

# Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors

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Hedgehog and canonical Wnt/ $\beta$ -catenin signaling are implicated in development of the osteoblast, the bone matrix-secreting cell of the vertebrate skeleton. We have used genetic approaches to dissect the roles of these pathways in specification of the osteoblast lineage. Previous studies indicate that *Ihh* signaling in the long bones is essential for initial specification of an osteoblast progenitor to a *Runx2*<sup>+</sup> osteoblast precursor. We show here that this is a transient requirement, as removal of Hh responsiveness in later *Runx2*<sup>+</sup>, *Osx1*<sup>+</sup> osteoblast precursors does not disrupt the formation of mature osteoblasts. By contrast, the removal of canonical Wnt signaling by conditional removal of the  $\beta$ -catenin gene in early osteoblast progenitors or in *Runx2*<sup>+</sup>, *Osx1*<sup>+</sup> osteoblast precursors results in a similar phenotype: osteoblasts fail to progress to a terminal osteocalcin<sup>+</sup> fate and instead convert to a chondrocyte fate. By contrast, stabilization of  $\beta$ -catenin signaling in *Runx2*<sup>+</sup>, *Osx1*<sup>+</sup> osteoblast precursors leads to the premature differentiation of bone matrix secreting osteoblasts. These data demonstrate that commitment within the osteoblast lineage requires sequential, stage-specific, *Ihh* and canonical Wnt/ $\beta$ -catenin signaling to promote osteogenic, and block chondrogenic, programs of cell fate specification.

**KEY WORDS:** Osteoblast specification, Hedgehog signaling, Canonical Wnt signaling, Lineage commitment, Terminal osteoblast differentiation

## INTRODUCTION

The mammalian skeleton forms from three distinct cell lineages (Nakashima and de Crombrughe, 2003). Facial bones and the cranium are derived from the neural crest. The base of the skull, the parietal bones and the axial elements of the ribs and vertebrae are derived from paraxial mesoderm. The sternum and long bones are formed from lateral plate mesoderm. Although several cell lineages contribute to skeletal structures, each gives rise to a common bone matrix-secreting cell type, the osteoblast. The requirement of this cell type for the production of bone is clearly demonstrated by the targeted mutation of *Runx2* (Komori et al., 1997; Ducy et al., 1997; Otto et al., 1997) and *osterix1* (*Osx1*; *Sp7* – Mouse Genome Informatics) (Nakashima et al., 2002), transcriptional regulators expressed in osteoblast progenitors where both mutants lack all bone.

Bone can be produced by two distinct mechanisms, direct differentiation of osteoblasts from mesenchymal progenitors (intramembranous ossification, e.g. skull and face) or by formation of bone on a cartilage scaffold (endochondral ossification, e.g. remainder of the skeleton). Endochondral ossification, the principle focus of this study, is initiated by the condensation of multipotent mesenchymal progenitor cells into structures that anticipate skeletal elements of the adult (reviewed by Kronenberg, 2003). Chondrocytes are the first cell type to form, starting out as immature proliferative cells that express type 2 collagen (*Col2a1*) that subsequently mature into postmitotic type 10 collagen (*Col10a1*)-expressing hypertrophic chondrocytes. Osteoblast progenitors can first be identified in the inner layer of perichondrial cells that lie

immediately adjacent to the zone of hypertrophic chondrocytes, the periosteum, where the first bone matrix is deposited. Death of hypertrophic chondrocytes and vascular invasion result in the formation of a new area of mineralization, the primary spongiosa, within the shaft of the long bones.

Several lines of evidence implicate Hedgehog (Hh) and canonical Wnt signaling in the regulation of endochondral ossification (reviewed by Kronenberg, 2003; Karsenty, 2003). Indian hedgehog (*Ihh*) is expressed by prehypertrophic chondrocytes and plays an essential role in coordinating the growth and differentiation of chondrocytes, both directly and through the control of other factors, notably parathyroid hormone-related peptide (*Pthrp*; *Pthlh* – Mouse Genome Informatics). In addition, *Ihh* appears to act directly on perichondrial mesenchyme to initiate an osteogenic program in osteoblast progenitors; in the absence of an *Ihh* input, these cells adopt an alternate chondrogenic fate (Long et al., 2004). The failure of activation of *Runx2*, a crucial early determinant of the osteoblast lineage, indicates that Hh signaling acts to initiate an osteogenic program. Whether Hh signaling is required at later stages of the osteogenic program has not been addressed.

Initial in vivo evidence for canonical Wnt activity in osteogenesis came from studies of human and mouse mutations in low-density lipoprotein receptor-related protein 5 (*Lrp5*) which encodes a co-receptor (together with the frizzled family of multi-pass membrane proteins) for Wnt ligands. Human genetic analysis identified mutations in *LRP5* where bone mass was increased (activating mutations) (Boyden et al., 2002), or decreased, in osteoporosis pseudoglioma syndrome (OPPG; null mutations) (Gong et al., 2001). These observations have been supported by parallel mouse studies (Kato et al., 2002). Together, these findings pointed to a role for a Wnt-mediated process in regulating bone mass. More recently, manipulation of the canonical Wnt pathway by regulating the activity of  $\beta$ -catenin, which together with members of the *Lef/Tcf* family forms a transcriptional effector

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complex for this arm of the Wnt signaling pathway, has shed light on this mechanism. Canonical Wnt signaling in osteoblasts appears to regulate the production of osteoprotegerin (OPG), a factor that acts on the other key cell type of bone metabolism, the osteoclast, inhibiting osteoclast-mediated bone resorption (Glass, 2nd et al., 2005).

In addition to Wnt function in the maintenance of bone homeostasis, several recent developmental studies have suggested that Wnts play a role in the specification of osteoblasts. Specifically, Hill et al. (Hill et al., 2005) used a *Prx1-cre* line to conditionally inactivate  $\beta$ -catenin function, where *Prx1-cre* is active in the developing head and limb mesenchyme at E9.0 prior to skeletogenesis (Hill et al., 2005). This resulted in a dramatic reduction in the size of long bone skeletal elements and in an overall failure to develop bone. Characterization of osteoblast development in this model demonstrates that *Runx2* expression was detectable; however, these mutants failed to express *Osx1*, a *Runx2*-dependent transcriptional regulator that, like *Runx2*, is essential for all bone development. In a second study, Day et al. (Day et al., 2005) used *Dermo1-cre* and *Col2a1-cre* mouse lines to remove  $\beta$ -catenin function in mesenchymal condensations prior to chondrocyte and osteoblast development (a later stage than *Prx1-cre*), and in cartilaginous condensates prior to the specification of osteoblasts, respectively. In both models, an overall reduction in the size of skeletal elements was observed that was accompanied by an apparent arrest of osteoblast development at a *Runx2*<sup>+</sup>, *Osx1*<sup>+</sup> precursor stage. By contrast, a third study using the same *Dermo1-cre* and  $\beta$ -catenin conditional mouse lines reported an earlier arrest at a *Runx2*<sup>+</sup>, *Osx1*<sup>-</sup> stage (Hu et al., 2005). Thus, although there is some disagreement as to the phenotypical outcomes, all studies indicate that  $\beta$ -catenin activity within skeletal elements is required for formation of mature osteoblasts. Furthermore, the failure to complete an osteogenic program was associated with the appearance of ectopic chondrocytes, suggesting a potential link between canonical Wnt signaling and inhibition of a chondrogenic pathway within osteoblast progenitors (Hill et al., 2005; Day et al., 2005).

As these models remove  $\beta$ -catenin function broadly within the skeletal anlage, not specifically in the osteoblast lineage, the issue of whether canonical Wnt/ $\beta$ -catenin activity acts directly within the osteoblast lineage to promote an osteogenic program remains to be resolved. Here, we have used a novel *Osx1-GFP::Cre* mouse strain to investigate the direct roles of hedgehog and canonical Wnt signaling in early *Runx2*<sup>+</sup>, *Osx1*<sup>+</sup> osteoblast precursors and their derivatives. These data demonstrate an essential role for canonical Wnt signaling, but not for Hh signaling, in the progression of osteoblast precursors to mature, matrix-secreting osteoblasts. Interestingly, cell fate analysis demonstrates that removal of  $\beta$ -catenin activity in *Runx2*<sup>+</sup>, *Osx1*<sup>+</sup> osteoblast precursors gives rise to ectopic chondrocytes, suggesting, along with earlier data (Hill et al., 2005; Day et al., 2005), an extended role for canonical Wnt signaling in the suppression of an alternate chondrocytic fate within osteoblast precursors. In contrast to loss-of-function studies, enhanced  $\beta$ -catenin activity rapidly accelerates this program leading to a dramatic expansion of osteoblast precursors and the premature synthesis of a mineralized bone matrix in the long bones. However, differentiation to a terminal osteocalcin<sup>+</sup> (*Oc*<sup>+</sup>; *Bglpal* – Mouse Genome Informatics) osteoblast is blocked by stabilization of  $\beta$ -catenin. Thus, canonical Wnt/ $\beta$ -catenin signaling plays crucial roles at specific stages of the osteogenic program.

## MATERIALS AND METHODS

### Mouse strains

*Col2a1-cre3* (Long et al., 2001),  *$\beta$ -catenin<sup>cl</sup>* (Brault et al., 2001), *Canblos<sup>ex3</sup>/lox<sup>ex3</sup>* (Harada et al., 1999),  *$\beta$ -catenin<sup>+/-</sup>* (Haegel et al., 1995), *Smo<sup>cl</sup>*, *Smo<sup>+/-</sup>* (Long et al., 2001) and *Sox2-cre* (Hayashi et al., 2002) mouse lines have been described previously. The *Wnt7b* conditional allele will be described elsewhere.

The *Osx1-GFP::Cre* mouse line was generated by pronuclear injection of a bacterial artificial chromosome (BAC) containing the *Osx1* gene targeted at exon 1, using standard BAC recombination methods (Lee et al., 2001). Specifically, primers *Osx5* (CTC TCC CTT CAC CCT CTC CCA CTG GCT CCT CGG TTC TCT CCA TCT GCC TGA CTC CTT GGG ACC CGG TCC CCA GCT CGA GGA GAA TTC GCT GTC TGC GAG G) and *Osx9* (TAG GCA TGG ATT AGG ACC AGG AAG ATT GTA GCT GGT TTC TTA AGG AAG GGA ACA GTT ACC TCA AGC AGA GCT ATT CCA GAA GTA GTG AGG) containing 80- and 70-nucleotide *Osx1*-specific homology arms (underlined), respectively, were used to PCR amplify the transgenic tTA regulated GFP::Cre construct from the plasmid pTGCK (M. T. Valerius and A.P.M., unpublished), a derivative of plasmids triTAUBi (Kistner et al., 1996), pBS592 (Le et al., 1999) and pIGCN21 (Lee et al., 2001), using high-fidelity platinum *pfx* polymerase, following the manufacturer's directions (Invitrogen).

