the joint and the most mature hypertrophic chondrocytes at 18.5 dpc. Cell death was not evident in the proliferative and resting chondrocytes (Fig. 7B). By contrast, in the *Catnb$^{c/-}$; Col2a1-Cre* embryo at 18.5 dpc, extensive cell death was detected in both proliferative and resting chondrocytes, and this cell death phenotype was completely suppressed in the *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* embryo (Fig. 7B). These data demonstrate that Wnt/β-catenin acts upstream of the Hh signaling pathway in promoting chondrocyte survival, and further suggest that p57 and p21 may act downstream of canonical Wnt and Hh signaling in this process.

Loss of Wnt/β-catenin signaling synergizes with activation of Ihh signaling in causing synovial joint fusion

The synovial joint fusions in *Ptch1$^{c/-}$; Col2a1-Cre* embryos (Fig. 2H) prompted us to investigate whether Ihh signaling is activated during normal synovial joint formation by examining the expression of *Hip1*, a transcriptional target of Hh signaling, at 14.5 dpc. We found that *Hip1* was expressed strongly in the joint region (Fig. 8A), as well as *Gli1* and *Ptch1* (data not shown). These results indicate that Hh signals to the joint cells directly and that Hh signaling may need to be kept at a low level to allow normal joint formation. The joint cells may be more responsive to Hh signaling, as the Hh signaling transcriptional targets *Gli1* and *Hip1* were activated more strongly in the joint region than in the neighboring chondrocytes in *Ptch1$^{c/-}$; Col2a1-Cre* embryos (Fig. 3B). Interestingly, we found extensive synovial joint fusions in the *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* double mutant, which were much more severe than each of the single mutants that only exhibited mild fusions in some joints (Fig. 2H) (see also Guo et al., 2004). For instance, the elbow joint was normal in both *Ptch1$^{c/-}$; Col2a1-Cre* and *Catnb$^{c/-}$; Col2a1-Cre* single mutant embryos, but was completely fused in the double mutant (Fig. 8B). To test whether the joint ever forms in the double mutant, we examined the earliest elbow joint development at 12.5 dpc when the elbow joint interzone has just formed (see Fig. S1 in the supplementary material). We found that the elbow joint interzone failed to form only in the double mutant embryos, based on both morphological and molecular criteria (see Fig. S1 in the supplementary material). These results indicate that the inhibition of canonical Wnt signaling was strongly enhanced by the activation of Ihh signaling, thus causing synovial joint fusion.

Consistent with previous findings in *Col2a1-Gal4; UAS-Ihh* mouse embryos (Minina et al., 2002), we observed that the expression of *Bmp7*, and to a lesser extent, *Bmp2* and *Bmp4*, but not the joint marker *Gdf5*, was activated in chondrocytes, perchondrium and the forming joint region in both *Ptch1$^{c/-}$; Col2a1-Cre* and *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* mutant embryos (Fig. 8C). Because Bmp signaling acts to inhibit synovial joint formation (Brunet et al., 1998; Tsumaki et al., 2002; Zou et al., 1997), it is likely that the Bmp genes mediate the activity of Ihh signaling in regulating synovial joint formation and that canonical Wnt signaling interacts with Ihh signaling indirectly through Bmps. We tested this possibility directly by culturing the limb buds from 12.5 dpc wild-type and *Ptch1$^{c/-}$; Col2a1-Cre* mutant embryos. Bmp signaling was inhibited in the culture by the addition of noggin, a Bmp inhibitor. The humerus-

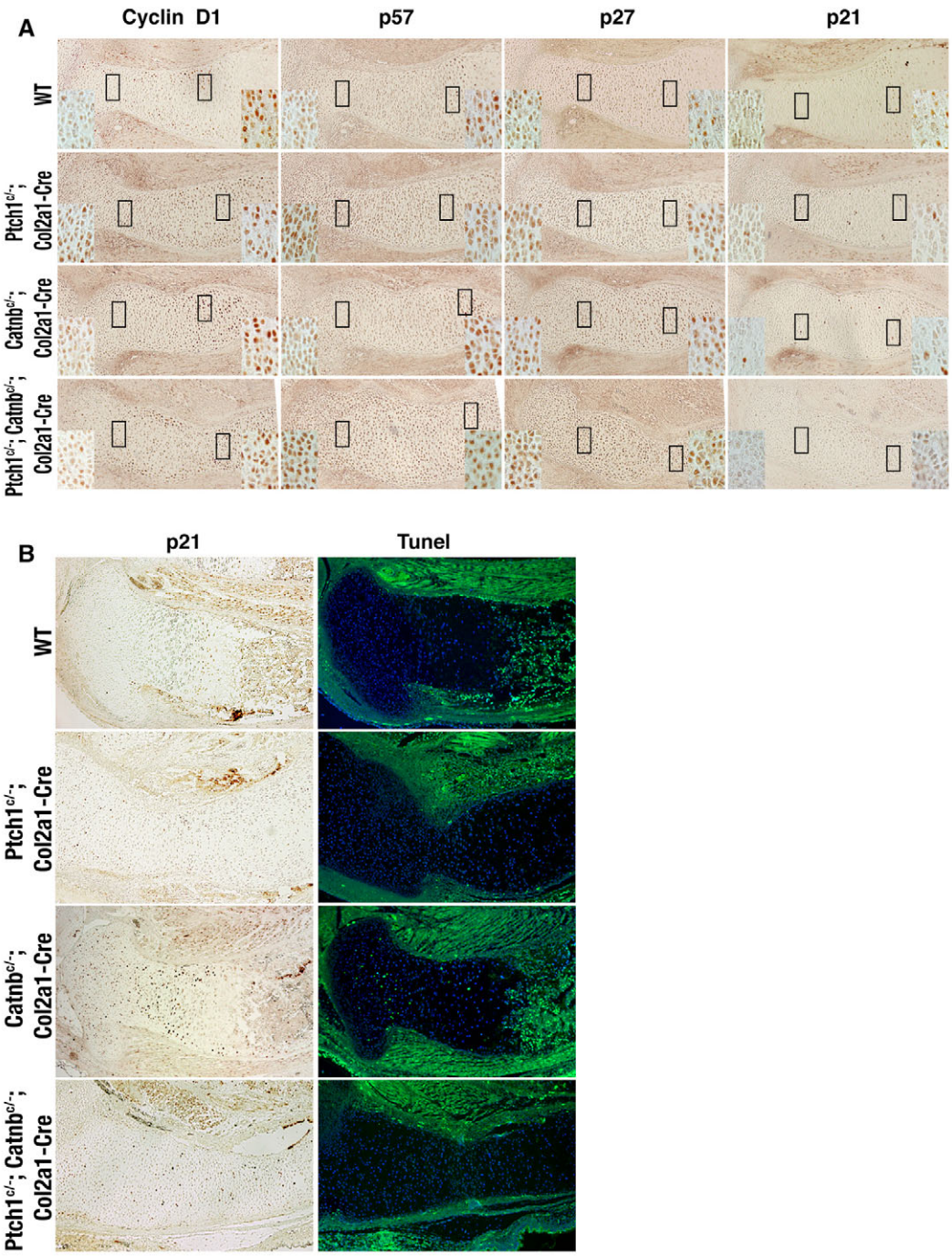

Fig. 7. Expression of cell cycle regulators and apoptosis in β-catenin and *Ptch1* mutant embryos. Results of immunohistochemistry with the indicated antibodies on limb sections of the indicated genotypes is shown. (**A**) Expression of the indicated cell cycle regulators in the distal humerus at 14.5 dpc. Boxed areas representing negative (left) and positive (right) staining regions in the wild-type sample and corresponding regions in every mutant are shown at higher magnification as insets in the lower left and right corners, respectively. (**B**) Expression of p21 and detection of cell death by fluorescent TUNEL assays on tibia sections at 18.5 dpc. Apoptotic cells are stained green; nuclei are stained blue by DAPI. p21 expression and apoptosis were greatly increased in the *Catnb$^{c/-}$; Col2a1-Cre* mutant.