The PCR product was gel purified prior to electroporation into EL250 bacteria containing the BAC clone RP23-399N14 (Children's Hospital Oakland Research Institute, Oakland, CA). Recombinants were selected on bacterial plates containing kanamycin and PCR screened for homologous recombination events. The kanamycin selection cassette was removed from correctly targeted BAC clones by L-arabinose induction of flpe recombinase prior to being grown up and nucleobond purified (BD Biosciences). *Osx1-GFP::Cre* mice were genotyped using primers *Osx10* (CTC TTC ATG AGG AGG ACC CT) and TGCK-3' (GCC AGG CAG GTG CCT GGA CAT). Activity of *Osx1-GFP::Cre* was assessed by mating the *Osx1-GFP::Cre* mouse to *R26RlacZ* reporter mice; embryos were collected and *lacZ* stained as described previously (Soriano, 1999). Alternatively, limbs were removed, fixed, cryo-embedded, sectioned and then stained for *lacZ* activity.

### Skeletal analysis

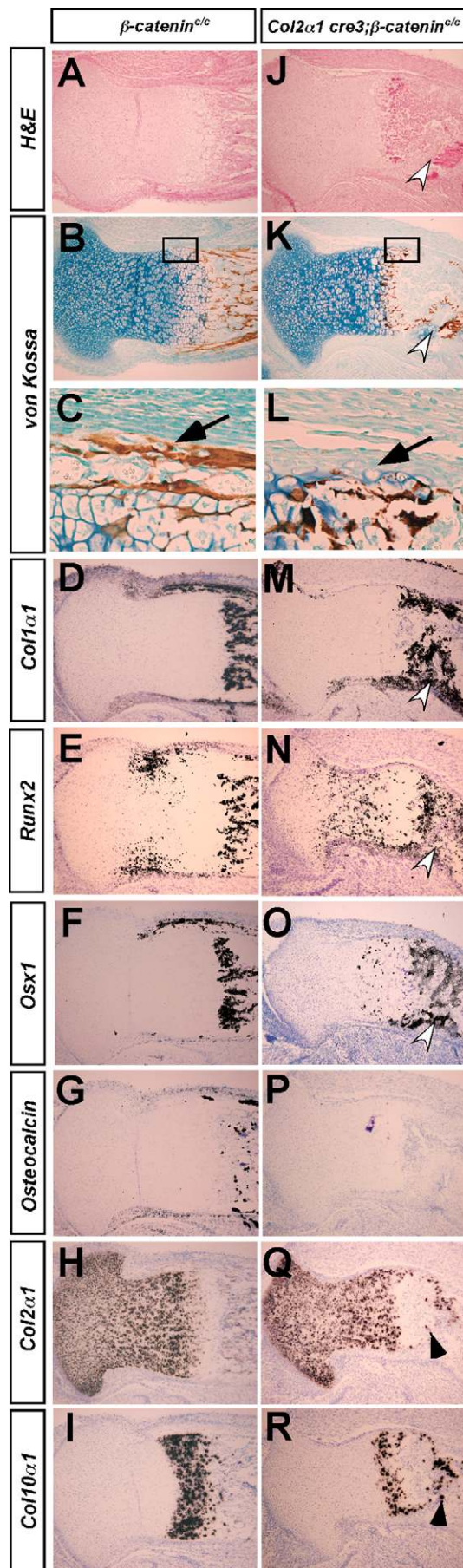
In our studies, we failed to observe any significant differences between skeletons of *Osx1-GFP::Cre* and  *$\beta$ -catenin<sup>cl</sup>* embryos and those of wild-type litter mates. Data are shown where  $\beta$ -catenin removal is compared with  *$\beta$ -catenin<sup>cl</sup>* individuals to control for reduced levels of  $\beta$ -catenin activity where only one allele is active. Skeletons were stained as described previously (Long et al., 2001). Embryonic limbs were dissected and fixed in sodium phosphate-buffered 4% paraformaldehyde over night at 4°C. Limbs were then washed in phosphate-buffered saline and either transferred to and stored in 70% ethanol prior to being paraffin-wax processed, embedded and sectioned, or cryo-protected in 30% sucrose prior to being frozen mounted in OCT and sectioned. In situ hybridization with <sup>35</sup>S-labeled probes and BrdU analysis of cell proliferation were carried out as described previously (Long et al., 2001). Hematoxylin/eosin, von Kossa and Safranin O staining were performed using standard histological methods. TUNEL analysis was performed using the ApopTag in situ Apoptosis Detection Kit (Chemicon), as described in the manufacturer's instructions.

## RESULTS

### Removal of canonical Wnt signaling prior to osteoblast specification

Earlier reports (Hu et al., 2005; Day et al., 2005; Hill et al., 2005) where  $\beta$ -catenin activity was removed prior to or after cartilage condensation have come to somewhat different conclusions about the requirement for canonical Wnt/ $\beta$ -catenin signaling in the initial specification of osteoblast progenitors. In a similar approach, we generated *Col2a1-cre3*; *$\beta$ -catenin<sup>cl</sup>* conditional knockout embryos in which  $\beta$ -catenin function was specifically removed from the mesenchyme-derived cartilaginous condensates that give rise to both the cartilage and the bone of the skeleton (Long et al., 2001). Hence *Col2a1-cre3* is active both in osteoblasts and in chondrocyte lineages.





**Fig. 1. *Col2α1-cre3* removal of  $\beta$ -catenin function results in a failure to develop terminally differentiated osteoblasts.**

Histological analysis and in situ hybridization of  $^{35}\text{S}$ -labeled riboprobes to sections of E18.5 tibia from (A–I)  $\beta$ -catenin<sup>cl/c</sup> (wild type) and (J–R) *Col2α1-cre3*;  $\beta$ -catenin<sup>cl/c</sup> (mutant) embryos, with specific anti-sense riboprobes for marker genes that identify states of osteoblast and chondrocyte differentiation. Osteoblast: (D,M) *Col1α1*, (E,N) *Runx2*, (F,O) *Osx1* and (G,P) *osteocalcin*. Chondrocyte: (H,Q) *Col2α1* and (I,R) *Col10α1*. Boxed areas in B and K are presented at higher magnification in C and L, respectively; black arrows indicate the absence of ossification in the mutant periosteum. White arrowheads indicate the invading wedge of mesenchymal cells into the marrow cavity; black arrowheads indicate sites of ectopic expression of chondrocyte marker genes.

Although the size and structure of the mutant growth plate was comparable between  $\beta$ -catenin<sup>cl/c</sup> or  $\beta$ -catenin<sup>cl/+</sup> littermates, and vascular invasion and seeding of the marrow cavity was evident from the presence of red blood cells within the forming marrow cavity, no histologically identifiable bone matrix was observed in the mutant long bones (Fig. 1).

These observations were corroborated by molecular analysis. *Col1α1* and *Runx2*, markers of early osteoblast progenitor cells, were expressed in mutant tibia at E18.5, albeit at lower levels than in  $\beta$ -catenin<sup>cl/c</sup> littermates. Specifically, *Col1α1* was expressed by cells within a wedge-like structure of mesenchyme invading into the presumptive marrow cavity. Furthermore, *Osx1*, a marker for osteoblast precursor cells whose function is downstream of *Runx2* (Nakashima et al., 2002), was expressed in the perichondrial region where osteoblast progenitors first arise, coincidental with the appearance of *Col1α1*-expressing cells in the invading mesenchymal wedge. By contrast, *Oc*, a definitive marker gene of terminally differentiated osteoblasts, was absent from most long bones; the occasional *Oc*<sup>+</sup> cell observed most likely reflected mosaicism in the activity of the *cre* transgene (Fig. 1; data not shown). These results are in general agreement with recent studies (Hu et al., 2005; Day et al., 2005; Hill et al., 2005). However, our observation that, despite robust *Osx1* expression, no mineralized bone matrix is formed suggests that canonical Wnt signaling may be required at a later stage downstream of *Osx1* in the osteogenic program.

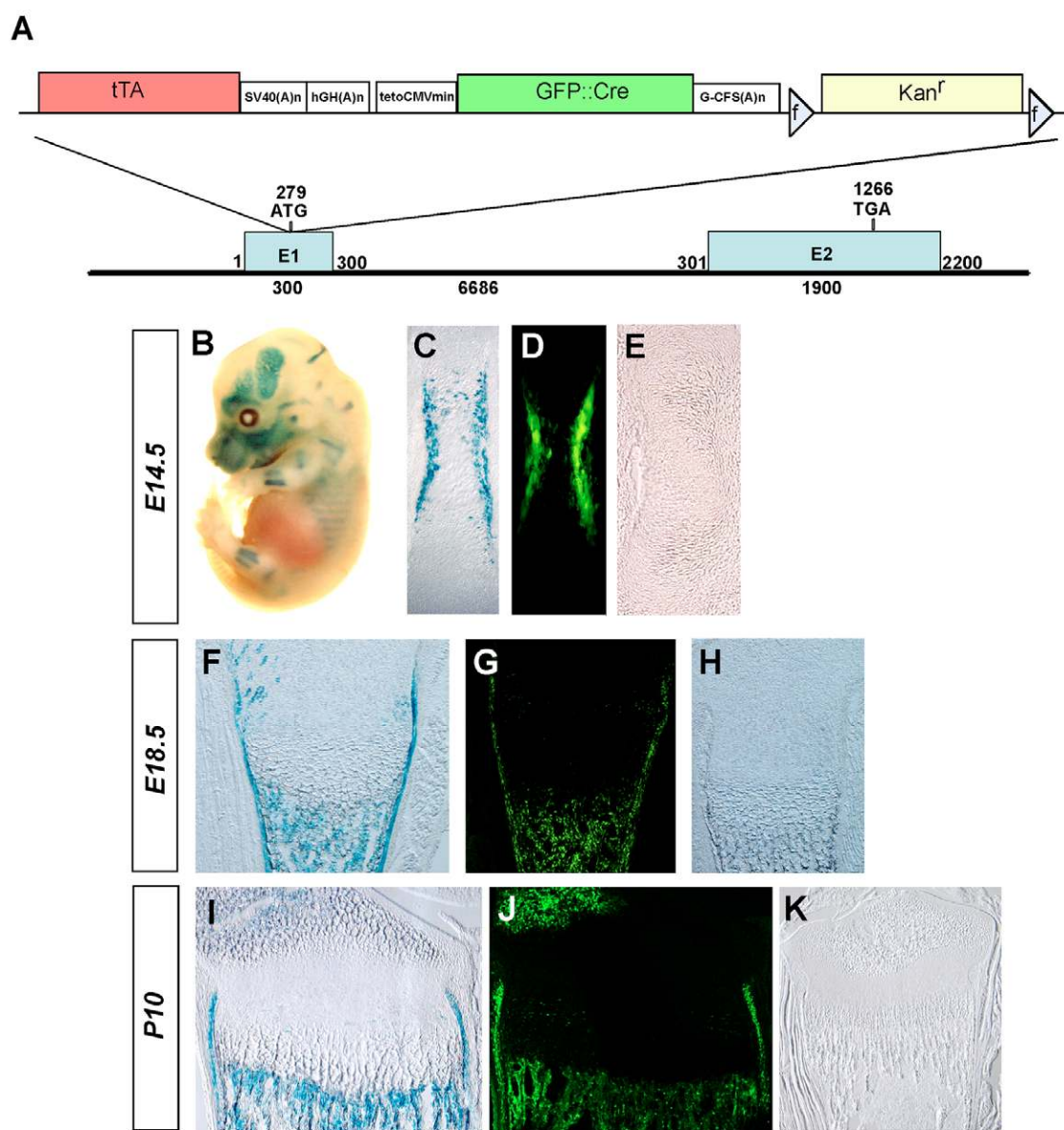
### Generation of a mouse line expressing a GFP::Cre fusion protein under the regulation of the *Osx1* promoter

Although these experiments are consistent with an intrinsic requirement for canonical Wnt/ $\beta$ -catenin signaling within the developing osteoblast lineage, we cannot rule out an alternative role for canonical Wnt/ $\beta$ -catenin signaling in the cartilage component that might indirectly regulate the osteoblast pathway. Indeed, cartilage development is perturbed in this and other similar models in which early growth and hypertrophic differentiation are disrupted (Hu et al., 2005; Day et al., 2005; Hill et al., 2005) (data not shown), and Wnt signaling is itself implicated in the chondrogenic program (Day et al., 2005). Furthermore, hypertrophic chondrocyte-derived factors (notably *Ihh*) play a crucial role in endochondral osteoblast development (reviewed by Kronenberg, 2003). To address signaling directly in the osteoblast lineage, we generated a BAC transgenic mouse line in which expression of a Tet-off regulatable GFP::Cre fusion protein is placed under the transcriptional regulation of the *Osx1* promoter.

The Tet-off cassette provides an additional level of potential temporal control of GFP::Cre activity within the osteoblast lineage that has not been examined in this study. The data herein and our unpublished data (S.J.R. and A.P.M.) indicate that this is an effective strategy for the dual regulation of gene activity. However, unrelated studies in which this regulatory system has been introduced by gene targeting into several loci suggest that this strategy can lead to dominant phenotypes. These are not always observed, as in this strategy, suggesting a gene-, cell type- and/or context-dependent mechanism, the nature of which is unclear.

As an initial step to characterize expression and activity of the *Osx1-GFP::Cre* transgene, transgenic mice were mated to *Rosa26lacZ* reporter (*R26R*) mice (Soriano, 1999). *lacZ* expression, indicative of GFP::Cre activity, was observed in all bony elements

(endochondral and membranous) consistent with endogenous *Osx1* expression (Nakashima et al., 2002) (Fig. 2B). Bone-specific activity was evident when GFP::Cre fluorescence and *lacZ* expression were examined in tibial sections (Fig. 2C,D). GFP::Cre and *lacZ* expression were observed in the inner bone-forming perichondrium, adjacent to hypertrophic chondrocytes, and sporadically in hypertrophic chondrocytes. Similarly, analysis at E18.5 (Fig. 2F-H) and postnatal day 10 (Fig. 2I-K) demonstrated that expression of *lacZ* and GFP::Cre fluorescence were largely restricted to the periosteum and primary spongiosa. Thus, *Osx1-GFP::Cre* is active specifically within the osteoblast lineage and expression is maintained throughout embryonic and early postnatal development. The fact that GFP::Cre activity is largely absent from chondrocytes in which *Osx1* normally shows weak expression (Nakashima et al.,



**Fig. 2. Generation of an *Osx1-GFP::Cre* transgenic mouse line.** (A) Schematic outlining the linear configuration of the pTGCK cassette used for homologous recombination to the first exon of the osterix1 locus contained within the BAC: RP23-399N14. The correctly targeted BAC was subsequently used to generate a transgenic mouse line by pro-nuclear injection. (B-K) Founder *Osx1-GFP::Cre* male transgenic mice were crossed to the female *Rosa26lacZ* reporter line and activity of the transgenic line was observed by way of whole-mount *lacZ* assay at (B) E14.5, and by *lacZ* assay or direct fluorescence microscopy on 15  $\mu$ m cryosections of tibia from (C,D) E14.5, (F,G) E18.5 and (I,J) postnatal day 10 mice. (E,H,K) Negative control specimens for littermates, at each corresponding age, that do not carry the transgene.



2002) suggests either that low-level GFP::Cre activity is inefficient in initiating recombination, or, alternatively, that chondrocyte-specific regulatory elements are absent in the BAC transgene.

### An intrinsic requirement for $\beta$ -catenin in the progression of *Osx1*<sup>+</sup> cells to bone-producing osteoblasts

To determine whether  $\beta$ -catenin is required downstream of *Osx1* in the osteoblast lineage, *Osx1-GFP::Cre;  $\beta$ -catenin<sup>cn</sup>* conditional knockout embryos were collected at E14.5, E16.5 and E18.5, and their gross skeletal organization analyzed (Fig. 3). At E14.5, *Osx1-GFP::Cre;  $\beta$ -catenin<sup>cn</sup>* mutant skeletons were comparable in size to wild-type embryos. At E16.5, no Alizarin red staining was observed despite clear zones of hypertrophy in mutant limbs. By E18.5, mineralization of the endochondral skeletal elements was visible; however, as in the *Col2 $\alpha$ 1-cre3* removals, the remaining mineralization was associated with hypertrophic chondrocytes. Importantly, in contrast to the *Col2 $\alpha$ 1-cre3* removal of  $\beta$ -catenin activity, which is specific to the endochondral skeleton, *Osx1-GFP::Cre;  $\beta$ -catenin<sup>cn</sup>* embryos lacked the membranous bone of cranial ossification centers. Hence, there is a complete loss of bone deposition that is reminiscent of the loss of osteoblast determinants, such as *Runx2* (Komori et al., 1997; Ducy et al., 1997; Otto et al., 1997) and *Osx1* (Nakashima et al., 2002).

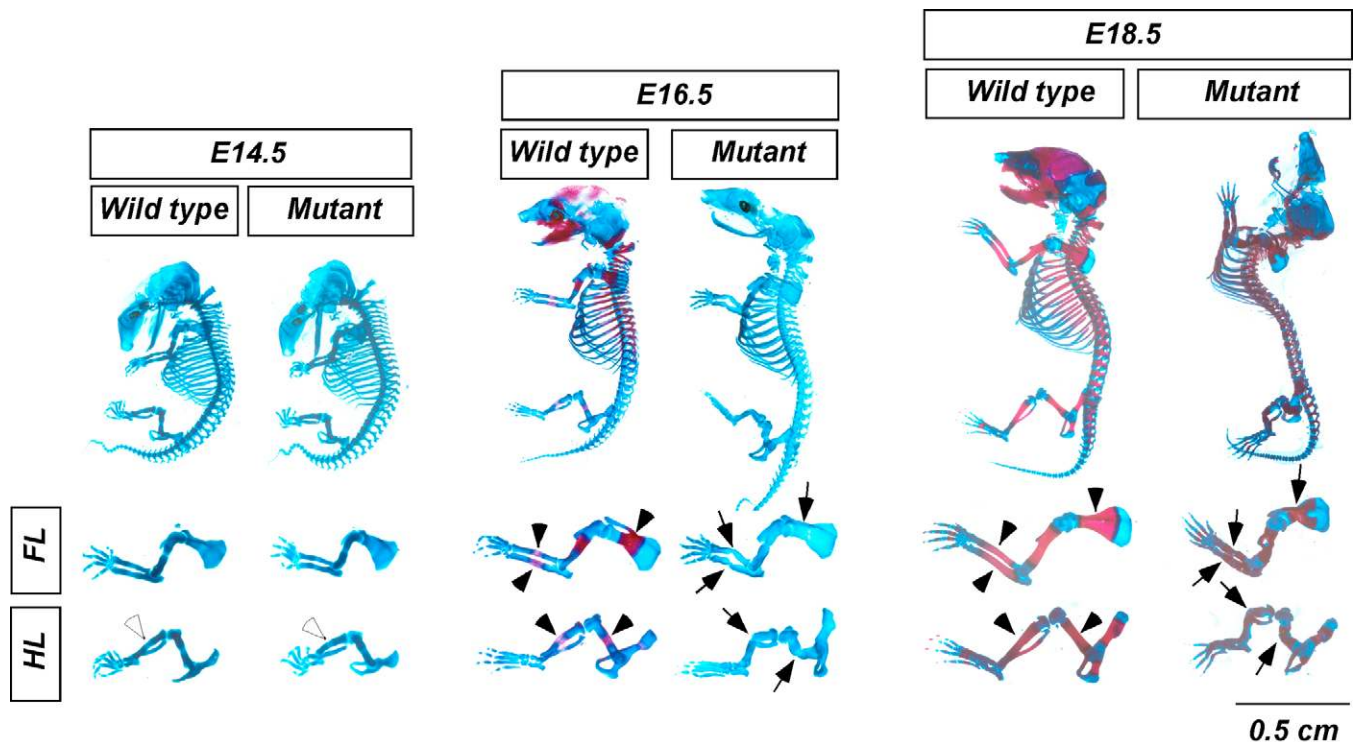
Histological analysis of long bones at E14.5 showed that the size and organization of the developing tibia was comparable to wild type with respect to chondrocyte differentiation and proliferation, as we had expected from the demonstrated specificity of the *Osx1-GFP::Cre* transgene (data not shown). Analysis of tibial elements at E16.5 and E18.5 indicated that the size and structure of the growth

plate was comparable to wild type. The only detectable mineralized matrix was associated with hypertrophic chondrocytes and, in addition to the normal growth plate, hypertrophic chondrocytes ectopically lined the periosteal region (Fig. 4 and data not shown). Vascular invasion is critically linked with osteogenesis; however, the adjacent forming marrow cavity was well vascularized, and *Mmp9*, *Mmp13* and *Vegf*, which are associated with vascular invasion were expressed normally (Fig. 4). Together, these observations support a primary role for  $\beta$ -catenin downstream of *Osx1* in osteoblast specification.