scapula joint was fused in the *Ptch1$^{c/-}$; Col2a1-Cre* mutant embryos in vivo and in vitro, and cells in the presumptive joint area maintained chondrocyte-specific Safranin O staining. Importantly, in the noggin-treated *Ptch1$^{c/-}$; Col2a1-Cre* embryos (n=3), many cells in the humerus-scapula joint were elongated and had much lighter Safranin O staining, thereby resembling those joint cells found in the wild-type embryos (Fig. 8D). Furthermore, we found that the expression of the joint marker *Gdf5* was lost in the humerus-scapula joint of the *Ptch1$^{c/-}$; Col2a1-Cre* mutant, but was significantly rescued by noggin treatment (see Fig. S2 in the supplementary material), demonstrating that joint formation was rescued in the *Ptch1$^{c/-}$; Col2a1-Cre* mutant by blocking Bmp signaling. Noggin did not affect joint formation in the wild-type limbs (n=3; Fig. 8D). These results demonstrate that joint fusion in *Ptch1$^{c/-}$; Col2a1-Cre* mutant embryos is caused by upregulated Bmp signaling.

To further understand the synergistic interaction between Bmp signaling and loss of β-catenin in joint fusion, we performed micromass cultures to mimic the in vivo chondrogenesis in vitro, as joint fusion is caused by enhanced chondrocyte differentiation. We found that Bmp2 could further promote cartilage nodule formation already enhanced by inactivating β-catenin (Fig. 8E), indicating that Bmps can promote chondrocyte differentiation independently of canonical Wnt signaling. Furthermore, we found that inactivation of β-catenin was sufficient for extensive chondrocyte differentiation even in the presence of noggin, which