To characterize osteoblast development, we examined expression of the osteoblast cell state marker genes *Col1 $\alpha$ 1*, *Runx2*, *Osx1* and *Oc* (Fig. 4). Expression of *Col1 $\alpha$ 1*, *Runx2* and *Osx1* was observed in the periosteal region and in a mesenchymal wedge invading the marrow cavity in E18.5 *Osx1-GFP::Cre;  $\beta$ -catenin<sup>cn</sup>* mutant tibia (Fig. 4). Thus, as expected *Osx1*<sup>+</sup> osteoblasts were present. However, no *Oc*<sup>+</sup> osteoblasts were detected indicating a failure of osteoblast progression to terminal *Oc*<sup>+</sup> osteoblasts in mutant embryos. No apparent difference was observed in either cell death or cell proliferation between wild-type and mutant tibia (data not shown).

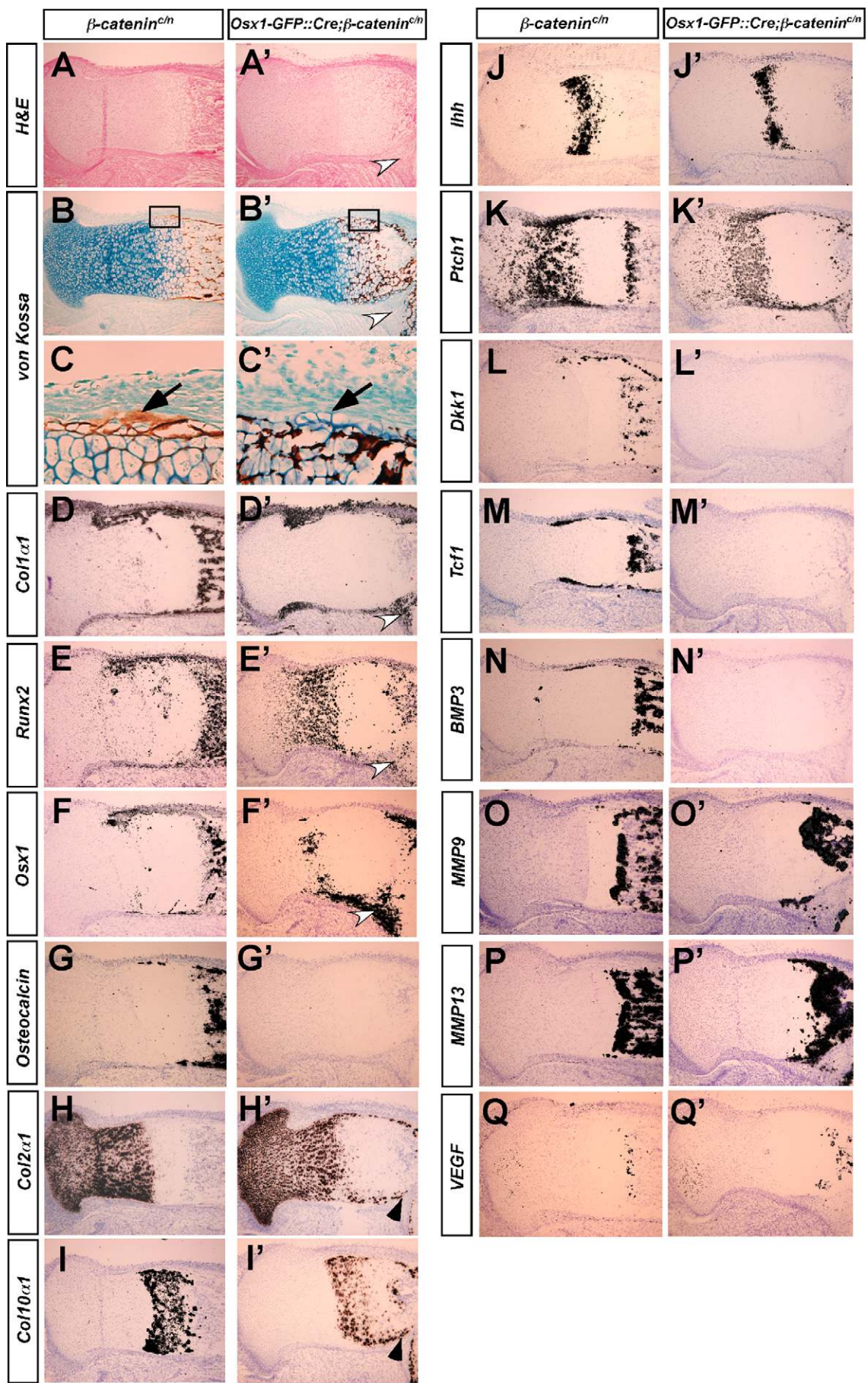
### A transient requirement for *Ihh* upstream of canonical Wnt/ $\beta$ -catenin signaling in osteoblast development

Several signaling systems are known to play important roles during osteoblast development (reviewed by Kronenberg, 2003; Karsenty, 2003). *Ihh* null mice fail to differentiate osteoblasts, but arrest in this pathway appears to occur at an initiating step: the transition of an unspecified cell to a pre-osteoblast (St-Jacques et al., 1999; Long et



**Fig. 3. *Osx1-GFP::Cre* removal of  $\beta$ -catenin function affects skeletal development.** Skeletal preparations of  *$\beta$ -catenin<sup>cn</sup>* (wild type) and *Osx1-GFP::Cre;  $\beta$ -catenin<sup>cn</sup>* (mutant) embryos at E14.5, E16.5 and E18.5. Higher magnifications of the fore- and hindlimbs are presented below each embryo. White arrowheads indicate comparable levels of hypertrophy between wild-type and mutant tibia. Arrows indicate the absence of mineralized bone matrix in mutants when compared with wild-type embryos (black arrowheads).





**Fig. 4. *Osx1*-GFP::Cre removal of  $\beta$ -catenin function results in a failure to develop terminally differentiated osteoblasts.** (A-Q') Histological analysis and in situ hybridization of <sup>35</sup>S-labeled riboprobes to sections of E18.5 tibia from (A-Q)  $\beta$ -catenin<sup>cn</sup> (wild type) and (A'-Q') *Osx1*-GFP::Cre; $\beta$ -catenin<sup>cn</sup> (mutant) embryos. Specific anti-sense riboprobes were used that identify marker genes for: states of osteoblast and chondrocyte differentiation, (D,D') *Col1 $\alpha$ 1*, (E,E') *Runx2*, (F,F') *osterix1*, (G,G') *osteocalcin*, (H,H') *Col2 $\alpha$ 1* and (I,I') *Col10 $\alpha$ 1*; components of Hh signaling, (J,J') *Ihh* and (K,K') *Ptch1*; components of canonical Wnt signaling, (L,L') *Dkk1* and (M,M') *Tcf1*; the early osteoblast marker, (N,N') *Bmp3*; matrix remodeling, (O,O') *Mmp9* and (P,P') *Mmp13*; and vascularization, (Q,Q') *Vegf*. Boxed areas in B and B' are presented at higher magnification in C and C', respectively. Black arrows indicate the absence of ossification in the mutant periosteum. White arrowheads indicate the invading wedge of mesenchymal cells into the marrow cavity, whereas black arrowheads indicate sites of ectopic expression of chondrocyte marker genes.



al., 2004). As expected, the *Ihh* expression domain of prehypertrophic chondrocytes was present, consistent with  $\beta$ -catenin<sup>c/n</sup> embryos, and active *Ihh* signaling was evident from the upregulation of *Ptch1* (the Hh receptor and primary target of Hh signaling) in the periosteal region where osteoblast specification initiates (Fig. 4).

Dickkopf homolog 1 (*Dkk1*), a negative regulator of Wnt signaling, and T-cell factor 1 (*Tcf1*; *Tcf7* – Mouse Genome Informatics), a transcriptional mediator of Wnt signaling, are transcriptional targets of canonical Wnt signaling (Roose et al., 1999; Cong et al., 2003). Within the developing long bone, these genes were expressed within the osteoblast-forming region in the perichondrium and the primary spongiosa of  $\beta$ -catenin<sup>c/n</sup> embryos, but were absent from *Osx1-GFP::Cre*;  $\beta$ -catenin<sup>c/n</sup> mutants (Fig. 4), consistent with regulation by a  $\beta$ -catenin-mediated Wnt signaling pathway. In addition, bone morphogenic protein 3 (*Bmp3*) expression, which is normally detected throughout the periosteum and primary spongiosa, was absent in the mutant, suggesting that *Bmp3* signaling lies downstream of canonical Wnt signaling (Fig. 4). Thus, *Osx1-GFP::Cre* removal of the  $\beta$ -catenin gene leaves Hh signaling intact in the bone forming region but terminates canonical Wnt signaling and, with this, progressive osteoblast development.

Further insight into the relationship between Hh and Wnt function comes from the osteoblast-specific removal of Hh signaling in *Osx1*<sup>+</sup> osteoblast precursors and their descendants. As discussed earlier, *Ihh* is required for the specification of early *Runx2*<sup>+</sup> osteoprogenitors, prior to the requirement for canonical Wnt signaling. However, whether this represents a transient signal input or the onset of a more extended role for *Ihh* signaling in osteoblast development has not been addressed. We generated *Osx1-GFP::Cre*; *Smo*<sup>c/n</sup> embryos to remove *Smoothened* (*Smo*) activity (and, consequently, all responsiveness to Hh signaling) after specification of a *Runx2*<sup>+</sup>; *Osx1*<sup>+</sup> osteoblast progenitor. Analysis of *Osx1-GFP::Cre*; *Smo*<sup>c/n</sup> embryos at E18.5 by whole-mount skeletal preparation, histological analysis and in situ hybridization with chondrogenic and osteogenic marker genes demonstrated a normal program of chondrocyte and osteoblast development (Fig. 5; data not shown). Thus, Hh signaling does not appear to play an essential role in the terminal differentiation of osteoblasts beyond an *Osx1*<sup>+</sup> cell state.