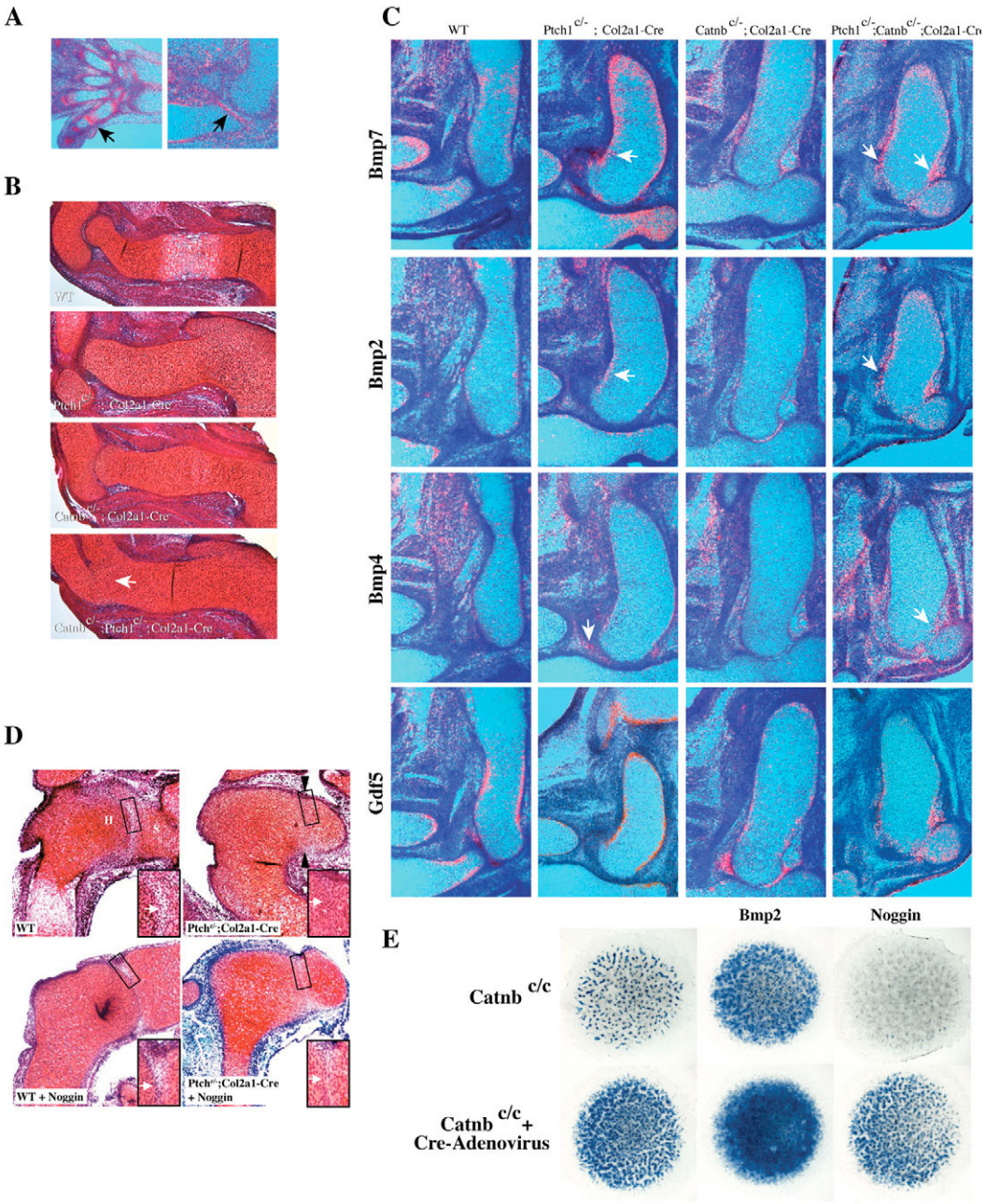

Fig. 8. Analysis of joint formation in β-catenin and *Ptch1* mutant embryos. (**A**) Expression of *Hip1* in the developing joint (arrows) at 14.5 dpc, as shown by in situ hybridization with ^{35}S-labelled riboprobes. (**B**) Safranin O staining. Complete fusion of the humerus-ulna joint was observed only in the *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* mutant (arrow). (**C**) Consecutive sections of the developing humerus-ulna region at 14.5 dpc were examined by in situ hybridization with the indicated ^{35}S-labelled riboprobes. Upregulation of *Bmp2*, *Bmp4* and *Bmp7* (arrows), but not *Gdf5*, was observed in both *Ptch1$^{c/-}$; Col2a1-Cre* and *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* mutants. (**D**) Safranin O staining of sections of the forelimbs that were isolated from 12.5 dpc embryos and cultured in vitro for 4 days. Boxed regions in the humerus-scapula joint are shown at higher magification in the insets. Joint cells are indicated (arrows). The fused joint in the *Ptch1$^{c/-}$; Col2a1-Cre* limb was rescued by noggin treatment. H, humerus; S, scapula. (**E**) Micromass culture with cells from *Catnb$^{c/c}$* embryonic limb bud mesenchyme at 12.5 dpc. Cartilage nodules were stained blue with Alcian Blue. β-catenin was deleted by Cre-Adenovirus infection. Bmp2 and noggin were added to the culture on day 2 of the 5-day culture period.

almost completely inhibited chondrocyte differentiation in wild-type progenitor cells (Fig. 8E). These results suggest that when the canonical Wnt pathway is inhibited, a much lower level of Bmp signaling is needed for chondrocyte differentiation. It is possible that the canonical Wnt and Bmp signaling pathways act in parallel with opposite activities in regulating the expression of a common target, such as *Sox9*, that is required for chondrocyte differentiation.

DISCUSSION

Here, we report that the canonical Wnt and Ihh signaling pathways interact with each other in a cell context-dependent manner in regulating different aspects of endochondral skeletal development, including osteoblast differentiation, chondrocyte proliferation, hypertrophy, survival and synovial joint formation. Different relationships between the two signaling pathways in these developmental processes reveal the unique genetic hierarchy in each developmental process and highlight the distinct contributions of each signaling cascade.

Canonical Wnt signaling is required downstream of Ihh signaling and *Osx* in regulating osteoblast differentiation during endochondral ossification

Previous studies have uncovered the requirement of the Ihh and canonical Wnt signaling pathways in osteoblast differentiation during endochondral bone formation (Day et al., 2005; Hill et al., 2005; Hu et al., 2005; Long et al., 2004). In this study, by activating the Hh signaling pathway while inactivating β-catenin simultaneously in the same cells in *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* double mutant embryos, we clarified two issues that were not settled before. First, the genetic relationship between Ihh signaling and β-catenin in promoting osteoblast differentiation was not clearly

demonstrated in vivo. It has been suggested that β-catenin may act downstream of Ihh signaling based on correlations of gene expression. In particular, expression of *Tcf1* and *Wnt7b* was suggested as a downstream target of Ihh signaling in activating the canonical Wnt signaling (Hu et al., 2005). However, the lack of *Tcf1* or *Wnt7b* expression in the perichondrium of $Ihh^{-/-}$ embryos could be a consequence of a loss of the right cell types in which *Tcf1* and *Wnt7b* are normally expressed. Second, it was not clear whether β-catenin could act downstream of *Osx* in controlling osteoblast differentiation, as *Osx* expression was reduced, but not abolished, in *Catnb$^{c/-}$; Dermo1-Cre* and *Catnb$^{c/-}$; Col2a1-Cre* mutant embryos (Day et al., 2005) (this study). The reduced expression of *Osx* could be a result of reduced Ihh signaling and thinner perichondrium in the mutant embryos. In this study, the thickness of perichondrium, the expression of both *Runx2* and *Osx*, and the final bone formation were greatly increased by activating Hh signaling in the *Ptch1$^{c/-}$; Col2a1-Cre* mutant. Yet, further removal of β-catenin in the *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* double mutant reduced bone formation to the same degree as that in the *Catnb$^{c/-}$; Col2a1-Cre* mutant, despite the fact that expression of *Runx2* and *Osx* in the perichondrium of the double mutant was similar to that in the wild type and stronger than that in the *Catnb$^{c/-}$; Col2a1-Cre* mutant. Therefore, *Osx*-expressing cells fail to give rise to bone-forming mature osteoblasts in the absence of β-catenin. β-catenin is required not only downstream of the Ihh signaling pathway, but further downstream of *Osx* in osteoblast maturation. Our results, together with previous studies, indicate that *Runx2* is a transcriptional target of Ihh signaling. As *Osx* is downstream of *Runx2*, β-catenin is required at least three steps downstream of Ihh signaling. Because ectopic chondrocyte differentiation occurred in the periosteum of the developing long bones of the *Catnb$^{c/-}$; Col2a1-Cre* and $Osx^{-/-}$ embryo (Day et al., 2005; Nakashima et al., 2002), it is likely that loss of β-catenin not only blocked osteoblast differentiation, but also caused *Osx*-expressing cells to lose *Osx* expression later and become chondrocytes. Consistent with this, we found that in the absence of β-catenin, *Osx* expression was much weaker in older *Catnb$^{c/-}$; Col2a1-Cre* embryos than in wild type (Fig. 4A,B), suggesting that β-catenin may even be required for the maintenance of *Osx* expression.

Ossification in the *Ptch1* conditional mutant is directional along the longitudinal axis

During normal long bone development, mature ossification is initiated from the middle of the cartilage. This is thought to be a result of the chondrocyte hypertrophy that is required for ossification and which occurs in the middle segment first (reviewed by Kronenberg, 2003). In the *Ptch1$^{c/-}$; Col2a1-Cre* mouse embryo, hedgehog signaling was activated cell autonomously in all chondrocytes and throughout the perichondrium independently of Ihh ligand and chondrocyte hypertrophy. However, mature ossification was still initiated from the middle of the long bones, although ectopic ossification did occur throughout the perichondrium, including the joint, eventually. The directional ossification along the longitudinal axis cannot be a consequence of localized chondrocyte hypertrophy as, in the developing humerus of the *Ptch1$^{c/-}$; Col2a1-Cre* mouse embryo, chondrocyte hypertrophy was inhibited by upregulated *Pthrp* expression (Fig. 4). The directional ossification was not a result of a differential response to Hh or Bmp signaling along the longitudinal axis of the developing long bones either, as we found that the expression of Hh signaling targets, *Runx2* and Bmps was upregulated strongly throughout the perichondrium, including the joint region. The directional osteoblast maturation might be caused by some unidentified inhibitor(s) of osteoblast differentiation localized in the joint region. The earliest sign of directional ossification in the *Ptch1$^{c/-}$; Col2a1-Cre* mutant is the expression of *Osx* that was expressed at the strongest level in the middle segment (Fig. 4A). Because osteoblast maturation eventually occurred in the joint, we favor another explanation: that the directional osteoblast maturation may simply reflect the time required by the *Runx2* expressing cells to proceed to the next stages of osteoblast maturation, including activating the expression of *Osx*. In the *Col2a1-Cre* mice, *Cre* is expressed in early osteochondral progenitor cells and chondrocytes, and not in late perichondral cells in the middle segment of the long bones. However, the effects of Cre-mediated recombination, which is inactivation of *Ptch1*, are inherited in all descendants of the early *Cre* expressing cells. As the development of chondrocytes and osteoblasts proceed from the joint to the middle segment of long bones, many osteoblasts in the middle segment are descendants of the earlier *Col2a1-Cre* expressing cells, and they have been activated by Hh signaling for a longer period of time.