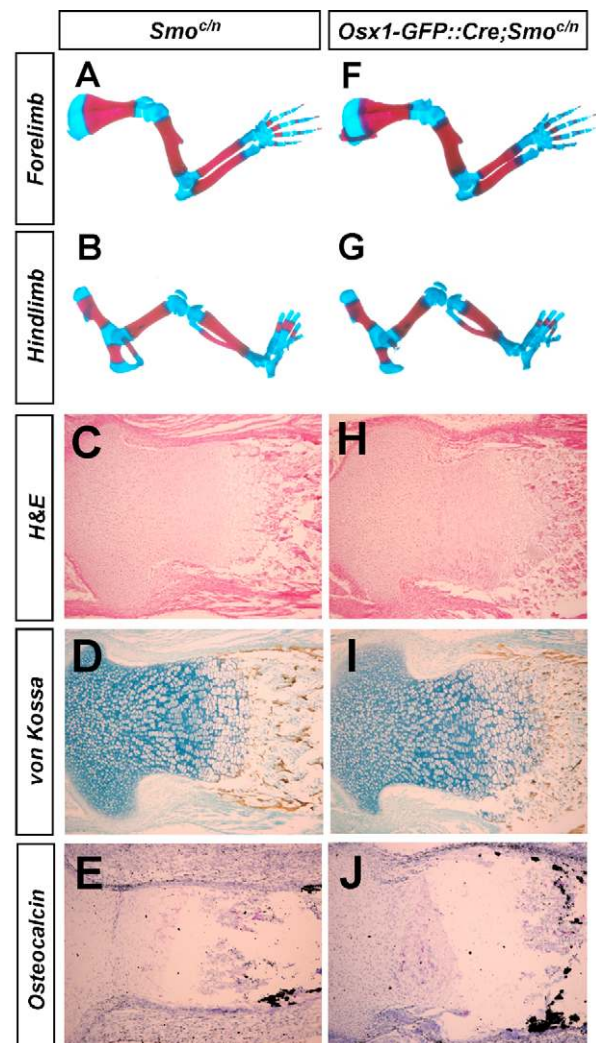
### Osteoblast to chondrocyte cell fate changes in the absence of $\beta$ -catenin activity in the osteoblast lineage

Previous studies have indicated that when osteoblast differentiation is arrested prior to *Osx1* expression the loss of a terminally differentiated osteoblast is accompanied by the appearance of ectopic chondrocytes (Nakashima et al., 2002; Long et al., 2004; Hill et al., 2005; Day et al., 2005). Consistent with this view, analysis of the expression of collagen 2 $\alpha$ 1 (*Col2 $\alpha$ 1*) and collagen 10 $\alpha$ 1 (*Col10 $\alpha$ 1*), markers of proliferating and postmitotic hypertrophic chondrocytes, highlight ectopic chondrocytes lining the periosteal region and an invading mesenchymal wedge adjacent to *Col1 $\alpha$ 1* (a marker of early osteoblast progenitors), *Runx2* and *Osx1* expressing cells (Figs 1, 4). Thus, the presence of *Runx2* and *Osx1* within an osteoblast precursor does not appear to be sufficient to maintain an osteogenic program in the absence of  $\beta$ -catenin; osteoblast precursors convert to a chondrocyte fate upon  $\beta$ -catenin removal. This conclusion was further supported by performing double-labeled fluorescent in situ hybridization on wild-type and mutant tibia, comparing the expression of *Col2 $\alpha$ 1* and *Col10 $\alpha$ 1* with the activity

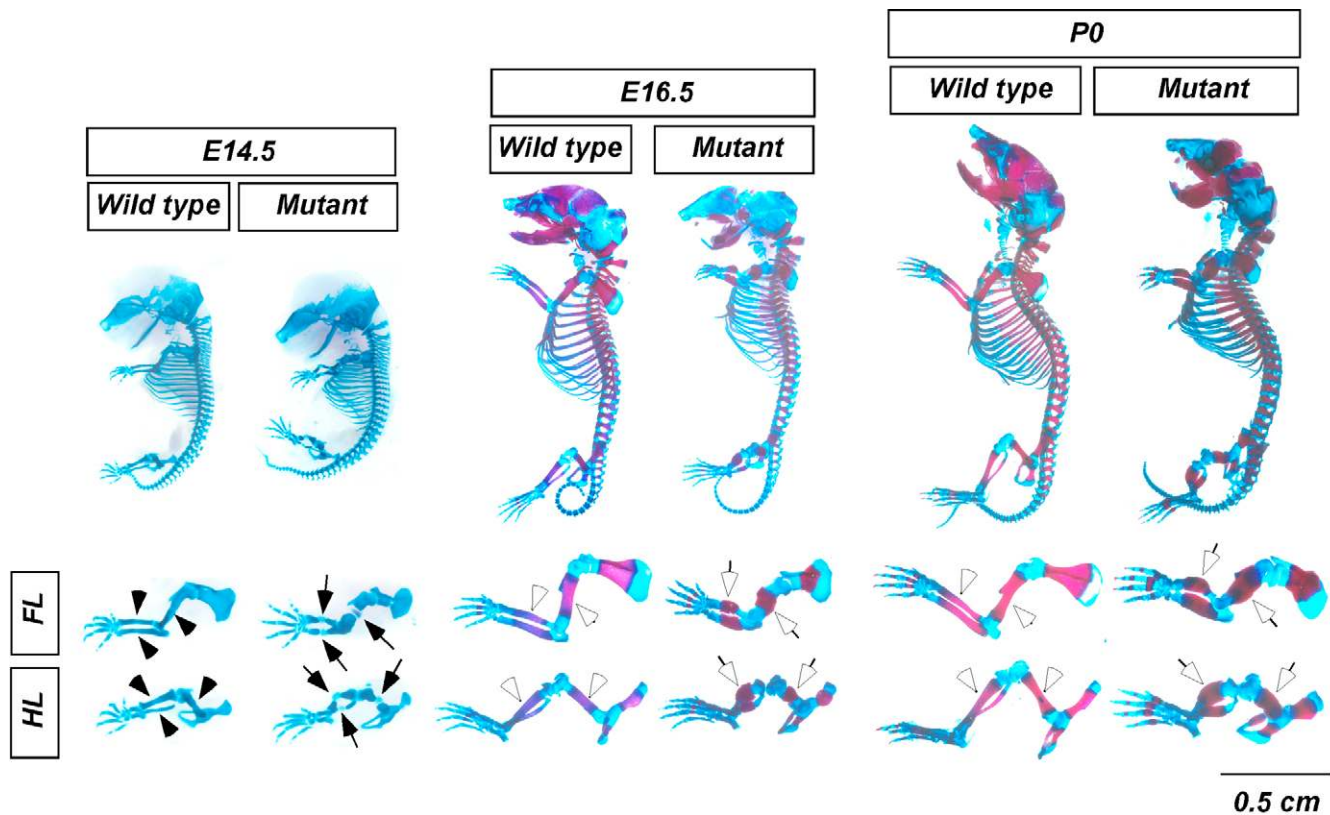
of the *Osx1-GFP::Cre* transgene. Furthermore, *Osx1-GFP::Cre*-mediated activation of the R26R allele in *Osx1-GFP::Cre*;  $\beta$ -catenin<sup>c/n</sup>; R26R mice demonstrates that the ectopic chondrocytes arise from *Osx1-GFP::Cre*-expressing osteoblast precursors (data not shown).

### Stabilization of $\beta$ -catenin in osteoblast precursor cells results in premature mineralization and increased proliferation of pre-osteoblasts

We next addressed whether activation of the canonical Wnt/ $\beta$ -catenin signaling pathway in osteoblast precursor cells influenced osteoblast differentiation. To address this, we mated *Osx1-GFP::Cre* mice to those carrying a conditional  $\beta$ -catenin allele ( $\Delta$ ex3; designated *Catnb*<sup>lox(ex3)/lox(ex3)</sup>) (Harada et al., 1999). Cre-mediated excision of exon 3 encoded N-terminal regulatory sequences produces a stabilized form of  $\beta$ -catenin, cell autonomously upregulating canonical Wnt signaling within the osteoblast lineage.



**Fig. 5. *Osx1-GFP::Cre* removal of *Smo* function does not affect osteoblast differentiation.** Skeletal preparations of (A,B) *Smo*<sup>c/n</sup> (wild type) and (F,G) *Osx1-GFP::Cre*; *Smo*<sup>c/n</sup> (mutant) fore- and hindlimbs. Histological analysis (C,D,H,I) and in situ hybridization with specific anti-sense <sup>35</sup>S-labeled riboprobes for the terminal osteoblast differentiation marker osteocalcin (E,J) to sections of E18.5 tibia from wild-type (C-E) and mutant (H-J) embryos.



**Fig. 6. Conditional stabilization of  $\beta$ -catenin by *Osx1-GFP::Cre* affects skeletal development.** Skeletal preparations of *Catnb<sup>lox(ex3)/+</sup>* (wild type) and *Osx1-GFP::Cre;Catnb<sup>lox(ex3)/+</sup>* (mutant) embryos at E14.5, E16.5 and P0. Higher magnifications of the fore- and hindlimbs are presented below each embryo. Black arrows indicate the presence of a poor Alcian blue-stained discontinuous matrix in mutants in comparison with wild-type embryos at E14.5 (black arrowheads). White arrows indicate the expansion in mineralized bone matrix observed in mutants when compared with wild-type embryos (white arrowheads).

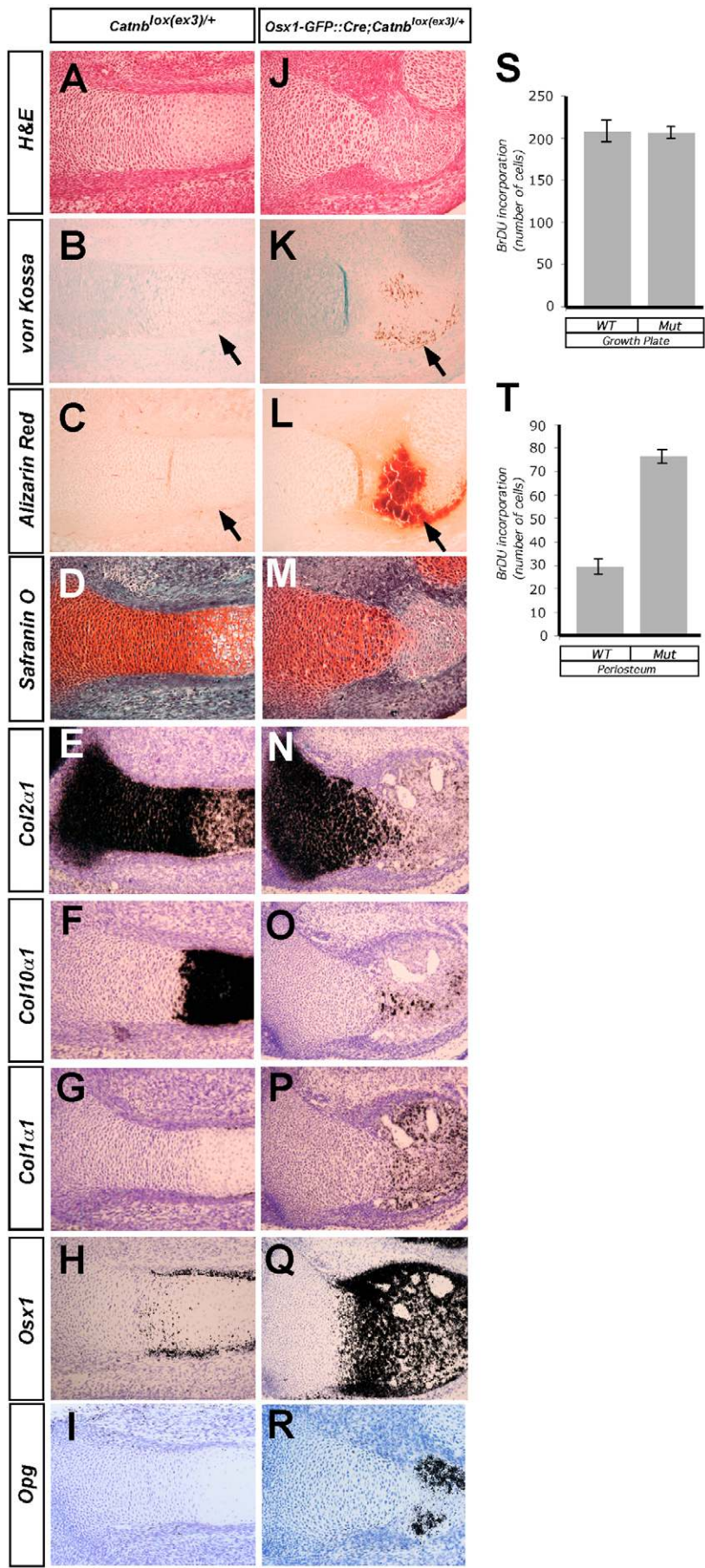
*Osx1-GFP::Cre;Catnb<sup>lox(ex3)/+</sup>* mutants died at birth. Skeletal preparations at E14.5, E16.5 and P0 revealed that mutant embryos, overall, had shorter limbs in comparison with *Catnb<sup>lox(ex3)/+</sup>* wild-type littermates (Fig. 6). Alizarin red staining of the mineralized bone matrix was first evident at E16.5 in whole-mount skeletons. At this time, mutants appeared to have an intense and broader ossification center in the long bones in comparison with their wild-type counterparts, although ossification in the skull bones was delayed. By P0, a thick bony matrix characterized all long bones in the mutants, and bone formation was now visible in several cranial regions.

Histological analysis of the long bones at E14.5 and E16.5 showed, an abnormal wedge-shaped growth plate with very few identifiable hypertrophic chondrocytes when compared with wild-type tibias (Figs 7, 8). Analysis of histological sections at E13.5 (data not shown) and E14.5 (Fig. 7) using von Kossa and Alizarin red staining techniques revealed extensive premature mineralization of the mutant tibia at this time; no mineralization was detected in the wild type, even at E14.5 (Fig. 7). At E16.5, the primary spongiosa was readily identifiable in the wild-type tibia but no primary spongiosa-like matrix was observed in the mutant. Rather, the matrix resembled the dense matrix normally restricted to the cortical bone-forming region that generates the bone collar (Fig. 8).

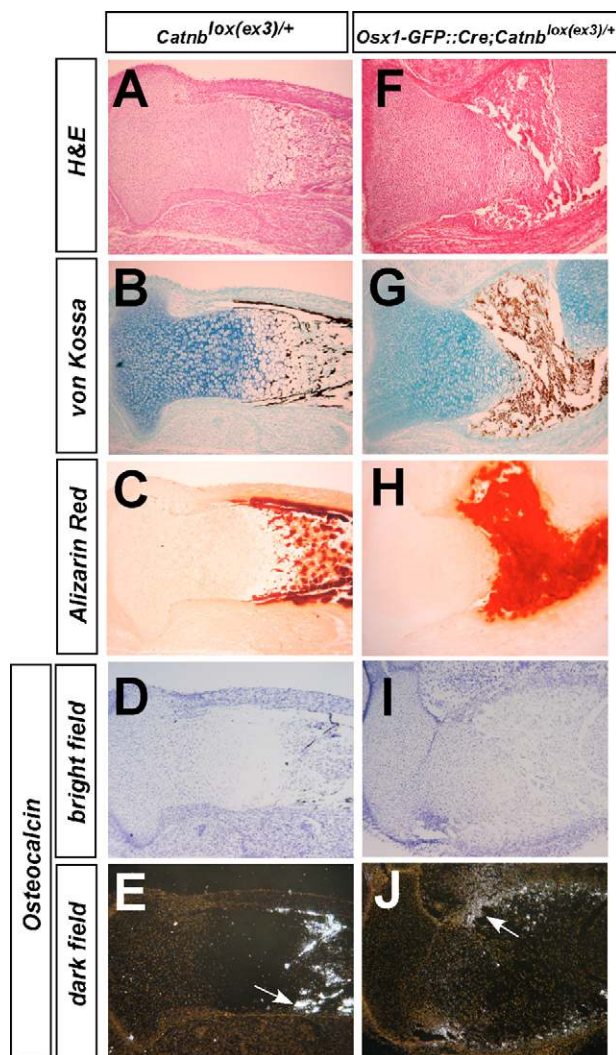
To characterize the observed premature ossification of the mutants, we examined expression of the osteoblast differentiation markers *Colla1*, *Osx1* and *Oc*. While *Colla1* was expressed in the

periosteal region of the wild type at E14.5, and the primary spongiosa at E16.5, expression of *Colla1* was observed throughout the periosteal region and entire central region of the tibia in the mutants (Fig. 7; data not shown). A similar dramatic expansion was also observed in the expression of *Osx1*, suggesting that the stabilization of  $\beta$ -catenin in osteoblast precursor cells resulted in the promotion of osteoblast development. Although the synthesis of bone matrix was activated prematurely at E13.5, we failed to observe *Oc*<sup>+</sup> terminal osteoblasts prior to the normal onset of *Oc* expression at E16.5 (data not shown). Furthermore, although *Oc*<sup>+</sup> osteoblasts were clearly identifiable in the wild-type tibia at E16.5, *Oc*<sup>+</sup> osteoblasts were rare in the mutant; when present, these cells were exclusively restricted to the periosteal region and expressed low levels of *Oc* relative to their wild-type counterparts (Fig. 8). The expansion of the osteoblast lineage was accompanied by a 3-fold increase in proliferation in osteoblast-forming regions along the length of the periosteum in mutants at E14.5 (Fig. 7). Together, these observations suggest that the stabilization of  $\beta$ -catenin results in a marked increase in proliferation of an *Osx1*<sup>+</sup> precursor population and an accelerated progression of an osteoblast program to mature bone-secreting osteoblasts. Although these cells actively synthesize a bone matrix, they do not progress to a terminal state, characterized by high *Oc*<sup>+</sup> expression. Thus, the cessation of canonical Wnt/ $\beta$ -catenin signaling may normally accompany this progression in vivo. As most *Oc*<sup>+</sup> cells are normally observed in the marrow cavity, *Oc*<sup>+</sup> expression may reflect an absence of local canonical Wnt signaling in this region.





**Fig. 7. Conditional stabilization of  $\beta$ -catenin by *Osx1-GFP::Cre* promotes premature mineralization of skeletal elements.** Histological analysis and in situ hybridization of  $^{35}\text{S}$ -labeled riboprobes to sections of E14.5 tibia from (A-I) *Catnb<sup>lox(ex3)/+</sup>* (wild type) and (J-R) *Osx1-GFP::Cre; Catnb<sup>lox(ex3)/+</sup>* (mutant) embryos, with specific anti-sense riboprobes for marker genes that identify states of chondrocyte and osteoblast differentiation. Chondrocyte: (E,N) *Col2 $\alpha$ 1* and (F,O) *Col10 $\alpha$ 1*. Osteoblast: (G,P) *Col1 $\alpha$ 1*, (H,Q) osteonin and (I,R) osteopontin. Arrows indicate premature mineralization, identified as silver deposition by (K) von Kossa and (L) Alizarin red staining, present in mutants compared with (B,C) wild types. (S,T) Analysis of BrDU incorporation in wild-type and mutant embryos at E14.5. Graphs represent the relative number of proliferative cells within the (S) growth plates or (T) periosteal region of mutant and wild-type tibia. Error bars represent the s.d. of the mean of the results ( $n \geq 3$ ).



**Fig. 8. *Osx1-GFP::Cre* conditional stabilization of  $\beta$ -catenin does not promote expression of osteocalcin.** Histological analysis and in situ hybridization of an osteocalcin-specific anti-sense  $^{35}\text{S}$ -labeled riboprobe to sections of E16.5 tibia from (A-E) *Catnb<sup>lox(ex3)/+</sup>* (wild type) and (F-J) *Osx1-GFP::Cre;Catnb<sup>lox(ex3)/+</sup>* (mutant) embryos. Arrows indicate sites of high osteocalcin expression in the wild type and low-level osteocalcin expression in the mutant in the periosteum.