Opposite and independent functions of canonical Wnt and Ihh signaling in chondrocyte hypertrophy

We observed that the phenotype of delayed chondrocyte hypertrophy in the *Ptch1$^{c/-}$; Col2a1-Cre* mutant embryo was enhanced by loss of β-catenin. As canonical Wnt signaling promotes chondrocyte hypertrophy, and Ihh signaling regulates chondrocyte hypertrophy mainly indirectly through activating and maintaining the expression of *Pthrp*, the canonical Wnt signaling pathway may also antagonize Pthrp signaling in controlling chondrocyte hypertrophy. It is likely that the canonical Wnt and Pthrp signaling pathways exert opposite activities in regulating common targets required for the execution of chondrocyte hypertrophy. The common targets may be *Runx2* or *Sox9*, or both, as they are both required to control chondrocyte hypertrophy (Bi et al., 2001; Enomoto et al., 2000; Kim et al., 1999). Consistent with the role of *Runx2* in promoting chondrocyte hypertrophy, *Runx2* expression was reduced in chondrocytes in *Ptch1$^{c/-}$; Col2a1-Cre* embryos, although its expression was upregulated in the perichondrium (Fig. 4A). But a reduction of *Runx2* expression in chondrocytes was not obvious in the *Catnb$^{c/-}$; Col2a1-Cre* mouse (Fig. 4A). It is also possible that the reduction of *Runx2* expression in *Ptch1$^{c/-}$; Col2a1-Cre* cartilage is only secondary to the initial delay of chondrocyte maturation caused by a *Runx2*-independent mechanism. This is supported by a recent finding that *Pthrp*, a major target of Hh signaling in controlling chondrocyte hypertrophy, regulates chondrocyte hypertrophy through both *Runx2*-dependent and -independent pathways (Guo et al., 2006). In contrast to *Runx2*, *Sox9* inhibits chondrocyte hypertrophy (Bi et al., 2001). Loss of β-catenin increases Sox9 activity in chondrocytes (Akiyama et al., 2004), whereas Pthrp potentiates Sox9 activity by activating PKA, which phosphorylates Sox9 (Huang et al., 2001). As *Sox9* is expressed in all *Col2a1* expressing chondrocytes at all developmental stages, the regulation of Sox9 activity is at least one of the underlying mechanisms for the opposite activities of canonical Wnt and Pthrp signaling in regulating chondrocyte hypertrophy.

Canonical Wnt and Hh signaling inhibit chondrocyte apoptosis

Our results indicate that both the canonical Wnt and Hh signaling pathways regulate chondrocyte survival. It has been demonstrated that the oxygen-sensitive hypoxia-inducible factor 1 alpha (Hif1α) and Vegfa are required for chondrocyte survival and the regulation

of chondrocyte proliferation in cartilage, which is an avascular tissue (Schipani et al., 2001; Zelzer et al., 2004). The phenotypes of *Catnb$^{c/-}$; Col2a1-Cre* embryos bear some similarities to those of *Hif1a* and *Vegfa* cartilage mutants in chondrocyte survival, although they are not as severe. It will be interesting to further investigate whether the canonical Wnt and Hh signaling pathways interact with the Hif1α and Vegfa pathway in controlling chondrocyte survival.

We have previously shown that inactivation of β-catenin promotes chondrocyte differentiation (Day et al., 2005; Guo et al., 2004). Our finding here that β-catenin inactivation also resulted in a dramatically increased cell death in differentiated chondrocytes further indicates that just inhibiting Wnt/β-catenin signaling alone is not sufficient to maintain healthy cartilage. The rescue of chondrocyte apoptosis and the inhibition of chondrocyte hypertrophy by Hh signaling in *Ptch1$^{c/-}$; Catnb$^{c/-}$; Col2a1-Cre* mouse embryos is significant from the therapeutic standpoint: to maintain healthy joint cartilage or to treat degenerating joint cartilage, inhibiting canonical Wnt signaling needs to be combined with the manipulation of other signaling pathways, such as activating Hh signaling or perhaps its downstream Pthrp signaling.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/18/3695/DC1

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