Recent evidence indicates that canonical Wnt/ $\beta$ -catenin signaling positively regulates bone matrix formation by suppressing osteoclast development (Glass et al., 2005). Thus, a failure of osteoclast development, and, consequently, a loss of bone matrix turnover, could explain the premature formation of a bone matrix following osteoblast-specific stabilization of  $\beta$ -catenin. However, when wild-type limbs were examined, it was evident that *Trap*-positive osteoclasts were not present at E14.5 (data not shown). Thus, the accelerated bone matrix phenotype precedes any role for osteoclasts in bone remodeling. However, when osteoclasts were normally detected in the wild type at E16.5, they were completely absent from the mutant (data not shown). Consistent with the findings of Glass et al. (Glass et al., 2005), that the osteoclast inhibitor osteoprotegerin (*Opg*; *Tnfrsf11b* – Mouse Genome Informatics) is regulated by canonical Wnt signaling, the failure of osteoclast formation correlated with a dramatic

upregulation of *Opg* upon osteoblast-specific stabilization of  $\beta$ -catenin relative to wild-type osteoblasts at E14.5 (Fig. 7; data not shown).

Analysis of *Dkk1* and *Tcf1* expression (Wnt pathway modulators and targets) indicated that active Wnt signaling was clearly upregulated in the osteoblast population in response to the activation of  $\beta$ -catenin (data not shown), which is consistent with the activation of canonical Wnt signaling and direct regulation of *Dkk1* and *Tcf1* transcription by  $\beta$ -catenin. Despite over-mineralization of the tibia, vascular invasion was evident, and mutants expressed *Mmp9* and *Mmp13* (data not shown). In addition, the population of *Col10 $\alpha$ 1*-expressing hypertrophic chondrocytes was clearly reduced (Fig. 7; data not shown). TUNEL analysis revealed that mutants displayed a significant increase in cell death at the interface of hypertrophic chondrocytes and the mineralized matrix, suggesting that TUNEL-positive cells were likely to represent hypertrophic chondrocytes (data not shown). Finally, we examined the expression of indicators of *Ihh* signaling. These appeared to be largely unaltered, suggesting that the observed increase in bone matrix does not result from an overt change in the *Ihh*-regulation of osteoblast precursors (data not shown).

### Wnt7b is not essential for the terminal differentiation of osteoblasts

The nature of the putative Wnt ligand that would mediate Wnt action in the osteoblast lineage is unclear. A recent report has highlighted *Wnt7b* as a candidate (Hu et al., 2005). *Wnt7b* expression was reported in the osteoblast-forming region of the long bone; furthermore, expression was dependent upon *Ihh* signaling, and was upregulated in response to *Ihh* signaling in *Ihh*-stimulated osteoblast specification of C3H10T1/2 mesenchymal progenitor cells (Hu et al., 2005). However, we recently generated embryos in which all *Wnt7b* function was removed and we have not observed any defects in skeletal development (S.J.R. and A.P.M., unpublished).

### DISCUSSION

We have used genetic approaches to modify *Ihh* and Wnt signaling in the developing osteoblast lineage of the mouse. These data point to sequential and genetically separable roles for these signaling pathways in the progressive specification of osteoblasts within the endochondral skeleton (Fig. 9).

Osteoblast progenitors can first be identified within the inner perichondrium adjacent to, and coincident with, the first appearance of hypertrophic chondrocytes. This tight linkage reflects a crucial role for *Ihh* signaling (St-Jacques et al., 1999; Chung et al., 2001). *Ihh* is produced by pre-hypertrophic chondrocytes and appears to act directly on perichondrially located osteoblast progenitors to specify the osteoblast precursors (St-Jacques et al., 1999; Long et al., 2004). To date, all markers of this program in the endochondral-, but not the membranous bone-, derived skeleton are dependent on an initial *Ihh* input. Furthermore, *Ihh* activates osteoblast development in a variety of mesenchymal and skeletogenic cell types in vitro (Nakamura et al., 1997; van der Horst et al., 2003; Long et al., 2004). In the absence of signaling, perichondrial osteoblast progenitors in the perichondrium adopt a chondrocyte fate, as evidenced by the layer of immature chondrocytes that surround the hypertrophic chondrocytes in *Ihh* mutants (St-Jacques et al., 1999), and by the ectopic chondrogenesis exhibited by perichondrially localized *Smo* mutant cells in chimeric mice (Long et al., 2004). Although this suggests that a potential cell, frequently termed an osteochondroprogenitor (OCP), resides within the perichondrium, a conclusion supported by clonal analysis in vitro (Nakase et al.,



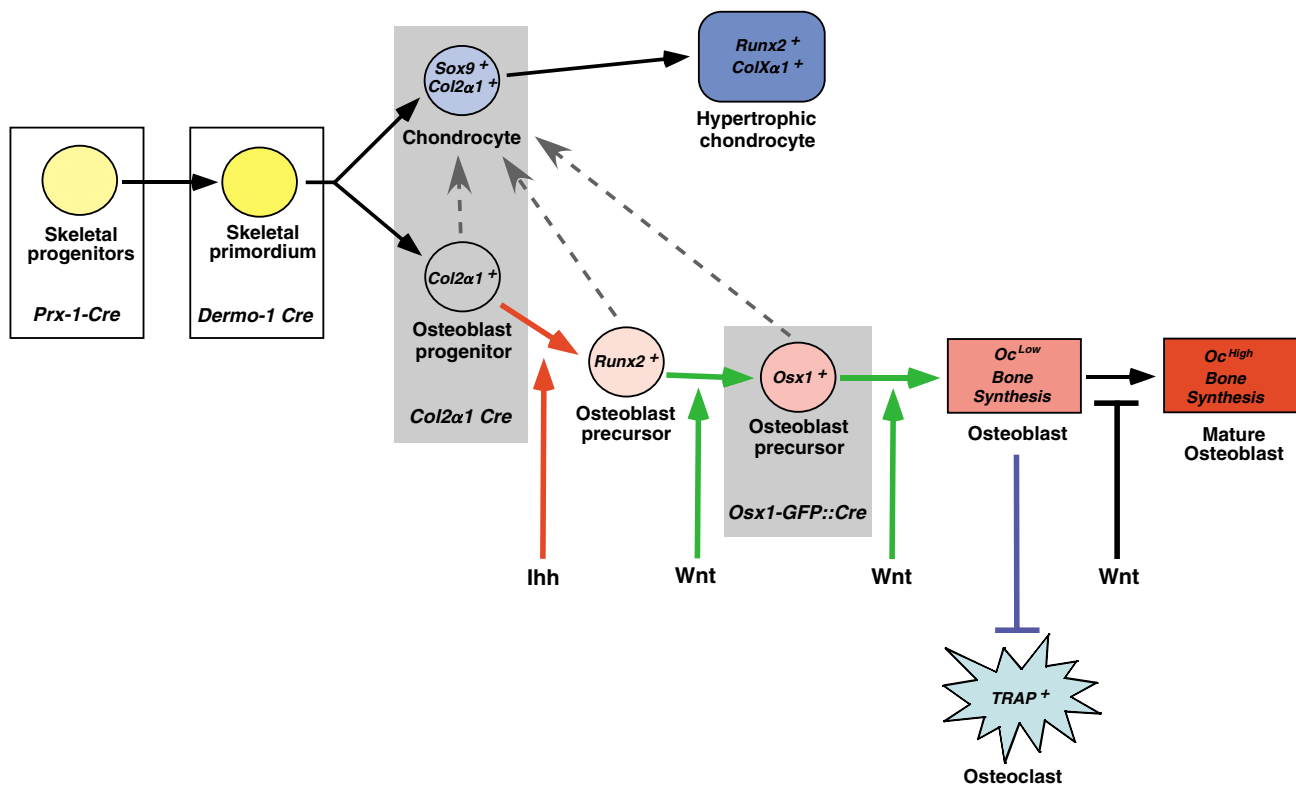
1993), there is currently no evidence that these cells ordinarily give rise to chondrocytes. Thus, during normal development, *Ihh* signaling appears to act as a switch within a specific population of inner perichondrial mesenchyme to initiate a program of bone formation. Failure to activate this switch results in cells adopting an alternative chondrocyte pathway of development (Fig. 9). Given the crucial role for *Ihh* signaling in regulating the temporal and spatial program of early osteoblast commitment, what role does *Ihh* play beyond this stage? Our results do not support an on-going role for *Ihh* signaling in progression along the osteoblast pathway of differentiation (Fig. 5). When *Smo* activity is removed in *Osx1*<sup>+</sup> osteoblast precursors, normal bone secreting *Oc*<sup>High</sup> osteoblasts are generated, and the endochondral skeleton at birth is indistinguishable from wild type. Whether this is also true in the adult is currently under investigation. Clearly, *Ihh* continues to be expressed postnatally within prehypertrophic chondrocytes, and the upregulation of *Ihh* signaling at sites of fracture repair implicates *Ihh* in skeletal homeostasis, repair and regeneration (Vortkamp et al., 1998; van der Eerden et al., 2000; Le et al., 2001).

In contrast to *Ihh*, the manipulation of  $\beta$ -catenin activity provides evidence against an essential role for canonical Wnt signaling in early osteoblast specification. When  $\beta$ -catenin activity was removed in skeletal progenitors with *Prx1-cre*, *Runx2*<sup>+</sup> prior to specification of the skeletal anlage in the limbs, osteoblast precursors were specified, but these failed to progress to *Osx1*<sup>+</sup> precursors and no bone matrix was laid down (Hill et al., 2005) (Fig. 9). Although the precise kinetics of the inactivation of the  $\beta$ -catenin conditional allele

and turnover of existing  $\beta$ -catenin protein in this model are not clear, it seems reasonable to conclude from the early expression of the Cre driver that  $\beta$ -catenin is not essential for the specification of a *Runx2*<sup>+</sup> osteoblast precursor. Furthermore, the results are consistent with a role for canonical Wnt/ $\beta$ -catenin signaling in the transition to an *Osx1*<sup>+</sup> osteoblast precursor. Other studies using later active Cre driver mice that, like *Prx1-cre*, are active in both the chondrocyte and osteoblast compartments of the endochondral skeleton give conflicting results. Although all reports fail to observe a role for  $\beta$ -catenin in the initial specification of *Runx2*<sup>+</sup> osteoblast precursors, using an identical *Dermo1-cre* transgenic line to remove  $\beta$ -catenin from pre-cartilaginous endochondral skeletal condensates, Hu et al. (Hu et al., 2005) observed an arrest of osteoblast specification at a *Runx2*<sup>+</sup>, *Osx1*<sup>-</sup> stage, whereas Day et al. (Day et al., 2005) reported a progression, albeit at reduced levels, to a *Runx2*<sup>+</sup>, *Osx1*<sup>+</sup> precursor and arrest at this stage, suggesting a continuing role for  $\beta$ -catenin in osteoblast differentiation beyond the *Osx1*<sup>+</sup> osteoblast precursor.

This interpretation is also supported by *Col2 $\alpha$ 1-cre*-mediated removal (Day et al., 2005) (this study; Fig. 9). However, the occasional appearance of *Oc*<sup>High</sup> osteoblasts (Day et al., 2005) (this study) and the broad activity of these Cre strains within both osteoblast and chondrocyte lineages prevents a rigorous assessment of the specific direct roles for canonical Wnt/ $\beta$ -catenin signaling in osteoblast differentiation.

Our data, in which  $\beta$ -catenin activity is removed specifically from *Runx2*<sup>+</sup>, *Osx1*<sup>+</sup> osteoblasts, provides compelling evidence that  $\beta$ -catenin is essential within the osteoblast lineage for the specification



**Fig. 9. Proposed model for the role of Hh and canonical Wnt signaling in regulating the differentiation of skeletal progenitors.** Various lines of evidence implicate the importance of canonical Wnt signaling at multiple stages along the osteoblast differentiation pathway, from the specification of early skeletal progenitor cells to a terminally differentiated osteoblast. Observations from these studies (Hu et al., 2005; Day et al., 2005), together with results presented in this study, have been used to synthesize a working model for the specific functions of Hh and canonical Wnt signaling during osteoblast specification and differentiation. See text for details. Red and green arrows indicate the requirement for Hh and canonical Wnt signaling, respectively; the blue line represents the negative regulation of osteoclasts by OPG.

of an *Osx1*<sup>+</sup> osteoblast to a bone-secreting osteoblast (Fig. 9). Furthermore, the demonstration that the stabilization of  $\beta$ -catenin, and, consequently, the activation of canonical Wnt signaling, within osteoblast precursors expands this population and accelerates the progression to a bone matrix-secreting osteoblast indicates that canonical Wnt/ $\beta$ -catenin signaling may regulate both the proliferation and maturation of the osteoblast precursor pool. Interestingly, continued activation of canonical Wnt/ $\beta$ -catenin signaling arrests osteoblasts at an *Oc*<sup>Low</sup> stage, suggesting that progression to an *Oc*<sup>High</sup> state may require the downregulation of this signaling input in the primary spongiosa where the majority of *Oc*<sup>High</sup> cells are located (Fig. 9).

In addition to a proposed cell autonomous role for  $\beta$ -catenin in promoting a bone-secreting osteoblast pathway, recent studies suggest that canonical Wnt/ $\beta$ -catenin signaling may play a non-cell autonomous role in suppressing the development of bone-matrix-degrading osteoclasts through the production of OPG (Glass et al., 2005). Our data lend further support to this view. We observed a dramatic upregulation of *Opg* upon stabilization of  $\beta$ -catenin in *Osx1*<sup>+</sup> osteoblast precursors. However, as the normal appearance of osteoclasts occurs after the onset of bone matrix secretion in this model, the premature bone matrix deposition observed at E14.5 precedes any role for osteoclast suppression in this early phenotype. By contrast, OPG-mediated inhibition of osteoclast development may contribute to the massive expansion of a cortical bone-like matrix in E16.5 long bones of *Osx1-GFP::Cre; Catnb*<sup>lox(ex3)/+</sup> embryos.

Interestingly, in earlier reports, in which Cre removed  $\beta$ -catenin broadly in skeletal structures, ectopic chondrocytes were observed in perichondrial regions where cortical bone first arises (Hill et al., 2005; Day et al., 2005). Here, we show that the osteoblast-specific removal of  $\beta$ -catenin results in osteoblast precursors in which Cre was active, adopting a chondrocyte-like fate. These ectopic chondrocytes appear to undergo a transition from early *Col2a1* immature chondrocytes to *Col10a1* hypertrophic chondrocytes, as in the normal growth zone. Thus,  $\beta$ -catenin acts both to promote an osteoblast program and to block an alternative program of chondrogenesis within osteoblast precursors: an *Ihh*-mediated activity at an earlier stage in the osteoblast lineage.

How do *Hh* and Wnt signals act at the molecular level to sequentially regulate osteoblast differentiation? *Hh* signaling has been shown to modify cellular responsiveness to other signals, most notably several members of the Bmp family (Murtaugh et al., 1999). Bmps are implicated in the specification of both chondrocytes and osteoblasts (reviewed by Hoffmann and Gross, 2001), as well as in the subsequent modification of the osteogenic program, where some Bmps promote bone formation [such as Bmp2, Bmp7, Bmp6 and Bmp9 (Peng et al., 2003)], although Bmp3 acts as a negative regulator of bone formation (Daluiski et al., 2001). In vivo, Bmp signaling is essential for the normal induction of endochondral anlage, and, both in vivo and in vitro, Bmps can promote an osteoblast program of mesenchymal cell differentiation. One possible role for *Ihh* signaling is to alter the responsiveness of perichondrial osteoblast progenitors to a Bmp input, modifying the response from chondrocyte inducing to osteoblast inducing. Consistent with this model, Bmp-mediated induction of an osteoblast phenotype in vitro requires prior *Ihh* signaling (Long et al., 2004), and has been shown to function, at least in part, by induction of a Wnt autocrine loop (Rawadi et al., 2003). In addition, Bmp2 signaling has been shown to upregulate the expression of *Sox9* (Healy et al., 1999; Zehentner et al., 1999), a key early determinate of chondrocytes, whereas *Sox9* is downregulated in the

perichondrial region where osteoblast differentiation initiates (Yamashiro et al., 2004). Whether *Sox9* downregulation is *Ihh* dependent is unclear but, given the ectopic chondrocyte phenotype adopted by perichondrially localized *Smo* mutant cells, it is reasonable to presume that this, as with normal chondrogenesis, requires high levels of *Sox9* activity.

The transcriptional activation domain of *Sox9* has also been reported to directly interact with specific armadillo repeats in  $\beta$ -catenin. On the basis of these interactions, it has been proposed that *Sox9* inhibits  $\beta$ -catenin activity, promoting  $\beta$ -catenin degradation (Akiyama et al., 2004), such that overexpression of *Sox9* generates a similar phenotype to loss-of-function of  $\beta$ -catenin. Thus, the balance of *Sox9* and  $\beta$ -catenin may regulate alternate programs of chondrocyte and osteoblast development, respectively. When *Sox9* levels are high and  $\beta$ -catenin levels are low, a chondrocyte program may be favored. By contrast, high levels of  $\beta$ -catenin and low levels of *Sox9* may act on appropriately specified progenitors to promote an osteoblast fate. Interestingly, *Prx1-cre*-mediated stabilization of  $\beta$ -catenin in skeletal progenitors leads to a dramatic loss of the endochondral skeleton, suggesting that the timing of action of  $\beta$ -catenin (downstream of *Ihh* in perichondrial osteoblast precursors) may be crucial to its normal osteoblast role (Hill et al., 2005).

Canonical Wnt ligands have been shown to stimulate *Runx2* expression and *Runx2* is itself essential for osteoblast development (Gaur et al., 2005). However, in our study, and in other studies discussed earlier,  $\beta$ -catenin is not essential for the initial activation of *Runx2*. These findings do not exclude the possibility that the Wnt input may normally regulate either the level or duration of *Runx2* expression in the osteoblast lineage. By contrast, *Prx1-cre* removal of  $\beta$ -catenin suggests that *Osx1* expression is  $\beta$ -catenin dependent. Analysis of the expression of each gene in osteoblast precursors in *Runx2* and *Osx1* mutants indicates that *Osx1* activation lies downstream of *Runx2* (Nakashima et al., 2002); thus *Runx2*, although required in vivo for *Osx1* activation, is not sufficient in the absence of  $\beta$ -catenin. Furthermore, Bmp2 activates *Osx1* in *Runx2* mutant cells (Lee et al., 2003); thus, the molecular hierarchies and interactions underlying *Osx1* activation are uncertain, although its crucial role in the specification of all osteoblasts has been clearly demonstrated (Nakashima et al., 2002).

After the initial appearance of a bone matrix, a subset of osteoblasts activates *Oc*; *Oc* is considered a late marker of terminal osteoblasts. *Oc* is itself a direct target of *Runx2* regulation, where *Runx2* binds a cis-regulatory region within the *Oc* promoter (Paredes et al., 2004). Our data indicate that when  $\beta$ -catenin levels remain high in the osteoblast, secretion of a bone matrix is promoted, but osteoblasts express only low levels of *Oc* (*Oc*<sup>Low</sup>, Fig. 9). Thus, a loss of a canonical Wnt input appears to accompany the progression to an *Oc*<sup>High</sup> state. Interestingly, *Lef1*, a  $\beta$ -catenin-binding partner and target of canonical Wnt/ $\beta$ -catenin signaling in the osteoblast lineage (Hu et al., 2005), has been shown to inhibit *Runx2*-mediated activation of the *Oc* promoter; *Lef1*- and *Runx2*-binding sites lie adjacent to each other in the relevant cis-regulatory region within *Oc* (Kahler and Westendorf, 2003). Thus, the direct integration of distinct regulatory inputs on the *Oc* promoter may explain the observed block in *Oc* activation in our study.

Finally, what is the identity of the postulated Wnt signal(s) mediating the proposed canonical Wnt signaling? Several Wnts have been reported to be expressed in the developing skeletal anlage (Hu et al., 2005). Of particular interest is *Wnt7b*. *Wnt7b* is expressed in the bone-forming region downstream of *Ihh*, providing a potential link between the *Ihh* and Wnt pathways (Hu et al., 2005). However,



although *Wnt7b* may play a redundant role with another factor, our data indicate that *Wnt7b* is not essential for osteoblast development. Thus, as with other regions of the embryo where studies of  $\beta$ -catenin action have made a strong case for a canonical Wnt input, most notably in stem cell maintenance in the mammalian gut (reviewed by Pinto and Clevers, 2005), the regulatory ligand(s) remains to be identified.

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